Proteins involved in embryo-maternal interaction around the signalling of maternal recognition of pregnancy in the horse

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During maternal recognition of pregnancy (MRP), a conceptus-derived signal leads to the persistence of the corpus luteum and the maintenance of gestation. In the horse, the nature of this signal remains to be elucidated. Several studies have focused on the changes in gene expression during MRP, but little information exists at the protein level. The aim of this study was to identify the proteins at the embryo-maternal interface around signalling of MRP in the horse (day 13) by means of mass spectrometry.

A distinct influence of pregnancy was established, with 119 proteins differentially expressed in the uterine fluid of pregnant mares compared to cyclic mares and with upregulation of several inhibitors of the prostaglandin synthesis during pregnancy. By creating an overview of the proteins at the embryo-maternal interface in the horse, this study provides a solid foundation for further targeted studies of proteins potentially involved in embryo-maternal interactions, MRP and pregnancy loss in the horse.

Maternal recognition of pregnancy (MRP) covers the series of events leading to the persistence of the corpus luteum and a receptive uterine environment to support the maintenance of gestation\textsuperscript{1}. In the cycling mare, pulsatile release of prostaglandin F\textsubscript{2α} (PGF\textsubscript{2α}) causes luteolysis, resulting in a decline in progesterone. This mechanism is inhibited during pregnancy by the presence of the conceptus\textsuperscript{2}. In pigs, the conceptus derived signal which initiates MRP has been identified a long time ago as oestrogen\textsuperscript{3} and in ruminants as interferon tau\textsuperscript{4,5}. However, the nature of this signal remains to be elucidated in the horse despite several decades of elaborate research on this topic\textsuperscript{6,7}. Initial studies have focused on the identity of specific candidate signalling molecules and while the equine embryo produces substantial quantities of oestrogen as well as prostaglandins (PG) and limited amounts of interferons, no convincing evidence exists for their signalling role in MRP\textsuperscript{7}. Potential embryonic signal targets involved in the luteostatic mechanism in the horse are prostaglandin-endoperoxide synthase 2 (PTGS2), an enzyme in the biosynthesis of PGF\textsubscript{2α}, and oxytocin, which stimulates endometrial PGF\textsubscript{2α} secretion through a positive feedback loop\textsuperscript{8}. Both PTGS2 and oxytocin receptor expression (OXTR) are repressed during early pregnancy compared to cycling mares, with downregulation of PTGS2 at the RNA level and of OXTR at the protein level\textsuperscript{9–13}.

During the last years, the topic of MRP in the horse has been broadened to all pathways involved in embryo-maternal communication around the timing of MRP. Signalling of MRP is a continuum of events, estimated to occur between days 12 and 14. Recipient mares can still get pregnant when an embryo is transferred to their uterus at day 12, but not at day 14 after ovulation\textsuperscript{14}, while repression of PTGS2 occurs by day 13 of pregnancy\textsuperscript{11}. By day 16, clear differences between pregnant and cyclic horses are observed. Transcriptomics of the equine endometrium and equine conceptuses have substantially contributed to the knowledge on pathways affected around the timing of MRP in the horse\textsuperscript{14–19}. Technological advantages, including sequencing, favoured development of genomics and transcriptomics compared to proteomics\textsuperscript{20}. However, mRNA abundances can only

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explain 40% of the variation in protein levels and the actual protein profile is influenced by post-transcriptional regulation mechanisms. This appeals for complementing transcriptomics knowledge on MRP with quantitative proteomics. This can now be achieved through mass spectrometry (MS). Recent improvements in MS technologies, including data-independent acquisition, allow reproducible label-free quantification of proteins in complex biological samples.

Mass spectrometry of the embryo-maternal interface around MRP has been performed in several farm animals including pigs, sheep and cattle. In the horse, specific molecules with a potential role in MRP have been targeted by immunohistochemistry and global screening of uterine proteins has been performed in the context of endometritis. However, the effect of pregnancy on the uterine secretome has not been assessed by means of high-throughput proteomics in the horse up to now. In a recent study, equine blastocysts were collected by uterine lavage on day 8 and an MS analysis was performed of the proteins secreted during culture of these embryos for 24 h and 48 h and of proteins present in the blastocoeel fluid and the embryo capsule. The authors detected prostaglandin F2 receptor inhibitor (PTGFRN) and a progesterone potentiating protein, FK506 binding protein 4 (FKBP4), in the blastocoeel fluid, but it remained to be determined whether these proteins were actively secreted into the uterine lumen.

The aim of this study was to gain new insights into the embryo-maternal communication around the signaling of MRP in the horse. Since signalling of MRP is estimated to occur between Day 12 and Day 14, sampling was performed at Day 13 (±0.5 day). We hypothesize that high-throughput proteomics can provide complementary information to the transcriptomic reports. To this end, proteomics was performed by high definition data independent mass spectrometry (HDMSE) with ion mobility drift time-specific collision-energy. In this way, proteins were identified and quantified in uterine fluid of pregnant and cyclic mares as well as in the yolk sac fluid of the pregnant mares.

### Results

#### Sampling.

Only reproductively sound mares with negative bacteriology and cytology of the uterine fluid were used for the sampling. In two cycles, namely one pregnant (P) and one control cyclic (C) cycle, a double ovulation occurred. Response to hCG resulted in ovulation 24–36 h after administration. In four cycles, ovulation only occurred 3 days after hCG; once in a P cycle, where artificial insemination (AI) was performed at the same time and in this case, the mare was inseminated a second time 48 h after the first time and she ovulated the day after. In all other P cycles, ovulation occurred within 48 h after AI. In one mare, a line of fluid was noticed by ultrasound of the uterus 1 day after AI and she was treated by intramuscular administration of oxytocin.

#### Identification of proteins.

The average protein concentration was similar in the uterine fluid (UF) of P (9.2 g/mL) and C (9.8 g/mL) mares, while the average protein concentration in the yolk sac (YS) was only 7.8 g/mL.

For the first time, an overview was created of the proteins present in the UF and the YS at day 13 after ovulation in the horse. In the UF samples, a total of 10,489 peptides were identified, accounting for 41% of all peptide like ions. Protein identification resulted in 11,531 identifiable proteins (Supplementary file 1). After filtering and normalization, a total of 707 normalized proteins with at least two unique peptides were assessed for differential expression.

Differential expression of proteins was assessed for P versus C mares and pregnancy was associated with upregulation of 62 proteins (Table 1) and downregulation of 57 proteins (Table 2). For all proteins in this comparison, the log fold change, the adjusted p-value and the number of peptides are listed in Supplementary file 3.

In the YS samples, a total of 6,500 peptide ions were identified, representing 51% of all peptide like ions and resulting in 903 identifiable proteins (Supplementary file 2). For the YS proteins, the primary goal was identification, rather than quantification, as different nature of the fluids impedes assessment of differential expression of proteins in YS versus UF.

#### Gene Ontology enrichment and pathway analysis.

Categorization in the Gene Ontology (GO) terms ‘molecular function’, ‘biological process’ and ‘cellular component’ is provided for all quantified proteins in the comparison of P versus C in Supplementary file 3.

Figure 1 summarizes the GO categories in which the differentially expressed proteins are involved. The main category to which most proteins are assigned is ‘cellular process (GO:0009987)’ for the biological processes and ‘binding (GO:0005488)’ for the molecular functions. This coincides with the results in porcine uterine fluid, but these are also the major categories when all proteins are taken into account. Overall, the differences in categorization between the groups are small.

Gene Ontology (GO) enrichment revealed no statistical overrepresentation when a Bonferroni correction for multiple testing was used (FDR < 0.05). No up- or downregulated KEGG pathways were detected either at a Benjamini-Hochberg corrected p-value of 0.05.

#### Embryo-maternal interaction.

Comparison of the proteins identified in the UF of the P mares and in the YS of the corresponding embryo revealed 347 common proteins, 806 proteins which were only detected in the UF and 556 proteins which were only found in the YS. Figure 2 represents an overview of these UF specific proteins, YS specific proteins and common proteins, with specific display of the proteins up- and downregulated during pregnancy and of the proteins categorized in the extracellular space.

A list of the 347 common proteins is provided in Supplementary file 4, including the functions in which these proteins are involved. Figure 3 summarizes the GOs in which these common proteins were involved. Similar to the results for the UF in Fig. 1, the main GO categories in which the common proteins are involved are also ‘cellular process (GO:0009987)’ and ‘binding (GO:0005488)’ and differences in categorization are small. Common proteins in YS and UF which were also upregulated in P versus C, showed a higher representation in the biological
| Protein Symbol | Log FC | Adj. p-value | Gene Symbol | Gene Description |
|----------------|-------|--------------|-------------|------------------|
| F7BAa0         | 2.27  | 0.03617      | GSTO1*      | Glutathione S-transferase omega 1 |
| F6z2a9         | 2.02  | 0.01980      | RAC1*       | Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1) |
| F6vvu1; F6yMx5 | 1.96  | 0.02370      | MOb1A*      | MOb kinase activator 1A |
| F6y2h3; F6y2v7 | 1.89  | 0.02907      | PEPD*       | Peptidase D |
| F7ccf5         | 1.81  | 0.01425      | Lxn*        | Latanix |
| P7di3          | 1.80  | 0.03253      | Sec14L3*    | Sec14 like lipid binding 3 |
| F6yaz9; F6yz9  | 1.79  | 0.01633      | Myl12a*     | Myosin light chain 12A |
| F6RH25         | 1.55  | 0.01371      | DCPS*       | Decapping enzyme, scavenger |
| F6y3v0         | 1.55  | 0.01980      | TBCA*       | Tubulin folding cofactor A |
| Q3s4d6         | 1.53  | 0.01915      | Gm2a*       | Gm2 ganglioside activator |
| F6Xki9         | 1.40  | 0.01142      | Dntt1ip2*   | DNA nucleotidetransferase terminal interacting protein 2 |
| F6rth0         | 1.39  | 0.01378      | TXNdc17*    | Thioredoxin domain containing 17 |
| F7cb0n         | 1.35  | 0.00336      | Akr1a1*     | Aldo-keto reductase family 1 member A1 |
| F6Pwc8         | 1.25  | 0.00112      | Ptgri1*     | Prostaglandin reductase 1 |
| F6xsn2         | 1.24  | 0.00106      | Cct7*       | Chaperonin containing TCP1 subunit 7 |
| F6xzq1         | 1.24  | 0.00026      | Caps*       | Calcyphosine |
| F7bar2         | 1.23  | 0.02275      | Tpmt*       | Thiopurine 3-methyltransferase |
| F6W8c8         | 1.20  | 0.00004      | SerpinB6*   | Serpin family B member 6 |
| F6Rgn2         | 1.19  | 0.04597      | Fab5*       | Fatty acid binding protein 5 |
| F6Rmm1         | 1.17  | 0.00336      | Sh3bgrl*    | Sh3 domain binding glutamate rich protein like |
| F7cb0r; F7dzd2 | 1.11  | 0.00336      | Loc100050322| Glutathione S-transferase |
| F7bhv8         | 1.11  | 0.00626      | Tub4a*      | Tubulin beta 4a class IVa |
| Q7Hzm6; F7a0t0| 1.09  | 0.00106      | Anxa1*      | Annexin A1 |
| F6Xa04         | 1.06  | 0.00001      | Ywhae*      | Tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein epsilon |
| F6xty8         | 1.05  | 0.02335      | Unassigned  | Unassigned |
| F7D3e3         | 1.03  | 0.00004      | Cmpk1*      | Cytidine/uridine monophosphate kinase 1 |
| F6W6b3         | 0.99  | 0.03617      | Gmd5*       | GDP-mannose 4,6-dehydratase |
| F7Db59         | 0.99  | 0.00106      | Pafah1b3*   | Platelet activating factor acetylhydrolase lb-catalytic subunit 3 |
| F6sQ49         | 0.97  | 0.00112      | Sms*        | Spermine synthase |
| F6rl46         | 0.96  | 0.01211      | Plgs*       | 6-Phosphogluconolactonase |
| F6W9b1         | 0.93  | 0.001371     | St13*       | Suppression of tumorigenicity 13 (colorectal carcinoma) (hsp70 interacting protein) |
| F7e0h3         | 0.91  | 0.01211      | Tubb4a*     | Tubulin beta class I |
| F6r8t8         | 0.90  | 0.01473      | Acy1*       | Aminoacylase 1 |
| F7D9j2         | 0.90  | 0.00053      | Tkt*        | Transketolase |
| F6W039         | 0.85  | 0.00024      | Arhgdia*    | Rho GDP dissociation inhibitor alpha |
| F6Zhq5         | 0.83  | 0.00336      | Clc1*       | Chloride intracellular channel 1 |
| F7D1r1         | 0.82  | 0.01371      | Pgtk1*      | Phosphoglycerate kinase 1 |
| F6Ts99         | 0.77  | 0.01633      | Tpi1*       | Triosephosphate isomerase 1 |
| F7C1x6         | 0.76  | 0.00591      | Eno2*       | Enolase 2 |
| F6W3t1         | 0.73  | 0.00106      | LdhA*       | Lactate dehydrogenase A |
| F7csg3         | 0.73  | 0.01378      | Psmd11*     | Proteasome 26s subunit, non-ATPase 11 |
| F6Ze54         | 0.72  | 0.04589      | Gpi*        | Glucose-6-phosphate isomerase |
| F7Bbw6         | 0.71  | 0.02824      | Vcp*        | Valosin containing protein |
| F7Czs6         | 0.71  | 0.01052      | Mdh1*       | Malate dehydrogenase 1 |
| F6Pi2         | 0.71  | 0.04256      | Lztrfl1*    | Leucine zipper transcription factor like 1 |
| F7Dmy1         | 0.70  | 0.04029      | Cbfb*       | Core-binding factor beta subunit |
| F6U3s5         | 0.69  | 0.02943      | Pfn1*       | Profilin |
| F6vsn2         | 0.69  | 0.00556      | Gstp1*      | Glutathione S-transferase pi 1 |
| F7Dxg8         | 0.69  | 0.01915      | Cfl1*       | Coltin 1 |
| F7Aps1; F6zws7 | 0.68  | 0.00106      | Cstb; Loc100050835* | Cystatin B |
| F7Be95; F6u2p8 | 0.67  | 0.03059      | Ube2v1*     | Ubiquitin conjugating enzyme E2 V1 |
| F7Alv0         | 0.67  | 0.00336      | Txn*        | Thioredoxin |
| F7Bpt4         | 0.61  | 0.00336      | Ezr*        | Ezrin |

Continued
processes ‘developmental process (GO:0032502)’ and ‘response to stimulus (GO:00050896)’. Molecular functions in which these proteins were more involved are ‘transporter activity (GO:0005215)’ and ‘transcription factor activity - protein binding (GO:0000988)’, while the common proteins which were downregulated in P vs C were rather represented in ‘structural molecule activity (GO:0005198)’.

The embryo-maternal interaction was further visualized by Cytoscape 3.3.0 in Fig. 4. The most prominent GO terms in this network are ‘embryo development’ (GO:0009790) and ‘embryo morphogenesis’ (GO:0048598), with a main contribution of proteins originating from the yolk sac, and embryo implantation (GO:0007566) with the involvement of both uterine and embryonic proteins. In Fig. 5, the contribution of growth factors and cytokines in equine embryo-maternal signalling is visualized. The most extensive networks with various proteins found in the yolk sac fluid and/or the uterine fluid of pregnant mares are involved in ‘regulation of cytokine production’ (GO:0001817), ‘response to cytokine’ (GO:0034097) and the downstream GO’s ‘cytokine receptor binding’ (GO:0005126), ‘cytokine mediated signalling pathway’ (GO:0019221) and ‘regulation of response to cytokine stimulus’ (GO:0006759).

**Discussion**

Maternal recognition of pregnancy is an intriguing subject in the horse and extensive research on the molecular processes involved has been performed in the field of transcriptomics. However, information on the downstream translation to proteins is scarce. In this study, quantitative proteomics of the uterine luminal fluid assessing the effect of pregnancy was performed for the first time in the horse. At the same time, proteins in the embryonic yolk sac fluid were mapped to provide insight into the embryo-maternal interaction.

With 119 proteins differentially expressed in the uterine fluid of P vs C mares, a distinct influence of pregnancy was established. In general, a function of more than 40% of the differentially expressed proteins in the UF was categorized as ‘binding (GO:0005488)’, coinciding with the findings in pigs and cattle, where the majority of proteins were also allocated to molecular binding processes. ‘Binding’ also represents the main category to which the common proteins in UF and YS were allocated, with subtly higher representation of proteins upregulated during pregnancy in categories linked to embryo-maternal interaction, namely ‘developmental process (GO:0032502)’, ‘response to stimulus (GO:00050896)’, ‘transporter activity (GO:0005215)’ and ‘transcription factor activity - protein binding (GO:0000988)’ at the expense of the more general GO term ‘structural molecule activity (GO:0005198)’. Cellular component categorization allocated 45% of the identified UF proteins to the extracellular space (Fig. 2). This coincides with the findings of Swegen et al., who specifically targeted secreted proteins by analysing embryo-conditioned medium. This supports the fact the proteins detected in our study mainly represent the proteins secreted in the uterine fluid rather than endometrial cells shed in the uterine lumen. This also accounts for the proteins which were found to be differentially expressed during pregnancy. Sixty four % of these proteins were categorized in the extracellular space; the other may have originated from occasional shedded endometrial cells. This also accounts for the proteins which were found to be differentially expressed during pregnancy. Sixty four % of these proteins were categorized in the extracellular space; the other may have originated from occasional shedded endometrial cells.

| Protein Symbol | Log FC | Adj. p-value | Gene Symbol | Gene Description |
|----------------|--------|--------------|-------------|-----------------|
| F6SJE7         | 0.59   | 0.01211      | TARS*       | Threonyl-tRNA synthetase |
| F6QXV2         | 0.58   | 0.01378      | PEBP1*      | Phosphatidylethanolamine binding protein 1 |
| F6XILG6; F7DY67| 0.57   | 0.04597      | PNP; LOC100058767 | Purine nucleoside phosphorylase |
| F7DZV9         | 0.57   | 0.00961      | YWHAB*      | Tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein beta |
| F7C132; F7ASU6; F7DK3 | 0.51 | 0.03433 | SELENBP1* | Selenium binding protein 1 |
| F7BSP1         | 0.49   | 0.03253      | CNDP2*      | CNDP dipeptidase 2 (metallopeptidase M20 family) |
| F6Y1Z3         | 0.48   | 0.03604      | S100A13*    | S100 calcium binding protein A13 |
| F6ZEV8         | 0.46   | 0.01618      | DBI*        | Diazepam binding inhibitor, acyl-CoA binding protein |
| F6X6A6; F6KX6; F6Z5Z4 | 0.45 | 0.00423 | LOC100052020; LOC100054282 | Peptidyl-prolyl cis-trans isomerase |

Table 1. Upregulated proteins in the uterine fluid of pregnant versus cyclic mares on day 13 after ovulation.
Table 2. Downregulated proteins in the uterine fluid of pregnant versus cyclic mares on day 13 after ovulation.

| Protein ID | Log FC | adj. P-Value | Gene Symbol | Gene Description |
|------------|--------|--------------|-------------|------------------|
| F6USV6     | −0.44  | 0.04220      | NOL11*      | Nucleolar protein 11 |
| F7BPJ1     | −0.52  | 0.01207      | SP12*       | alpha-1-antitrypsin |
| F6VZW6     | −0.57  | 0.01980      | PSM1A       | prospeosome subunit alpha 1 |
| F6TLA3     | −0.62  | 0.00626      | TXNRD1*     | thioredoxin reductase 1 |
| F6VTO0     | −0.71  | 0.01004      | RASGRP4*    | RAS guanyl releasing protein 4 |
| F6ZFH9     | −0.72  | 0.02902      | YWHAG*      | tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma |
| F7AED2     | −0.72  | 0.01528      | LOC100050100* | alpha-1-acid glycoprotein 2-like |
| F7CZW9     | −0.73  | 0.02003      | SERPING1*   | serpin family G member 1 |
| F6T7X3     | −0.75  | 0.00626      | LOC100065767 | membrane primary amine oxidase |
| F6RRV1     | −0.76  | 0.00368      | FETUB*      | fetuin B |
| F7BKK5     | −0.76  | 0.04705      | GSTM3*      | glutathione S-transferase mu 3 |
| F6Q46      | −0.78  | 0.01052      | CP*         | ceruloplasmin |
| F6R942; F6R47 | −0.79 | 0.00336      | A2M*        | Alpha-2-macroglobulin |
| F6SJ41     | −0.82  | 0.03640      | PPN2        | profilin 2 |
| F6XWM5     | −0.82  | 0.00041      | HP*         | haptoglobin |
| F6RMD0     | −0.87  | 0.00516      | CFB*        | complement factor B |
| F6ZD04     | −0.89  | 0.01443      | PYGB        | glycogen phosphorylase B |
| F69905     | −0.89  | 0.01010      | HBA1        | hemoglobin subunit alpha 1 |
| F7AIJ3     | −0.90  | 0.02643      | CHI3L1*     | chitinase 3 like 1 |
| F6VT27     | −0.91  | 0.00072      | CFAP58*     | cilia and flagella associated protein 58 |
| F6RDD3; F6VE37 | −0.91 | 0.00072      | HBB         | hemoglobin subunit beta |
| F7BPJ1     | −0.92  | 0.01242      | F2          | coagulation factor II, thrombin |
| F6RZ27     | −0.93  | 0.00336      | APOA4*      | apolipoprotein A4 |
| F6WMT7; F7C7Y1 | −0.93 | 0.00053      | KRT71; KRT73* | keratin 71; keratin 73 |
| F6Q541; F7BQS9 | −0.95 | 0.00336      | MBOH2A*     | maestro heat like repeat family member 2A |
| F6ZL5     | −0.96  | 0.00005      | APOA1*      | apolipoprotein A1 |
| F6XM13     | −0.98  | 0.01371      | APOD*       | apolipoprotein D |
| F6XLB1     | −0.99  | 0.00056      | LT*         | Lactotransferrin |
| F6XR11; F6YAV2 | −1.01 | 0.00217      | SERPINB11*  | serpin family B member 11 |
| F7D7V1     | −1.04  | 0.01115      | PON1*       | paraoxonase 1 |
| F6SGV9     | −1.07  | 0.00338      | TTF2*       | transcription termination factor 2 |
| Q29482     | −1.07  | 0.01298      | CLU         | clusterin |
| F6SR7      | −1.10  | 0.01010      | CAP1        | adenylate cyclase associated protein 1 |
| F6QX36     | −1.13  | 0.00119      | ITIH1*      | inter-alpha-trypsin inhibitor heavy chain 1 |
| F6TJX5     | −1.16  | 0.03059      | TPP1*       | tripeptidyl peptidase 1 |
| F7BZ41     | −1.19  | 0.02235      | CTSL*       | cathepsin L |
| F7BNQ2     | −1.20  | 0.00217      | C4BPA*      | complement component 4 binding protein alpha |
| F7AM7      | −1.20  | 0.02095      | STK38*      | serine/threonine kinase 38 |
| F6RM73     | −1.22  | 0.00366      | APOA2       | Apolipoprotein A-I |
| F6YNT8     | −1.27  | 0.02370      | PEBP4*      | phosphatidylethanolamine binding protein 4 |
| F6YUV2     | −1.30  | 0.00626      | CTSS*       | cathepsin S |
| F6TE92     | −1.33  | 0.02043      | AGL         | amylo-alpha-1,6-glucosidase, 4-alpha glucanotransferase |
| F7CO6E     | −1.36  | 0.00259      | PLS1*       | plastin 1 |
| F6X56      | −1.36  | 0.02043      | ADSL        | adenylosuccinate lyase |
| F7BCH1     | −1.37  | 0.00000      | INHBA       | Inhibin beta A chain |
| F6UX2      | −1.41  | 0.00026      | MSN*        | moesin |
| F7DXH4     | −1.44  | 0.00106      | VPS33B*     | VPS33B interacting protein, apical-basolateral polarity regulator, sre-39 homolog |
| P11008     | −1.54  | 0.00178      | SERPINC1    | serpin family C member 1 |
| F7CWG8     | −1.57  | 0.00119      | unassigned  | Amine oxidase [flavin-containing] |
| F7CW70     | −1.58  | 0.00056      | P19*        | P19 lipocalin |
| F6WKB2     | −1.59  | 0.00199      | MANBA*      | mannosidase beta |
| F7BLE3     | −1.69  | 0.01851      | unassigned  | unassigned |
| F6QYS3     | −1.75  | 0.01765      | ECM1*       | extracellular matrix protein 1 |
| F6RRP9; F6RM27 | −2.13 | 0.00112      | TTLL7*      | tubulin tyrosine ligase like 7 |
| F6SNJ4     | −2.22  | 0.00556      | UBOX5*      | U-box domain containing 5 |
| F7CHR8     | −2.23  | 0.01530      | CCDC36*     | coiled-coil domain containing 36 |
| F6VST0; F6W6H2 | −3.34 | 0.00004      | NEFL*       | neurofilament, light polypeptide |
While glutathione transferases are also generally involved in the biosynthesis of prostaglandins and leukotrienes, as well as progesterone and testosterone, a specific anti-inflammatory effect of GSTP1 by reduction of PTGS2, formerly known as cyclooxygenase-2 (COX-2), has been described. Furthermore, transport of GSTP1 across the plasma membrane was demonstrated. Based upon these observations with recombinant human GSTP1 in mice and the high homology of equine GSTP1 with other species, equine GSTP1 in uterine fluid might cross the plasma membrane and target intracellular PTGS2. Interestingly, GSTP1 was also detected in the YS of the equine conceptuses. In this regard, the pregnancy associated upregulation of GSTP1 observed in the equine uterine fluid could be involved in the luteostatic mechanism by inhibiting PTGS2. Further research is needed to examine this hypothesis, as this is the first report on the presence of GSTP1 in equine uterine fluid.

Another anti-inflammatory factor with an inhibitory effect on prostaglandin synthesis, more specifically on phospholipase A2, is annexin A1. Annexin A1 was upregulated in the uterine fluid of pregnant mares when compared to the cyclic mares and this association of annexins with pregnancy coincides with literature. An increase in ANXA1 was also reported in the uterine luminal fluid of pregnant ewes from day 10 to day 12. Several annexins have been linked to embryo-maternal interaction. Annexin 4 (ANXA4) was found to increase over time from day 10 to day 13 in both cyclic and pregnant pigs, and we previously reported greater quantities of ANXA4 in the oviductal fluid of pregnant mares, when compared to cyclic mares. In our study, we detected annexin 1, 2, 3, 4, 5, 7, 8 and 11 in the UF, while ANXA2 and ANXA5 were also found in the YS. Swegen et al. also reported the presence of ANXA2 in both the equine blastocoel fluid and the embryo-conditioned medium after 48 h. The only annexin found to be upregulated during pregnancy was ANXA1. Annexin 1 is an inhibitor of phospholipase A2, a rate-limiting enzyme which liberates arachidonic acid for the synthesis of prostaglandins and for which a lower enzyme activity of phospholipase A2 has been demonstrated in pregnant mares.

Figure 1. Categorization in Gene Ontology terms of all identified proteins in the uterine fluid (UF) and of differentially expressed (DE) proteins in the uterine fluid of pregnant (P) versus cyclic (C) mares. The main GO biological processes (a,b), molecular functions (c,d) and structural components (e,f) are represented for all quantified proteins in the equine uterine fluid (a,c,e) as well as for proteins found to be differentially expressed in the uterine fluid of pregnant versus cyclic mares (b,d,f).
compared to cyclic mares on day 14\(^41\). In our study, phospholipase A2 group IIA (PLA2G2A) tended to be downregulated in the uterine fluid of pregnant mares with a logFC of –1.31 compared to the cyclic condition, but it was not significant at a 0.05 FDR. Both PLA2G2A and phospholipase A2 group VII (PLA2G7) were detected in the YS; the latter was also found in the equine embryo-conditioned medium after 48 h\(^34\). Overall, our data suggest pregnancy associated interference with the luteolytic eicosanoid pathway with upregulation of inhibitory factors at different levels of the prostaglandin synthesis pathway.

A close interaction between prostaglandins and oxytocin has been described in the context of MRP in the horse with downregulation of the oxytocin receptor protein in the pregnant endometrium on day 14\(^13\). In the present study, the presence of oxytocin in the uterine luminal fluid was examined, but it was not detected. However, this does not mean it was not present in the original samples; the collection method might have retained some peptides and missing values are intrinsic to mass spectrometry\(^42,43\). Phosphoinositide phospholipase C (PLCD1), involved in the oxytocin receptor signalling pathway, was detected, but not significantly affected by pregnancy\(^44\). The reduced expression of OXTR during pregnancy has been hypothesized to be induced by an observed decrease in the gene expression of oestrogen receptor 1 (ESR1) in the pregnant equine endometrium\(^45\). Several proteins related to ESR1 were also found to be affected in the uterine fluid. Surprisingly, pregnant mares showed a strong upregulation of deoxynucleotidyltransferase terminal interacting protein 2 (DNTTIP2), previously known as oestrogen receptor binding protein (ERBP). Binding of DNTTIP2 to ESR1 enhances its transcription\(^45\). Uptregulation of DNTTIP2, which would lead to increased transcription of ESR1 in pregnant mares is contradictory to findings in literature and further targeted research is required to clarify this aspect. Downstream of the ESR1, the influence of oestrogen on the ezrin–radixin–moesin (ERM) family of actin-binding proteins has been studied, mainly in the context of breast cancer\(^46,47\). The distribution pattern of ERM-proteins in the blastocyst and the uterus has been linked to the implantation potential in mice. Protein analysis of uterine fluid has demonstrated the presence of ezrin (EZR) and moesin (MSN) in cattle\(^26,27\) and pigs\(^23\). In the horse, upregulation of EZR and downregulation of MSN was detected, while an inverse association with pregnancy was noted in cattle\(^26,27\). We detected both EZR and MSN in the YS and they were also found in the blastocoel fluid\(^34\).

Apart from the specific interest in proteins involved in prostaglandin synthesis, we also aimed to create a general overview of the proteins present at the embryo-maternal interface and potentially involved in signalling. Supplementary File 4 presents all proteins which were commonly found in the UF of P mares and in the YS and the functions of each protein are included. To visualize their role in embryo-maternal interaction and signalling, the proteins involved in GO terms including ‘embryo’, ‘maternal’ or ‘uterus’ are depicted in Fig. 4 and those linked to GO terms ‘growth factor’ and ‘cytokine’ in Fig. 5. The origin of the proteins can be distinguished in red (UF), yellow (YS) and pink (UF and YS) and up- and downregulation during pregnancy is represented by enlargement or shrinkage of the protein respectively. Interestingly, most proteins which were found to be upregulated during pregnancy were detected both in UF and in YS, while downregulation during pregnancy generally coincided with absence of these proteins in the YS, indicating a potentially important role of the embryo in the production of these proteins during pregnancy. Several common proteins were found at the embryo-maternal interface during MRP in cattle, including aconitase 1 (ACO1), which was specifically detected in the uterine fluid of pregnant and not in cyclic heifers, as well as glucose-6-phosphate isomerase (GPI), which has been detected in the uterine fluid of both pregnant and cyclic heifers and for which an embryonic source has been presumed based on transcriptomics\(^27\). In our study, both proteins were found in the YS and the UF of P mares, with significant upregulation of GPI in P versus C. Two other proteins which were commonly found in UF and YS, namely FK506 binding protein 4 (FKBP4) (Fig. 4) and heat shock protein 90 (HSP90AB1) (Fig. 5), have been elaborately discussed by Swegen et al.\(^34\) concerning their progesterone supportive role. Co-operation of both factors is necessary for activation of the progesterone receptor\(^48\). FKBP4 has shown to be crucial for uterine receptivity and implantation in mice\(^49\) and FKBP4 deficit has been associated with pregnancy loss in human\(^50\). While FKBP4 was detected in equine blastocoel fluid and speculated to be involved in signalling, it was not detected in the embryo-conditioned medium\(^34\). Interestingly, we did find both FKBP4 and HSP90AB1, not only in YS, but also in UF, even though their presence...
was not affected by pregnancy. While further confirmation of the role of specific proteins is required, the overview created in this study can be used as a basis for further targeted studies in the horse.

In addition to the role in prostaglandin and progesterone metabolism, involvement in proteolysis and lipid metabolism was also prominent in the commonly detected proteins in our study and the one of Swegen et al.\textsuperscript{34}, also coinciding with previous findings on transcriptomics around MRP 15. Several cathepsins (G, D, L and S) were detected in the UF with downregulation of cathepsin L (CTSL) and S during pregnancy. Pregnancy associated downregulation of \textit{CTSL1} was also found at the transcriptome level\textsuperscript{15}. Considering lipid metabolism, we detected differential expression of lipocalin (P19), apolipoprotein A1 (APOA1) and apolipoprotein D (APOD). These proteins are important transporters of essential lipids to the developing conceptus. Retinol binding protein (RBP), which also belongs to the lipocalin family, and APOA1 have been detected in uterine fluid of pregnant and cyclic pigs, cattle and sheep\textsuperscript{23–25,27,28}, with increasing amounts between day 10 and day 13 in both pregnant and cyclic pigs\textsuperscript{34}. In the horse, lipocalin (P19), apolipoprotein A1 (APOA1) and apolipoprotein D (APOD) were all downregulated in the uterine fluid of the pregnant mares. Pregnancy associated upregulation of APOA1 was reported at the transcriptome level\textsuperscript{15} and presence of P19 and APOA1 in the yolk sac fluid illustrates their role in the embryo-maternal dialogue. Therefore, lower amounts in the uterine fluid during pregnancy rather indicate the transport and binding to the conceptus. Lipocalin P19 or uterocalin is a progesterone induced protein, which is abundantly present in the equine uterine secretions during dioestrus and early pregnancy\textsuperscript{51,52}. While the early developing equine conceptus moves around the uterus, it entirely depends upon the uterine secretions for its nutrition and P19 can function as a carrier for essential lipids and amino acids\textsuperscript{53}. Coinciding with our findings, P19 has been detected in the trophoblast and the yolk sac fluid of the equine embryo\textsuperscript{51,52} and it is one of the most abundant proteins in the embryonic capsule\textsuperscript{54–56}. Therefore, the lower amount of P19 in P versus C is probably due to binding of substantial quantities to the embryo.

While a novel and informative overview is created, it has to be borne in mind that no statistically significant results were obtained at the level of molecular functions, biological processes and pathways. Differential expression of individual proteins was observed between the different UF conditions, and these proteins were categorized in GO terms, but statistical analysis showed no significant overrepresentation of any of the GO terms or KEGG pathways. Furthermore, it should be noted that MS intrinsically suffers from missing values and conclusions based on the absence of proteins cannot be made\textsuperscript{42,43}. However, the field of proteomics has greatly evolved in recent years, providing the possibility for statistically robust quantitative comparison of individual protein levels in complex biological samples, like uterine fluid\textsuperscript{35}. HDMSE specifically has been shown to provide good proteome coverage and reproducibility\textsuperscript{35}. At the same time, however, analysis of GO terms and pathways for proteomics is still in its infancy\textsuperscript{37,38}. As many of the here described bioinformatics approaches for proteomic analysis were originally developed for genomics, a similar but more matured field, their performance can be expected to show a similar growth as that of the genomic approaches. Moreover, the similarity between these fields potentially allows an integrated approach in which results from several omics studies can be combined.
In conclusion, proteins present in the equine uterine and embryonic yolk sac fluid around the signalling of MRP at day 13 were identified and quantified at large scale for the first time in the horse. We detected upregulation of several inhibitors of prostaglandin synthesis, including PTGR1, GSTP1 and ANXA1, in the uterine fluid of pregnant mares. Overall, an overview was created of the proteins playing a role at the embryo-maternal interface in the horse. This study provides a solid foundation for further targeted studies of proteins potentially involved in embryo-maternal interactions, maternal recognition of pregnancy and pregnancy loss in the horse.

Methods

Sampling. All animal handlings were approved by the Ethical Committee of the Faculty of Veterinary Medicine (EC2013/118) of Ghent University. All methods were performed in accordance with the relevant guidelines and regulations. A switch back design was followed with 5 mares undergoing two different types of cycles: a pregnant cycle (P) and a cyclic control cycle (C). In this way, the samples were paired using the same mare as its own control for pregnancy and the experimental unit was the mare. The order of P and C cycles was randomly altered for the different mares. No resting cycles were included. During the breeding season, five reproductively sound Warmblood mares between 4 and 13 years old were monitored by transrectal ultrasound. Reproductive soundness was confirmed by negative cytology and bacteriology. Mares displaying uterine oedema and a follicle exceeding 35 mm received 1500 IU hCG intravenously and were either inseminated the next day with fresh semen of the same stallion (P) or left unbred (C). Ovulation was evaluated twice daily by ultrasound. In both groups, sampling was performed 13 days after detection of ovulation. To recover undiluted uterine fluid in order to avoid negative effects of excessive Ringer's salts on MS59, intra-uterine application of a tampon (OB Mini; Johnson & Johnson, Beerse, Belgium) was performed based upon the method described by Wolf et al. A double gloved technique was used to avoid vaginal contamination. The tampon was left in the uterus during 10 minutes and upon removal it was placed in a Falcon tube at 4 °C until further processing. Subsequently, the mare's uterus was flushed with sterile Ringer's solution by means of a modified endotracheal tube to recover the embryo (P). To process the uterine fluid, 1 mL of sterile water (B60, Biosolve, Valkenswaard, The Netherlands) was infused on top of the tampon and the tampon was attached in the upper part of the Falcon tube by fixing the cord with the cap. Subsequently, the Falcon tube was centrifuged for 20 minutes at 1000 × g at 4 °C. The supernatant was
collected and stored in a Protein LoBind Eppendorf tube (Eppendorf AG, Hamburg, Germany) at −80 °C. Meanwhile, the embryo was isolated in a petri dish and the yolk sac fluid was collected by aspiration with a 21 G needle and stored in a Protein LoBind Eppendorf at −80 °C.

A total of 15 samples were collected, consisting of uterine fluid (UF) (n = 10) from five biological replicates coinciding with the five mares (1–5) for the P and C treatment cycles, as well as yolk sac fluid (YS) (n = 5) from the P cycles.

Sample preparation for mass spectrometry analysis. After thawing, protein concentration in each sample was determined using the Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific, San José, CA, USA) according to the manufacturer’s instructions. Further processing was performed for 10 µg protein of each uterine fluid sample and for 500 ng protein of the yolk sac samples. Samples were dissolved in 20 µL 0.5 M triethylammonium bicarbonate (TEABC; Sigma-Aldrich, St. Louis, MO, USA). Two µL of reducing agent (10 µM DTT; Invitrogen, Merelbeke, Belgium) were added followed by incubation for 1 h at 60 °C. Subsequently, 1 µL of alkylizing agent (200 mM methyl methanethiosulfonate (MMTS) in isopropanol; Sigma-Aldrich, St. Louis, MO, USA) was added and samples were incubated for 10 min at room temperature. Digestion was performed overnight at 37 °C with trypsin lys C (1:20, trypsin:protein w/w, Promega, Leiden, The Netherlands) in TEABC buffer with 1 mM CACL(2) and 5% acetonitrile (Biosolve, Valkenswaard, The Netherlands). Samples were vacuum-dried and stored at −20 °C until analysis.

Data acquisition by HDMS® analysis. The peptides were separated using a nanoscale UPLC system (nanoAcquityUPLC, Waters, Milford, USA) coupled to a Synapt G2-Si mass spectrometer (Waters). Peptides were first trapped in 0.1% formic acid on a 180 µm × 20 mm C18 Trap column. Separation was performed on a HSS C18 1.8 m, 100 m × 250 mm analytical column at a flow rate of 300 nL/min and a temperature of 45 °C. As mobile phase A a 0.1% formic acid with 4% DMSO in water solution was used and 80% ACN containing 0.1% formic acid constituted mobile phase B. Peptides were separated for 60 min at 1–40% solvent B and for 1 min
40–85% solvent B. Seven minutes of rinsing (85% solvent B) re-equilibrated the column to the initial conditions. Eluted peptides were analysed in positive mode ESI-MS using High Definition MS E (HDMS E) with a collision energy look up table as described in 22. The spectral acquisition time of low and elevated energy scans was 0.6 s over an m/z range of 50–2000. [Glu1]-Fibrinopeptide B was used for post-acquisition lock mass correction. All
Identification and quantification of peptides and proteins. All data were processed in Progenesis QIP (Progenesis QIP 2.0, Nonlinear Dynamics, Waters), including normalization and quality control. A database with UniProt IDs was created by conversion of Ensembl gene identifiers for Equus caballus (n = 22295) to Uniprot IDs using http://www.uniprot.org/uploadlists/ and including common contaminants (http://www.theqpm.org/crap/). As only secreted proteins are expected to be found, it can be argued that this database should be limited to only these secreted proteins. However, there is much debate on the accuracy of FDR calculations with such limited databases60-62 and as such a cautious approach was taken in which all proteins were assessed. Using Progenesis QIP, peptides were identified against this database with a FDR of 4%63 and allowing maximum one missciew. Protein quantification was based on the Hi-3 method64, which uses the average of the three most intense peptides of each protein for its quantification. Resulting normalized abundances for each protein, as well as unique peptide counts were further used for analysis of differential expression.

Analysis of differential expression. Analysis of differential expression was performed for the UF samples. Only normalized abundancies of proteins with at least two unique peptides (n = 707) were included in the analysis. Pairwise comparisons of differential expression were made for P versus C with the individual horses as a blocking factor, using R Bioconductor limma package65 and a FDR of 0.05.

Gene Ontology enrichment and pathway analysis. Gene Ontology (GO) terms (molecular functions, biological processes and cellular locations) were downloaded for each protein with the PANTHER Classification System66. For the pair-wise comparison of P and C, a statistical overrepresentation test against all quantified proteins was performed for all significantly up- and downregulated (FDR < 0.05) proteins. These tests were done for all primary GO classes: molecular functions, biological processes and cellular components. Pathways were analysed with Biocductor’s GAGE package68. LogFC values of all quantified proteins were used as input against Equus caballus background reference pathways from KEGG.

Proteins involved in the embryo-maternal interaction were visualized using Cytoscape 3.3.0. To visualize embryo-maternal signalling, all GO terms and pathways that include ‘cytokine’ or ‘growth factor’ in their description were selected, together with all identified proteins in either the yolk sac or uterine fluid belonging to these GO terms or pathways. The connection between these GO terms, pathways and proteins was then visualized using Cytoscape 3.3.0. The same methodology was used to create a network based on GO terms and pathways including ‘embryo’, ‘maternal’ or ‘uterus’ in their description.

Data availability. All data are available in the Supplementary files.

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Author Contributions
K.S. performed the experiments and wrote the manuscript. S.W. provided the bioinformatics analysis. K.V.S. was responsible for the mass spectrometry. M.V.D.V., V.D.L., C.V., K.R. and J.G. contributed to the sampling of the mares. F.V.N., L.P., D.D. and A.V.S. participated in the study design. All authors reviewed the manuscript.

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