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1 **Global proteomic characterisation of uterine histotroph recovered from beef heifers**  
2 **yielding good quality and degenerate Day 7 embryos**

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5

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15

## 16 **Abstract**

17 The objective was to analyse the proteomic composition of uterine flushes collected from  
18 beef heifers on Day 7 post-insemination. Oestrus was synchronised in cross-bred beef heifers  
19 using a Controlled Intravaginal Drug Releasing device (CIDR) protocol. Heifers detected in  
20 standing oestrus (within 24-48 h post CIDR removal) were inseminated (oestrus=Day 0) with  
21 frozen-thawed semen from a single ejaculate of a bull with proven fertility. Heifers from  
22 which an embryo was recovered (following slaughter on Day 7) were classified as either  
23 having a viable embryo (morula/blastocyst stage) or a degenerate embryo (arrested at the 2-  
24 to 16-cell stage). The overall recovery rate (viable and degenerate combined) was 64%.  
25 Global LC-MS/MS proteomic analysis of the histotroph collected identified 40 high  
26 confidence proteins present on Day 7; 26 proteins in the viable group, 10 in the degenerate  
27 group and four shared between both groups. Five proteins (Platelet-activating factor  
28 acetylhydrolase IB subunit gamma (PAFAH1B3), Tubulin alpha-1D chain, Tubulin beta-4A  
29 chain, Cytochrome C and Dihydropyrimidinase-related protein-2) were unique or more  
30 abundant in the histotroph collected from animals with a viable embryo and one protein  
31 (S100A4) was more abundant in the histotroph collected from animals with a degenerate  
32 embryo. Of interest, PAFAH1B3, detected only in histotroph from the group yielding viable  
33 embryos, belongs to the group of platelet activating factors that are known to be important for  
34 the development of the pre-implantation embryo in other species. To our knowledge this is  
35 the first report of PAFAH1B3 in relation to bovine early embryonic development.

36

37 **Keywords:** Proteomics, histotroph, embryo development

## 38 1. Introduction

39 Embryo mortality in cattle, reflected in reduced conception rate/calving rate per service, is a  
40 major cause of economic loss for the farming industry. In heifers, only 60% of single  
41 inseminations lead to a successful full term pregnancy despite a fertilisation rate of 90-95%  
42 [1]. Despite the fact that the period of greatest reproductive wastage in cattle occurs before  
43 Day 16 [1,2], the underlying molecular events that regulate early conceptus development up  
44 to the time of maternal recognition of pregnancy in cattle have not been clearly elucidated. It  
45 is clear, however, that the uterine endometrium plays a central role in early conceptus-  
46 maternal communication for establishment and maintenance of pregnancy. This involves  
47 dynamic changes in the uterine epithelium that are tightly regulated by changes in steroid  
48 hormones. The embryo leaves the oviduct and enters the uterus between Day 4 and 5 post  
49 fertilisation at the 8- to 16-cell stage [3]. From this point onwards until the start of  
50 implantation, which occurs around Day 19, the embryo is not attached in the uterus and is  
51 completely dependent on the uterine secretions for its further development [4,5]. As such it is  
52 of vital importance that the composition of the histotroph meets the requirements of a  
53 developing embryo. Indeed, changes in endometrial gene expression around this time, under  
54 the influence of progesterone, can lead to changes in the composition of the histotroph to  
55 which the developing conceptus is exposed [6,7]. The importance of histotroph for conceptus  
56 development has been demonstrated in the uterine gland knockout (UGKO) model in sheep  
57 in which embryos developed to Day 9 of gestation but then failed to develop beyond the  
58 blastocyst stage, i.e., Day 14, in adult UGKO ewes [8,9]. The process of conceptus  
59 elongation post-hatching is regulated mainly by histotroph-derived factors as evidenced by  
60 the fact that despite attempts to artificially induce this process, hatched bovine blastocysts fail  
61 to elongate *in vitro*, but will do so if transferred to the uterus of a recipient female [10,11].

62 The histotroph is composed of proteins, carbohydrates, sugars, lipids and ions produced by  
63 the endometrial glands that are necessary to sustain the conceptus. The protein components of  
64 the histotroph are important for conceptus-maternal interaction, specifically the processes of  
65 elongation of the trophoblast, recognition of pregnancy, implantation, and placentation  
66 [12,13]. Many components of the histotroph are secreted under the influence of progesterone  
67 and in the early luteal phase [14,15] also by oestradiol [16] but the optimum biochemical  
68 composition of the histotroph that supports the development of a healthy embryo/conceptus is  
69 not yet known. Recent studies have described the proteomic composition of uterine  
70 histotroph during the oestrous cycle and in comparison with plasma [17-19].

71 The objective of this study was to analyse the proteomic composition of uterine flushes of  
72 inseminated beef heifers with normal and degenerate embryos on Day 7 post insemination in  
73 order to elucidate what potential proteins are present in the uterus to support the embryo until  
74 blastocyst development. Our hypothesis is that there will be differences in composition  
75 between the animals with different types of embryos.

76

## 77 **1. Materials and Methods**

78 All experimental procedures involving animals were approved by the Animal Research  
79 Ethics Committee of University College Dublin and were licensed by the Department of  
80 Health and Children, Ireland, in accordance with the cruelty to animals act (Ireland 1876) and  
81 European Community Directive 86/609/EC.

82

### 83 *2.1 Animal management and treatments*

84 The experimental design used for this study has been described previously [20]. Cross-bred  
85 beef heifers, approximately 2 yrs old and weighing  $524 \pm 5.5$  kg housed in a slatted floor

86 facility in a commercial feedlot were used. All heifers were housed under the same  
87 management conditions with *ad-libitum* access to a total mixed ration designed to achieve an  
88 average live-weight gain of 1.3 kg/heifer/day. Oestrus (Day 0) was synchronised by insertion  
89 of a Controlled Internal Drug Release (CIDR, 1.36g Progesterone, Pfizer UK) device placed  
90 per vaginum for 8 days with a 2 mL injection of PGF2 $\alpha$  analogue (Prosolvin, Intervet Ireland  
91 Ltd., Dublin, Ireland) given on Day 7. Heifers were checked for signs of oestrus 4 times per  
92 day commencing 36 h after CIDR removal. Twelve to eighteen hours after onset of oestrus  
93 (Day 0) only those heifers recorded in standing oestrus within a narrow window were  
94 inseminated with frozen-thawed semen from a single ejaculate of a bull with proven fertility.  
95 Jugular blood samples were collected on Days 4, 6 and 7 post-oestrus from all heifers. Blood  
96 samples were stored at room temperature for 1 h and at 4°C for a further 16 h. Serum was  
97 decanted after centrifugation for 20 minutes at 1,600 x g and stored at -20°C until subsequent  
98 analyses. All heifers were slaughtered on Day 7 of pregnancy.

99

## 100 2.2 Progesterone assay

101 Serum progesterone concentrations were measured in all heifers on Days 4, 6 and 7 post  
102 oestrus using a time-resolved fluorescenceimmunoassay (FIA) with an AutoDELFIA™  
103 Progesterone kit (Perkin Elmer, Wallac Oy, Turku, Finland), as previously described (Carter  
104 *et al.* 2008). All samples were assayed within a single assay with a sensitivity of 0.01 ng/mL  
105 for the progesterone assay. The intra-assay coefficients of variation (% CV) were 4.6, 5.5 and  
106 4.6% for high, medium and low progesterone quality control sera, respectively.

107

## 108 2.3 Flush collection

109 Heifers from which an embryo was recovered were assigned to either (i) the viable group  
110 when the embryo was at the correct developmental stage for age (i.e. morula/early  
111 blastocyst), or (ii) the degenerate group when the embryo was arrested at the 2- to 16-cell  
112 stage. Heifers from which an unfertilised oocyte was recovered or from which no structure  
113 was recovered were omitted from the study. Within 30 min of slaughter the reproductive tract  
114 of all heifers was flushed with 20 mL of 10 mM Tris (pH 7.2, Sigma, Dublin, Ireland) by  
115 injecting this volume into the tip of the uterine horn and collecting it at the caudal end of the  
116 uterine body. All flushes were subsequently transported on ice to the laboratory and flushes  
117 were centrifuged at 4000 x g for 30min at 4°C prior to snap freezing in liquid nitrogen and  
118 storage at -80°C until further analysis.

119

#### 120 *2.4 Protein extraction from uterine flushes*

121 Proteins were extracted from six samples (three heifers per group) that were visibly free from  
122 blood (no red colour) using acetone precipitation as described previously [14]. Each sample  
123 was thawed on ice, split into 2 aliquots of 10 mL. Four volumes of ice cold acetone were  
124 added and samples stored at – 80 °C overnight. Samples were then thawed, centrifuged at  
125 4,000 x g for 30 min at 4 °C and the supernatant removed. The pellets were resuspended in  
126 100 µl of 100 mM Tris buffer, transferred into 1.5 mL Eppendorf tubes and sonicated briefly  
127 to aid resuspension. Samples were centrifuged at 12,000 x g for 30 min at 4 °C to remove  
128 insoluble material and the supernatant decanted and frozen at -80 °C for proteomic analysis.  
129 Total protein concentration of extracted uterine flush samples was determined using the  
130 Bradford assay, according to the manufacturers' protocol (Sigma Aldrich, Ireland).

131

132

133 *2.5 Protein digestion*

134 Extracted uterine proteins were vacuum dried and subjected to reduction and alkylation and  
135 proteolytic digestion as described by [21]. Cysteine residues were reduced by using 10 mM  
136 DTT in 100 mM ammonium bicarbonate for 1 h at 56 °C. The samples were alkylated with  
137 50 mM iodoacetamide in 100 mM ammonium bicarbonate for 30 min at room temperature  
138 and digested with sequencing grade modified porcine trypsin 100 ng (Sigma-Aldrich, Ireland)  
139 on a rotary shaker at 37 °C for 8 h. Samples were subsequently dried down and stored at -80  
140 °C until further analysis.

141

142 *2.6 Chromatography*

143 Proteolytic peptides were resuspended in 1 mL of strong cation exchange (SCX) buffer A (10  
144 mM K<sub>2</sub>PO<sub>4</sub>, pH 3.0 25% MeCN) and separated offline by strong cation exchange using a  
145 Dionex/LC Packings UltiMate. Samples were loaded onto a polysulfoethyl A column (The  
146 Nest Group, Southboro, MA, USA) and eluted with an increasing linear gradient (0% - 40%)  
147 of SCX buffer B (10 mM K<sub>2</sub>PO<sub>4</sub>, pH 3.0 + 25% MeCN with 600 mM KCl) over 70 min at a  
148 flow rate of 200 µl / min. Seventy SCX fractions of approximately 200 µl were collected into  
149 96-well microtitre plates. Eluted peptide fractions were pooled into 10 to 12 fractions  
150 according to the UV activity (214 nm) and desalted using Silica C18 columns (The Nest  
151 Group, Southborough, MA, USA), dried under vacuum and stored at -80 °C until further  
152 analysis.

153

154 *2.7 Proteomic analysis*

155 Tryptic peptides from pooled SCX fractions were resuspended in 1% ACN, 0.1% FA and  
156 analyzed on a Thermo Scientific LTQ linear ion trap mass spectrometer (Thermo Fisher

157 Scientific, Rockford, IL, USA) connected to a Surveyor, (Thermo Scientific) chromatography  
158 system with auto-sampler. Each sample was loaded onto a Biobasic C18 Picofrit™ column  
159 (100 mm length, 75 mm ID) and was separated by an increasing ACN gradient.  
160 Chromatography buffer solutions (Buffer A, 1% ACN, 0.1% formic acid; Buffer B, 100%  
161 ACN and 0.1% formic acid) were used to deliver a 72-min gradient (5 min sample loading,  
162 32 min to 40% Buffer B, 2 min to 80%, hold 11 min, 1 min to 0%, hold for 20 min, 1 min  
163 flow adjusting). A flow rate of 150 µl/min was used at the electrospray source.

164

### 165 *2.8 Database search and protein identification*

166 Protein identification was carried out using PEAKS (v 5.3). Parameters: enzyme, trypsin; two  
167 missing cleavages allowed; parent tolerance  $\pm 0.8$  Da and  $\pm 0.5$  Da for fragment ion masses;  
168 methionine oxidation and carbamidomethylation of cysteines were specified as variable  
169 modifications. MS/MS spectra were searched against the Uniprot-Swissprot/TrEMBL *Bos*  
170 *Taurus* v 7.6 database (25/04/12) containing 35,297 sequences.

171 Estimation of false positives was conducted by searching all spectra against decoy databases.  
172 The cut-off false discovery rate (FDR) for peptide spectrum matches was <1% and the  
173 maximum FDR observed for peptide sequences was 2.6 %. Only proteins with a PEAKS  
174 score of  $-10 \log P \geq 20$ , containing at least one unique peptide and only peptides containing  
175 an unbroken “b” or “y” ion series of a minimum of 4 amino acid residues were considered for  
176 further analysis.

177

### 178 *2.9 Gene Ontology (GO) and Ingenuity Pathway Analysis (IPA)*

179 GO analysis was carried out using AMIGO [22] (v1.8) and the DAVID (v6.7) bioinformatics  
180 resource [23,24] with Benjamini corrected and EASE score  $P$  values of  $< 0.05$  were  
181 considered significant, respectively.

182 For IPA (v9.0) analysis, the enriched protein data set containing Uniprot ID's and  
183 corresponding spectral count values was uploaded into the application. Each identifier was  
184 mapped to its corresponding object in the Ingenuity® Knowledge Base (build 171496;  
185 content version 14197757, release date 11-8-2012). These molecules, called Network Eligible  
186 molecules, were overlaid onto a global molecular network developed from information  
187 contained in the Ingenuity Knowledge Base. Networks of Network Eligible Molecules were  
188 then algorithmically generated based on their connectivity. Only IPA networks with a score  
189 of 4 or greater, equivalent to a significance value of  $P < 0.001$  were reported [25].

190

## 191 *2.10 Statistics*

192 Spectral counts (the number of mass spectra assigned to each protein) were normalised using  
193 the average total spectra obtained for each sample (4,228), an approach described and  
194 validated by [26] and utilised by several other studies [19, 27-30]. Due to non-normal  
195 distribution of spectral count data and to facilitate the use of parametric statistical analysis a  
196 fixed integer of 1 was added to all data values and subsequently log transformed. Both groups  
197 were then compared with each other using PROC MIXED (SAS v. 9.1; SAS Institute, Cary,  
198 NC, USA). Probability values less than or equal to 0.05 were considered significant.  
199 Correction for multiple testing was carried out using the Benjamini and Hochberg FDR as  
200 described previously [31].

201

202

## 203 **3 Results**

### 204 *3.1 Progesterone*

205 The progesterone concentration on Day 4 was  $0.43 \pm 0.05$  ng/mL for the degenerate group  
206 and  $0.52 \pm 0.04$  ng/mL for the viable group. On Day 5 the concentrations were  $0.95 \pm 0.09$   
207 ng/mL and  $0.99 \pm 0.06$  ng/mL respectively and on Day 7 they were  $1.03 \pm 0.1$  ng/mL versus  
208  $1.21 \pm 0.1$  ng/mL. There were no differences in area under the curve (AUC) for serum  
209 progesterone concentrations between both groups of heifers from Day 4 to Day 7.

210

### 211 *3.2 Protein identification*

212 To discern the most biologically pertinent proteomic profiles, and considering the low  
213 number of animals per group (n=3), thresholds were applied to characterise high confidence  
214 targets present in the viable or degenerate groups. These included: 1) signal presence in all  
215 three animals in either group; or 2) identification with at least one unique peptide per sample  
216 (as mentioned above); similar criteria as utilized in previous studies [30, 32, 33]. This  
217 classification identified a total of 40 high confidence proteins; 26 proteins in the viable group,  
218 10 in the degenerate group and 4 common to both groups (Table 1; Supplemental Table 1).

219 Five proteins were identified as more abundant in the viable compared with the degenerate  
220 group (Table 1; Supplemental Table 1) which included Platelet-activating factor  
221 acetylhydrolase 1b, catalytic subunit 3 (PAFAH1B3), Tubulin, beta 4A class IVa (TUBB4A),  
222 Tubulin, alpha 1d (TUBA1D), Cytochrome c-1 (CYC1) and dihydropyrimidinase-like 2  
223 (DPYSL2). One protein, S100 calcium binding protein A4 (S100-A4), was significantly  
224 increased in histotroph from the degenerate group but failed to maintain significance after  
225 multiple testing correction. However, it was retained in the subsequent analysis and

226 discussion to avoid loss of pertinent information and as prior information on its abundance in  
227 uterine flushes on Day 7 in cattle has been reported [19].

228

### 229 *3.3 Characterization of Day 7 histotroph proteomes*

230 GO slim analysis of the n=40 high confidence targets identified in histotroph on Day 7  
231 showed all GO terms identified, with the exception of cell death, were associated with more  
232 proteins in the viable compared with the degenerate group. The biological processes with the  
233 largest number of proteins associated were response to stress, cellular component assembly  
234 and macromolecular complex assembly (n=9), the latter two processes only associated with  
235 proteins identified from the viable group. In addition, three other processes were unique to  
236 the viable group and include protein complex assembly, cytoskeletal organization and cell  
237 cycle (Figure 1).

238

### 239 *3.4 IPA analysis*

240 IPA identified one network associated with proteins differentially expressed in histotroph  
241 between the viable and degenerate groups (n=6) on Day 7 involved in cellular assembly and  
242 organisation; cellular function and maintenance; and cell morphology (Figure 2).

243

## 244 **4. Discussion**

245 The aim of this study was to analyse the proteomic composition of uterine flushes of beef  
246 heifers with normal and degenerate embryos on day 7 post insemination in order to provide  
247 insights into what proteins are present in the uterus to support the embryo until its successful  
248 development as blastocyst. In this study we identified 40 proteins in histotroph collected on

249 Day 7 and these proteins were associated with a range of biological processes amongst which  
250 response to stress, cellular component assembly and macromolecular complex assembly. The  
251 latter two processes were only associated with proteins identified from the viable group. In  
252 addition, three other processes were unique to the viable group and include protein complex  
253 assembly, cytoskeletal organization and cell cycle. This was not unexpected given the  
254 number of proteins identified in the viable group was over double that identified in the  
255 degenerate group and suggests a greater provision of functionality in histotroph supportive of  
256 early embryo development. The results of the GO analyses were consistent with the network  
257 analysis identifying a significant relationship with cellular assembly and organisation;  
258 cellular function and maintenance; and cell morphology. GO analysis also identified immune  
259 system processes associated with the Day 7 proteome which could be associated with the fact  
260 that at a later stage of pregnancy embryo development is dependent on a tight regulation of  
261 the maternal immune system [34-36] with expression of components of the immune system  
262 in the endometrium and uterine histotroph implicated with successful or unsuccessful  
263 embryonic development. We recently found that a decreased expression of genes involved in  
264 the regulation of the immune response in the endometrium of heifers from which a viable  
265 embryo was recovered [20, 37], which is also supported by the suggestions and findings of  
266 Hansen [38] that the regulation of the uterine immune response is precise and that subtle  
267 changes can change the outcome of the developing embryo. The establishment of receptivity  
268 of the uterine luminal epithelium (LE) to the developing conceptus and the key role in  
269 regulating differentiated functions of the uterine glandular epithelium (GE) is very much  
270 regulated via indirect effects of progesterone on the endometrium [6, 39, 40], with the up and  
271 down regulation of the different genes being tightly regulated and a minor disruption of this  
272 regulation having major consequences on conceptus survival.

273

274 Although only six proteins were identified as differentially expressed between the groups,  
275 their presence in histotroph, during this critical time period may indicate an important role  
276 during early embryo development. Indeed, PAFAH1B3, unique to histotroph recovered from  
277 the viable group, belongs to the group of the Platelet-activating factors (PAF) that are one of  
278 the most potent phospholipids involved in a variety of physiological events including  
279 biological processes pre- and post-fertilisation such as spermatozoal function, fertilization,  
280 embryo development and implantation [41, 42]. In male reproduction PAF increases the  
281 sperm motility and improves the acrosome reaction [43], while in female reproduction the  
282 protein is secreted by pre-implantation embryos of a number of species and its secretion  
283 appears to be positively correlated with the viability of human embryos produced by IVF [44]  
284 PAF antibody inhibits mouse pre-implantation embryo development [45] and platelet  
285 activating factor produced by the rabbit embryo has been shown to increase during the pre-  
286 implantation phase [46].

287 The function of Tubulins, a group of proteins to which both TUBB4A and TUBA1D belong,  
288 in reproduction is not clear although these proteins have been found in the flagellum of  
289 mouse sperm where it appears to be located in the midpiece and terminal piece, as well as in  
290 the testes [47]). Tubulin, however, is more widely described in relation to cancer research.  
291 The protein can be found in the nucleus of cells and in mitochondria and downstream events  
292 that result from tubulin binding are critical events for the generation of apoptosis in malignant  
293 cells [48]. The identification of two members of the tubulin family unique to histotroph  
294 recovered from the viable embryo group during a stage of rapid embryo growth may support  
295 a role for these proteins in the regulation of cell proliferation and successful blastocyst  
296 development. Indeed, TBA1D has previously been identified in histotroph from highly fertile  
297 dairy cattle on Day 7 [19].

298 CYC1, also previously identified in histotroph on both Day 7 and Day 13 post oestrus by  
299 Mullen *et al.* [19], is a component of the electron transport chain in mitochondria. The protein  
300 is associated with the inner membrane of the mitochondrion and is involved in initiation of  
301 apoptosis when it is released. Li et al (2000) [49] report that cells lacking CYC1 show  
302 reduced caspase-3 activation and are resistant to the proapoptotic effects of UV irradiation  
303 and serum withdrawal. However, cells lacking CYC1 appear to demonstrate increased  
304 sensitivity to cell death signals triggered by TNF $\alpha$ . As such lack of CYC1 can lead to an  
305 altered stress induced apoptotic response. CYC1 is an essential component of an apoptotic  
306 pathway responsive to DNA damage and other forms of cell stress and interestingly mouse  
307 embryos that lack CYC1 die *in utero* by mid gestation [49]. The fact that this protein was  
308 more abundant in flushes from uteri that yielded a viable embryo indicates this protein may  
309 be important for early embryo survival. While CYC1 is typically classified as non-secretory,  
310 bioinformatic analysis using Secretome P predicted CYC1 to be secreted non classically (data  
311 not shown).

312

313 The remaining protein more abundant in the flushes of uteri of which a viable embryo was  
314 recovered was DPYSL2. DPYSL2 has been described in relation to neuronal development  
315 and axon growth, the family of dihydropyrimidinases is known to have a role in growth and  
316 development and deficiency of this protein in humans can lead to abnormalities of both a  
317 neurological and gastrointestinal nature [50]. DPYSL2 has also been identified as one of the  
318 proteins that are part of a group that appears to be a specific target of protein carbonylation.  
319 In the brain the carbonylation of DPYSL2 leads to shortening of dendritic length with  
320 consequent decreased interneuronal communication. In general, protein carbonyl content is  
321 the most general and well-used biomarker of severe oxidative protein damage in many human  
322 diseases. The role of reactive oxygen species (ROS) that cause this protein damage has

323 become more apparent in many disease processes and ROS have the potential to induce  
324 significant biological damage to cells [51]. Under physiological conditions, there is an  
325 established balance between formation and neutralisation of ROS, but this fine balance is  
326 disrupted, for instance by disruption in the anti-oxidant defence mechanism of the cell,  
327 oxidative stress and hence damage to the cell can occur. Protein carbonyls have been found in  
328 both placentas and decidua of women that suffered from pre-eclampsia, suggesting a role of  
329 damage done by ROS in this disorder [52]. The increased abundance of DPYSL2 in heifers  
330 from which subsequently a viable embryo was recovered suggests it might play a role in  
331 embryo protection regulating the oxidative damage at this stage of early pregnancy. Indeed,  
332 DPYSL2 has been reported approximately 3-fold more abundant on Day 7 compared with  
333 Day 13 [19], which may indicate a stage specific requirement for this protein during early  
334 embryo development.

335 Only one protein was identified as more abundant in the histotroph of heifers yielding  
336 degenerate embryos compared with viable embryos, S100-A4. We have previously identified  
337 S100-A4 unique to Day 7 uterine flushes compared with those on Day 13 [19]. S100A4 is a  
338 protein involved in the regulation of a number of cellular processes such as cell cycle  
339 progression and differentiation [53]. The protein belongs to a group of calcium binding  
340 proteins that tend to be highly expressed in pathological conditions. The group of S100  
341 calcium binding proteins has been associated with a number of aspects of the interaction  
342 between cancer cells and stromal cells, and contributes to the formation of an inflammatory  
343 tumor microenvironment [54]. It has also been associated with cancer cells and appears to  
344 contribute to the motility of tumor cells and as such the progression of metastasis [55, 56].  
345 The relative increase in abundance of S100-A4 in the flushes of animals that yielded a  
346 degenerate embryo may indicate a negative impact of excess S100-A4 on early embryo

347 development perhaps through the anti- and pro-inflammatory processes that occur during the  
348 pre-implantation phase as we previously hypothesised [37].

349 In conclusion, we identified 40 proteins in histotroph collected from the uterus on Day 7 of  
350 pregnancy from heifers that were inseminated and included five proteins more abundant in  
351 histotroph collected from animals with a viable embryo and one protein more abundant in the  
352 histotroph collected from animals with a degenerate embryo. While we are aware of the  
353 limited numbers per group and difficulties associated with any secretome analysis such as the  
354 potential for cellular contamination, in our opinion, these data may consist of markers of  
355 successful early embryo development and warrant further investigation. In support of these  
356 findings several proteins have previously been identified as expressed in the endometrium [6,  
357 57] and histotroph [19] by our group on Day 7 in cattle. Of particular interest, one protein  
358 PA1B3, belongs to the groups of platelet activating factors which are known to be very  
359 important for the development of pre-implantation embryos in other species, but to our  
360 knowledge has not been reported in relation to bovine early embryonic development and may  
361 warrant further investigation.

362

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371

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544 Table 1. 40 proteins identified on Day 7 of pregnancy in histotroph from beef heifers yielding viable or degenerate embryos.

Uniprot ID	Mass (Da)	Protein description <sup>a</sup>	Biological Function <sup>b</sup>	Viable				Degenerate			P value		
				86	144	177	Mean	18	205	203	Mean	LS	FDR
PA1B3_BOVIN	25865	Platelet-activating factor acetylhydrolase IB subunit gamma	multicellular organism reproduction	6	4	6	5 ± 1	0	0	0	0 ± 0	<.0001	0.000
TBA1D_BOVIN	50283	Tubulin alpha-1D chain	protein complex assembly	6	16	23	15 ± 5	0	0	0	0 ± 0	0.002	0.030
DPYL2_BOVIN	62278	Dihydropyrimidinase-related protein 2	differentiation	17	20	17	18 ± 1	0	3	0	1 ± 1	0.006	0.064
TBB4A_BOVIN	49586	Tubulin beta-4A chain	microtubule cytoskeleton organization	6	45	6	19 ± 13	0	0	0	0 ± 0	0.015	0.095
CYC_BOVIN	11704	Cytochrome c	generation of precursor metabolites and energy	6	57	46	37 ± 16	0	1	0	0 ± 0	0.013	0.102
S10A4_BOVIN	11807	Protein S100-A4	Regulation of kappa kinase cascade	6	8	6	7 ± 1	22	9	29	20 ± 6	0.054	0.293
TKT_BOVIN	67906	Transketolase	regulation of growth	6	8	23	12 ± 6	0	3	5	2 ± 1	0.105	0.418
HBA_BOVIN	15184	Hemoglobin subunit alpha	gas transport	153	201	209	187 ± 17	1458	340	320	706 ± 376	0.102	0.467
TRFE_BOVIN	77753	Serotransferrin	transition metal ion transport	232	197	133	187 ± 29	123	119	158	134 ± 12	0.161	0.470
GDIR1_BOVIN	23421	Rho GDP-dissociation inhibitor 1	intracellular signaling cascade	0	6	17	8 ± 5	45	24	16	28 ± 9	0.135	0.479
PRDX1_BOVIN	22210	Peroxiredoxin-1	response to reactive oxygen species	11	25	17	18 ± 4	0	9	8	6 ± 3	0.153	0.491
ACBP_BOVIN	10044	Acyl-CoA-binding protein	transport	0	142	0	47 ± 47	11	193	76	94 ± 53	0.267	0.534
LDHB_BOVIN	36724	L-lactate dehydrogenase B chain	response to reactive oxygen species	6	4	17	9 ± 4	0	0	11	4 ± 4	0.222	0.545
SERA_BOVIN	56452	D-3-phosphoglycerate dehydrogenase	glutamine metabolic process	6	16	12	11 ± 3	0	12	6	6 ± 3	0.312	0.554
TBA1C_BOVIN	49857	Tubulin alpha-1C chain	protein complex assembly	6	16	23	15 ± 5	0	21	0	7 ± 7	0.212	0.566
ENOA_BOVIN	47326	Alpha-enolase	glycolysis	0	6	57	21 ± 18	22	22	36	27 ± 4	0.336	0.566
TBB5_BOVIN	49671	Tubulin beta-5 chain	microtubule cytoskeleton organization	6	45	6	19 ± 13	0	25	0	8 ± 8	0.301	0.567
A1AG_BOVIN	23182	Alpha-1-acid glycoprotein	acute inflammatory response	45	4	17	22 ± 12	0	9	8	6 ± 3	0.266	0.568
TBA1B_BOVIN	50152	Tubulin alpha-1B chain	microtubule cytoskeleton organization	6	16	23	15 ± 5	0	0	26	9 ± 9	0.251	0.573
TERA_BOVIN	89330	Transitional endoplasmic reticulum ATPase	DNA damage and repair	11	8	12	10 ± 1	0	7	15	7 ± 4	0.396	0.576
NDKB_BOVIN	17316	Nucleoside diphosphate kinase B	apoptosis regulation	0	34	34	23 ± 11	45	44	23	37 ± 7	0.363	0.581

Uniprot ID	Mass (Da)	Protein description <sup>a</sup>	Biological function <sup>b</sup>	Viable <sup>c</sup>				Degenerate <sup>c</sup>				P value	
				86	144	177	Mean <sup>d</sup>	18	205	203	Mean <sup>d</sup>	LS	FDR
TPM3_BOVIN	32819	Tropomyosin alpha-3	No information	6	4	6	5 ± 1	0	3	8	4 ± 2	0.382	0.582
PPIA_BOVIN	17869	Peptidyl-prolyl cis-trans isomerase A	protein folding	0	74	51	42 ± 22	56	25	47	43 ± 9	0.528	0.604
APOA1_BOVIN	30276	Apolipoprotein A-I	regulation of cytokine production	6	29	6	13 ± 8	0	14	11	9 ± 4	0.520	0.617
IDHC_BOVIN	46785	Isocitrate dehydrogenase [NADP] cytoplasmic	cellular aldehyde metabolic process	0	23	6	9 ± 7	22	5	10	12 ± 5	0.505	0.622
HS90A_BOVIN	84731	Heat shock protein HSP 90-alpha	regulation of nitric oxide biosynthetic process	40	29	17	29 ± 6	0	46	61	36 ± 18	0.644	0.624
PNPH_BOVIN	32037	Purine nucleoside phosphorylase	nucleoside metabolic process	23	25	12	20 ± 4	0	16	31	15 ± 9	0.454	0.632
HBB_BOVIN	15954	Hemoglobin subunit beta	gas transport	210	135	342	229 ± 61	482	265	153	300 ± 97	0.613	0.633
ALDR_BOVIN	35919	Aldose reductase	oxidation reduction	11	61	81	51 ± 21	0	35	115	50 ± 34	0.595	0.635
B2MG_BOVIN	13677	Beta-2-microglobulin	regulation of leukocyte mediated cytotoxicity	11	0	11	8 ± 4	11	1	27	13 ± 8	0.675	0.635
TPIS_BOVIN	26690	Triosephosphate isomerase	monosaccharide metabolic process	28	12	12	17 ± 6	0	25	31	19 ± 9	0.635	0.635
HSP7C_BOVIN	71241	Heat shock cognate 71 kDa protein	protein foldin	17	25	52	31 ± 11	0	48	45	31 ± 16	0.579	0.639
HS90B_BOVIN	83253	Heat shock protein HSP 90-beta	placenta development	23	33	29	28 ± 3	0	35	44	26 ± 13	0.505	0.647
PRDX2_BOVIN	21946	Peroxiredoxin-2	MAPKKK cascade	0	23	11	11 ± 7	11	12	16	13 ± 2	0.488	0.651
TBB4B_BOVIN	49831	Tubulin beta-4B chain	protein polymerisation	6	45	6	19 ± 13	0	25	24	16 ± 8	0.757	0.654
KCRB_BOVIN	42719	Creatine kinase B-type	Ion homeostasis	23	4	29	19 ± 8	0	17	44	20 ± 13	0.717	0.656
AMPN_BOVIN	109276	Aminopeptidase N	angiogenesis	6	25	41	24 ± 10	0	38	47	28 ± 14	0.749	0.666
HSPB1_BOVIN	22393	Heat shock protein beta-1	response to temperature stimulus	0	40	17	19 ± 12	34	5	6	15 ± 9	0.864	0.728
ZA2G_BOVIN	33852	Zinc-alpha-2-glycoprotein	immune response	102	0	17	40 ± 32	11	4	19	12 ± 4	0.923	0.738
ALBU_BOVIN	69294	Serum albumin	cytolysis by symbiont of host cells	2180	1331	916	1476 ± 372	1693	1090	1294	1359 ± 177	0.905	0.743

545 <sup>a</sup>Proteins are listed with their Uniprot ID and description. <sup>b</sup>Functional annotation was performed using DAVID. <sup>c</sup>Spectral counts (the number of mass spectra assigned to each  
546 protein) reported normalised counts. <sup>d</sup>Mean ± s.e.m.

547 Figure 1. Gene Ontology (GO) slim terms and the numbers of proteins associated with each term  
548 on Day 7 in high confidence datasets (i.e. proteins (n=40) identified in at least all three animals in  
549 either viable or degenerate groups).

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551 Figure 2. Ingenuity pathway interaction network analysis. Proteins more abundant in histotroph in  
552 viable compared with degenerate groups on Day 7 post insemination involved in cellular assembly  
553 and organisation; cellular function and maintenance; and cell morphology (n=6),  $P < 10^{-15}$ . The  
554 network displays nodes (genes/gene products) and edges (the biological relationship between  
555 nodes). The colour intensity of the nodes indicates the spectral count increase associated with a  
556 particular protein in histotroph from the viable group (red) or the degenerate group (green) on Day  
557 7. A solid line indicates a direct interaction between nodes (genes/gene products) and a dashed line  
558 indicates an indirect relationship between nodes. The shape of the node is indicative of its  
559 function.

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