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Systemic Changes in Early Pregnancy in the Mare: An Integrated Proteomic Analysis of Blood Plasma, Histotroph, and Yolk Sac Fluid at Day 14 Post-Ovulation

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ABSTRACT

Purpose: Embryo-maternal signaling during the establishment of pregnancy in horses remains one of the biggest mysteries in large animal physiology. Early pregnancy loss represents a major source of economic loss to the breeding industry. This study aimed to investigate the systemic changes associated with early pregnancy by mapping the proteome of blood plasma at 14 days in pregnant and non-pregnant mares.

Experimental Design: Plasma proteomes were analysed in commercially bred pregnant (n = 17) and non-pregnant (n = 17) Thoroughbred mares at 14 days after ovulation, using high-resolution mass spectrometry. Day 14 histotroph and yolk sac fluid were also profiled and datasets were integrated through pathway analysis.

Results: We identified 229 total protein IDs, with 12 increased and 10 decreased significantly in pregnant versus non-pregnant plasma. To gain functional insight, these data were aligned with proteomes of 14-day pregnant mare uterine fluid (n = 4; 1358 IDs) and conceptus fluid (soluble proteins within the yolk sac fluid; n = 4; 1152 IDs), and further interrogated using gene ontology databases and pathway analysis.

Conclusions and Clinical Relevance: These analyses identified consistent systemic changes in the mare's proteome that indicate a profound and specific immune response to early pregnancy, which appears to precede the systemic endocrine response to pregnancy. Integrated pathway analysis suggests that embryo-maternal interactions in early pregnancy may mimic elements of the virus-host interaction to modulate the maternal immune response. Transthyretin (TTR) and uteroglobin (SCGB1A1) were respectively down- and upregulated in plasma while also present in uterine fluid, and are proposed to be key proteins in early pregnancy establishment. These findings contribute significantly to our knowledge of early pregnancy in the mare and identify potential new avenues for developing clinical approaches to reduce early embryo loss.

Aleona Swegen and David A. Skerrett-Byrne contributed equally to this study.

1 | Introduction

Early diagnosis of pregnancy and avoidance of pregnancy loss are critical aspects of equine breeding management. Pregnancy losses are higher during the first 8 weeks than the entire remaining 9 months of gestation [1, 2]. While conception rates are estimated to exceed 90% [1], pregnancy rates are much lower by the time the pregnancy can be reliably detected clinically (65%: [2]). The inability to detect pregnancy ultrasonographically prior to Day 9 makes it impossible to distinguish between failure of fertilization and embryo loss during this period. The physiological process of maternal recognition of pregnancy (MRP) is incompletely explained in the horse, with no definitive signal yet identified as being responsible for inhibiting luteolysis during Days 14–15 of pregnancy [3, 4]. As such, there are both clinical and fundamental reasons for wanting to better understand the physiology of early pregnancy in the mare.

Following ovulation, a corpus luteum (CL) forms in the evacuated follicle and becomes the defining feature of the subsequent diestrous period, which in the mare lasts 13–15 days. At this time, a non-pregnant mare will typically undergo PGF2α-induced lysis of the CL leading to a rapid fall in circulating progesterone concentrations accompanied by a return to oestrus, and an estrogen-dominated phase ultimately progressing to another ovulation. In a pregnant mare, luteolysis is averted and the CL will be retained and continue to secrete progesterone to support the pregnant state. Because dioestrus continues for 14–15 days irrespective of whether an embryo is present or not, and progesterone has long been known to be the major hormone of pregnancy, the current paradigm is that up until 14 days after ovulation, a pregnant mare is largely indistinguishable from a non-pregnant mare in terms of her systemic endocrinology and biochemistry. Remarkably, this assumption has not been directly challenged, despite some indications that hematological changes occur as early as Day 3 after conception in the mare [5].

The emergence of omics-based detection modalities offers new opportunities for investigating the systemic physiological changes associated with early pregnancy and, in turn, the mechanisms contributing to a healthy pregnancy or predisposing to pregnancy failure. Omics technologies have been harnessed previously by our group and others to investigate the proteins and lipids secreted by the early equine embryo [6–8], and those present in the uterus [9] and oviduct [10] during this critical early stage of gestation. Others have examined the systemic (serum) profile, but at later stages in pregnancy [11]. Here, we use high-resolution mass spectrometry to investigate changes in the systemic proteome associated with early pregnancy at Day 14 of the mare's ~330-day gestation.

MRP in the mare is thought to involve an as-yet-unknown biochemical signal released by the embryo that modifies the endometrial capacity to drive luteolysis, by preventing the cyclical upregulation in oxytocin receptor sensitivity and prostaglandin endoperoxide synthase 2 (PTGS2) expression. This abrogation of the ability of the endometrium to secrete prostaglandin F2α (PGF2α) ensures retention of the CL, instead of lysis and a return to oestrus (reviewed in Ref. [3]). The CL is critical to supporting ongoing pregnancy through its production of progesterone, the major hormone responsible for the development and maintenance of a receptive endometrium, and other systemic aspects of the pregnant state in eutherian mammals. By Day 14, MRP signaling should have been initiated with the presence of the conceptus triggering a cascade of endometrial responses [12, 13]. However, the CL is typically still active in both pregnant and non-pregnant mares at this timepoint, meaning that any systemic changes downstream of CL lysis and falling progesterone concentration have not yet been triggered. This is critical because any blood plasma protein changes are therefore likely to be predominantly the result of MRP itself, or other processes associated with early pregnancy, rather than the broad systemic changes related to falling progesterone concentrations. Thus, by choosing Day 14 for this study, we can ensure accurate sample classification and be confident that the differential abundance of plasma proteins is likely to reflect processes upstream (or independent) of progesterone. This is important because progesterone-independent changes are more likely to be those emerging at earlier timepoints in pregnancy, and, therefore, more promising as future biomarkers for detecting and assessing healthy progress of pregnancy.

In clinical practice, pregnancy is typically diagnosed around Days 12–16 post-ovulation via transrectal ultrasound. Detection of pregnancy as early as Days 9–10 is possible, albeit less reliable and therefore not desirable in a practical setting. Examining mare plasma at Day 14 post ovulation ensures that each mare could be confirmed definitively as pregnant or not using ultrasonography, at the time of sampling.

Another feature of equine reproductive physiology is an unusually prolonged pre-implantation period. The equine conceptus descends into the uterus at around Days 6–6.5 after ovulation and migrates around the lumen until around approximately Day 16. The major "implantation" reactions, endometrial cup formation, and interdigitation of the chorioallantois with the endometrium do not begin until Days 35 and 40–42, respectively. Until then, the only communication between the embryo and the maternal system occurs via the uterine luminal fluids or "histotroph," and the endometrial surface. It follows that any transfer of nutrients and pro-survival factors (from the mare) or signaling components (from the conceptus) would inevitably transit through the histotroph and likely be detectable in that fluid. It is, therefore, a useful medium to analyze, alongside the proteins found in the conceptus itself, when seeking to understand the relevance and origin of pregnancy-associated proteins detected in the blood plasma. We have previously conducted a preliminary analysis of pregnant mare histotroph at 14, 21, and 28 days post-ovulation [9]. We now hope to exploit these data further, alongside soluble proteins found within the yolk sac fluid of the corresponding conceptus, to generate deeper insights into the potential origin and distribution of pregnancy-associated plasma proteins.

In the current study, we aimed to investigate and identify systemic markers of early pregnancy in the mare. We used a comparative integration approach to investigate the biological pathways central to establishment of pregnancy, and to delve into the potential origins of plasma proteins altered in pregnant animals.

2 | Materials and Methods

2.1 | Mare Plasma: Sample Collection

For blood plasma analyses, thoroughbred mares aged 3–16 years ($n = 28$) in a commercial breeding program were randomly selected from two breeding farms in the Hunter Valley region of Australia. Procedures were approved by the University of Newcastle Animal Care and Ethics Committee (approval number A-2018-804). The animals were considered healthy based on veterinary records, physical examinations, and reproductive tract examinations performed by rectal palpation and ultrasonography. An experienced equine reproduction veterinarian used ultrasound scanning to monitor ovarian and uterine activity during oestrus. The mares were bred by live cover 1 day before or on the day of predicted ovulation, and ovulation was confirmed the following day. Mares were checked for pregnancy 12–14 days after ovulation to confirm their pregnancy status by ultrasonographic examination, and the blood samples were collected at Day 14 after ovulation. All mares, regardless of pregnancy status, were maintained in an outdoor paddock with the same diet (predominantly pasture with supplementary feeding) and ad libitum water. Blood samples were collected from the jugular vein of the mares and dispensed into 10-mL EDTA anticoagulant tubes. Within 1 h of collection, the samples were centrifuged at 3000 × *g* for 10 min at room temperature, and the obtained plasma was stored at −80°C until further processing. Pregnancy maintenance details and foaling dates were also recorded, allowing samples to be categorized as pregnant (P; $n = 17$) or non-pregnant (NP; $n = 17$), and any samples culminating in pregnancy loss were excluded from the study.

2.2 | Mare Plasma: Mass Spectrometry

The workflow for mare plasma analysis is summarized in Figure 1A–E. Samples were prepared for mass spectrometry runs in batch mode (12 samples at once, 6 per group) as previously described [14, 16]. In summary, plasma samples were lysed in 80 µL of ice-cold 0.1 M Na_2CO_3 supplemented with protease (Sigma) and phosphatase inhibitors (Roche, Complete EDTA free). These suspensions were subjected to probe tip sonication at 4°C for 3 × 10 s intervals. The protein concentrations were measured using BCA quantification and aliquoted to obtain 400 µg total protein per sample for the MS run preparation. To equalize sample volumes, 1:1 v/v of 12 M urea/4 M thiourea was added as required. Samples were then reduced using final concentrations of 10 mM DTT (30 min, room temperature), alkylated using 20 mM iodoacetamide (30 min, room temperature, in the dark), and subsequently digested with Lys-C/trypsin (1:30 ratio, protease: protein concentration) for 3 h at room temperature. Subsequently, the urea concentration was diluted below 1 M using 50 mM triethylammonium bicarbonate (TEAB; pH 7.8) and incubated overnight at 37°C. Lipids were precipitated by the addition of formic acid (2% v/v final concentration), and peptides were purified using desalting columns (Oasis, Waters). Quantitative fluorescent peptide quantification (Qubit Assay; Thermo Fisher Scientific) was employed to ensure even injections of peptide populations on an Orbitrap Exploris 480 MS coupled to a Dionex Ultimate 3000RSLC nanoflow HPLC system (Thermo Fisher Scientific). The samples were loaded onto an Acclaim PepMap 100 C18 75 µm × 20 mm trap column (Thermo Fisher Scientific) employing a linear gradient of ACN (2%–35%) over 120 min. The MS was operated in data-dependent acquisition (DDA) mode. The Orbitrap mass analyzer was used at a resolution of 60,000, to acquire full MS with an m/z range of 360 to 1500, incorporating a normalized target automatic gain control value of 300% and maximum fill times of 100 ms. The 20 most intense multiple-charged precursors were selected for higher-energy collision dissociation fragmentation with stepped collisional energy of 30% and 36%. MS/MS fragments were measured at an Orbitrap resolution of 15,000 with the automatic gain control target set to standard.

2.3 | Histotroph and Yolk Sac Fluid: Sample Collection

In an attempt to shed light on the origin and functional significance of Day 14 pregnant mare plasma proteins, we integrated our plasma data with proteomic datasets of Day 14 pregnant mare uterine luminal fluid (Histotroph; HS) and the soluble protein fraction of Day 14 yolk sac fluid (Yolk sac; YS) generated in a separate study. Histotroph and yolk sac fluid samples were obtained as follows. The animal procedures were approved by Utrecht University's Animal Experimentation Committee (permission no: 2007.III.02.036). Cycling Warmblood mares aged between 5 and 15 years, and maintained at pasture, were monitored by transrectal palpation and ultrasonographic examination of the reproductive tract. Upon signs of impending ovulation, mares were inseminated with a minimum of 5×10^8 progressively motile sperm from a single stallion of proven fertility. Insemination was repeated at 48 h intervals until ovulation, and the day of ovulation was determined by daily examination. Pregnancy was diagnosed on Day 12 after ovulation and repeated immediately before uterine sample collection on Day 14. Uterine luminal fluid (Histrotroph) was collected by aspiration (without introduction of any other "flushing" fluid) into a PTFE cannula introduced via the biopsy channel of a 1.7 m long strobed-light video-endoscope [Pentax EC 3870LK colonoscope with EPK-700 processor: Pentax Medical, Dodewaard, The Netherlands], connected to a 20 mL syringe. In pregnant mares ($n = 4$), uterine fluid collection was performed immediately prior to puncture of the conceptus with a fresh PTFE cannula with a sharpened tip to recover the yolk sac fluid; subsequently, the conceptus membranes and an endometrial biopsy sample were recovered for a separate study. After recovery, samples of uterine fluid (approximately 10 µL each) and yolk sac fluid (0.5–2 mL) were centrifuged at 500 × *g* to remove cells and cell debris, frozen in cryovials, and kept at −80°C during shipment and storage until proteomic analysis.

2.4 | Histotroph and Yolk Sac Fluid Samples: Mass Spectrometry

Samples were centrifuged (1000 × *g*) to remove cellular contamination, and assayed for protein content using a DC quant kit, as per the manufacturer's instructions (BioRad, Castle Hill, NSW, Australia). Depletion of high-abundance plasma proteins was achieved using a trichloroacetic acid/isopropanol precipitation: 400 µL of 1% trichloroacetic acid in isopropanol was added to

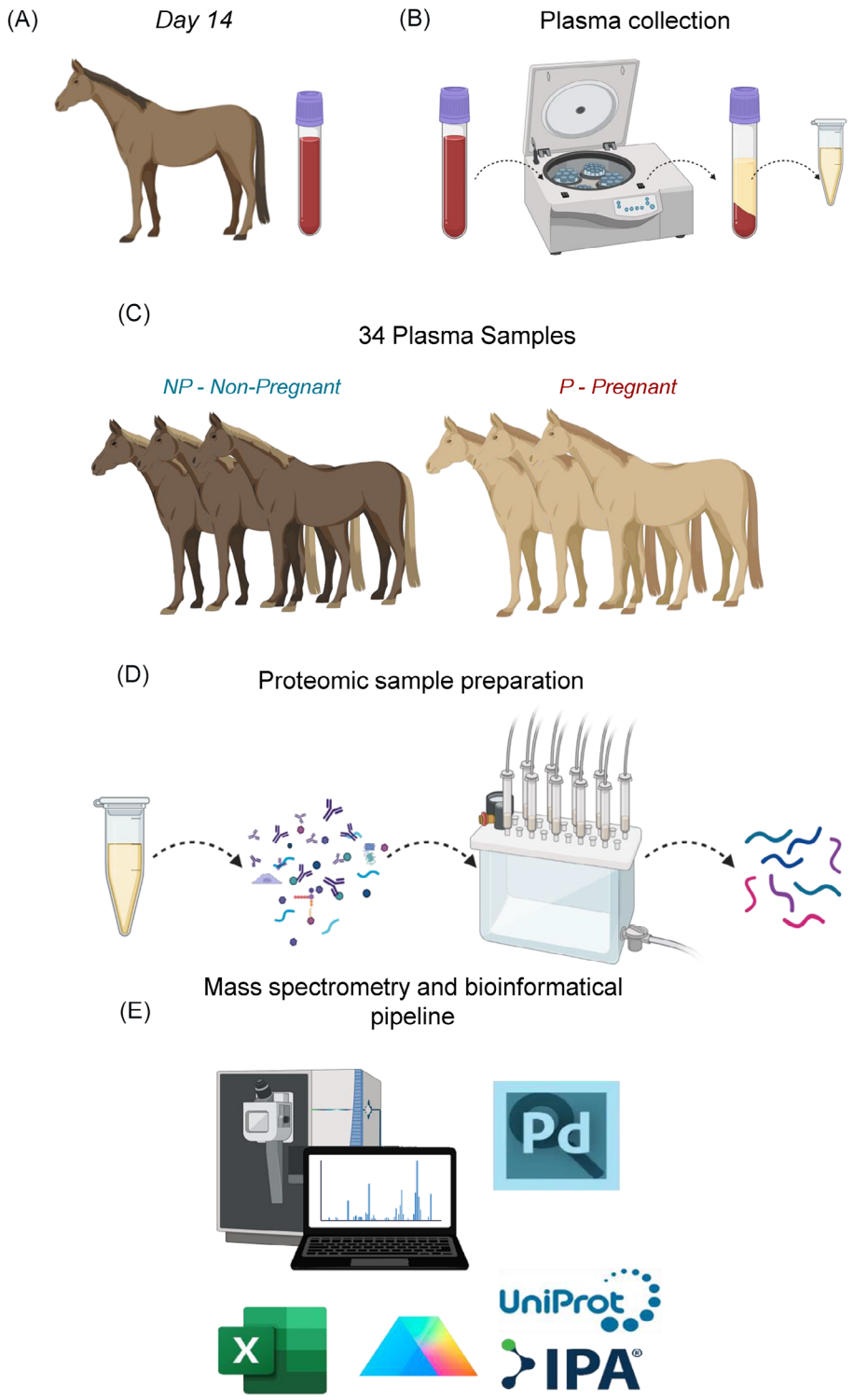

FIGURE 1 | Overview of the proteomics workflow. (A) Blood samples were collected from mares 14 days after ovulation and (B) centrifuged to obtain plasma. (C) Plasma samples were classified as "pregnant" or "non-pregnant" following confirmation of pregnancy status via ultrasound. (D) Plasma proteins were extracted, reduced, alkylated, and digested to peptides. (E) Samples were run on a high-resolution MS 480 Exploris, and then analyzed with a workflow of Proteome Discoverer 2.5, Perseus, Ingenuity Pathway Analysis, and UniProt. Some components of this figure were created with BioRender.com.

40 µL diluted uterine luminal fluid and mixed by vortexing for 5 min. Samples were centrifuged for 5 min at 2000 × *g* (4°C), the supernatant was removed, and the pellets washed with 200 µL methanol. Final pellets were resuspended in a 50 mM ammonium bicarbonate digestion buffer containing 400 ng trypsin and 50% acetonitrile. Digestion was performed for 18 h at 37°C on a shaker at 400 rpm and stopped by the addition of trifluoroacetic acid to a final concentration of 1%. Digests were centrifuged for 10 min at 14 000 × *g* to remove any undigested material, supernatants collected and vacuum

concentrated, then resuspended in 2% acetonitrile and 0.1% trifluoroacetic acid before transfer into Waters Autosampler vials for analysis.

Peptides were analyzed by Quadrupole-Orbitrap MS/MS as follows: Peptides were sequenced by nanoflow reversed phased liquid chromatography (Dionex Ultimate 3000 RSLCnano, Thermo Fisher Scientific) coupled directly to a high-resolution mode equipped, Q-Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific). Samples were loaded at 10 μL/min (2% Acetonitrile, 0.1% Trifluoroacetic acid in Water) for 5 min onto a 5 μm C18 nanoViper trap column (100 μm × 2 cm, Acclaim PepMap100, Thermo Fisher Scientific) for desalting and pre-concentration. Separation was then performed at 300 nL/min over an EASY-Spray PepMap column (3 μm C18, 75 μm × 15 cm) utilizing a gradient of 2%–40% Buffer B (80% acetonitrile, 0.1% formic acid) over 120 min. The gradient was then increased to 90% Buffer B briefly to wash the column before re-equilibrating at 2% Buffer B for a further 15 min (145 min total run time per sample). The compounds were eluted directly into the EasySpray nano-ESI Ion Source (Thermo Fisher Scientific) for high-resolution MS mass determination and MS/MS analysis of the top 20 ions in each MS scan. MS analysis scanned the mass range from 370 to 1400 m/z in positive ion mode with resolution set to 70,000 at m/z 200 (equals approximately 50,000 at m/z 400), an AGC target of 1e6, and a maximum injection time of 50 ms. Ubiquitous polysiloxane was utilized as a lock mass at m/z 445.12002. Top 20 MS/MS was performed on ions with intensities greater than 1.8e4 at a resolution of 17,500 with an AGC target of 2e5 and a maximum injection time of 110 ms. HCD collision energy was set to a value of 30, and dynamic exclusion time was set at 30 s. Singularly charged peptides were excluded from MS/MS selection.

2.5 | Data Processing and Analysis

Database searching of raw files was performed using Proteome Discoverer 2.5 software (Thermo Fisher Scientific), utilizing SEQUEST HT to search against the UniProt *Equus caballus* database (March 23, 2021). The parameters included up to two missed cleavages, a precursor mass tolerance of 10 ppm, and a fragment mass tolerance of 0.02 Da. Interrogation of the corresponding reversed database was also performed to evaluate the false discovery rate (FDR) of peptide identification using Percolator based on *q*-values, which were estimated using the target-decoy search approach. A fixed FDR of 1% was set at the peptide level to filter out the target peptide spectrum matches (PSMs) over the decoy-PSMs. LFQ was carried out as described previously [14]. The protein list with scaled data was exported as an Excel file and further refined to include only those with a quantitative value in >70% replicates within a group, with ≥1 unique peptide. Protein ratios were calculated by fold change, and statistical testing was completed using a Student's *t*-test. To ensure stringency of these protein findings, cut-offs for differential expression were set at a fold-change of ±1.5 and a p value of ≤0.05. Further, employment of Reactome was utilized for initial pathway enrichment to elucidate the biological processes governed by these significantly altered proteins, only pathways with an enrichment p value ≤ 0.05 and ≥2 associated proteins were considered.

To obtain an overview of the functional categories of the plasma proteins, we utilized the Ingenuity Pathway Analysis software (IPA; Qiagen, Hilden, Germany) as previously described [14, 17–20]. To facilitate the use of IPA, we initially mapped all the identified horse proteins into their closest human homologue using OmicBox (version 3.1.9, BioBam Bioinformatics, Valencia, Spain) (https://www.biobam.com/omicsbox) [19]. Horse accessions were uploaded to Omicbox and transformed into the closest human accession. To further profile the systemic state of early pregnancy in the mare, we used the full remit of plasma proteins identified in pregnant mares for downstream functional analysis using IPA. To elucidate the most significant changes in our analyses, we applied a stringency criterion of p value ≤ 0.05 to the function/pathway enrichment scores. Furthermore, we integrated this dataset with the proteomes generated from soluble fractions of Day 14 yolk sac and uterine luminal fluid from pregnant mares, with the same criterion above, in an attempt to better understand the protein dynamics of these compartments and the interactions between the embryo, uterine environment, and systemic proteome.

3 | Results

Proteomic analysis of mare plasma returned 229 identified between the non-pregnant (NP) and pregnant (P), with an average protein coverage of 54.2% and 37.2%, respectively (Figure 2A). A full list of protein IDs is presented in Tables S1 and S2. Initial analysis on UniProt revealed a mere seven proteins (3.1%) with evidence at the protein level, while the remaining 222 were annotated within the genome, but without previous protein confirmation (Figure 2A). The full list of protein IDs is presented in Tables S1 and S2. These comprised 54 proteins with only transcript evidence (23.9%), 82 inferred from homology (36.3%), and 86 predicted proteins (38.1%) (Figure 2B). A comparison of protein abundance in pregnant versus non-pregnant mares identified 22 significantly altered proteins (Fold-change of ±1.5, p value of ≤0.05; Figure 2C). Of these, 12 were increased and 10 decreased in abundance in pregnant mares (Table 1). Reactome analysis revealed 12 pathways enriched (p value of ≤0.05) in the upregulated cohort, and five pathways enriched in the downregulated cohort (Table 2).

Omicsbox successfully mapped 226/229 IDs (98.9%) to homologous human proteins (Figure 3A; Table S3). Humanized lists were subjected to analysis using IPA, allowing the categorization of proteins with enzymes (38 proteins) to be revealed as the dominant protein type (Figure 3B). The next most common protein type was transporters (22), while transmembrane receptors, transcription/translation regulators, cytokines, and growth factors each constituted <2% of the proteome (Figure 3B). Localization of proteins (Figure 3C) predominantly mapped to the extracellular space (109), cytoplasm (14), plasma membrane (18), and nucleus (2). Proteins assigned to the category of "other" were excluded from these figures. IPA analysis identified dominant molecular and physiological functional categories of embryonic development, lipid metabolism, and immunology enriched in the mare plasma proteome, including "*platelet aggregation*," "*immune mediated inflammatory disease*," "*complement component deficiency*," "*cholesterol transport*," and "*transport of lipids*," (enrichment p value of ≤0.05; Figure 3D; Table S4).

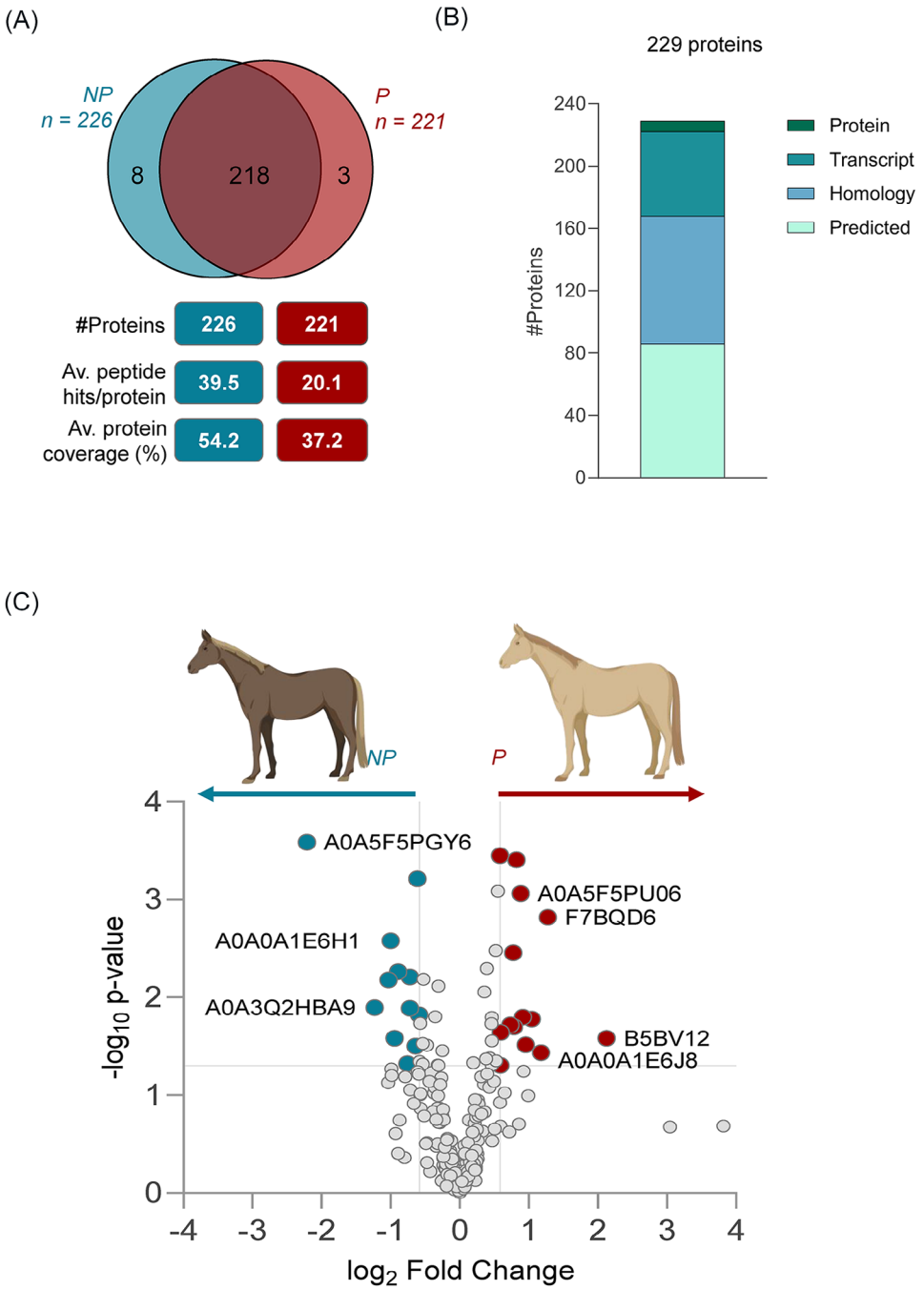

FIGURE 2 | Characterization of the equine plasma proteome related to early pregnancy. (A) Venn Diagram comparing the protein composition of the non-pregnant (NP) and pregnant (P) plasma proteomes. (B) Using UniProt, all identified proteins were assessed for their level of annotated evidence. (C) The volcano plot depicts the quantitative comparison between the P and NP groups. Red and blue dots represent upregulated and downregulated proteins, respectively (fold-change ≥1.5 and $p \leq 0.05$). Gray dots represent proteins that are not differentially expressed between the groups.

Integration of the Day 14 yolk sac fluid proteome (yolk sac) and Day 14 pregnant mare uterine fluid (histotroph) datasets (Figure 4A) showed that the majority of plasma proteins could also be detected in histotroph (132 shared proteins), while only a small proportion were shared with the yolk sac fluid (19 IDs). Only 17 proteins were common to all three datasets. Full yolk sac and histotroph proteomes and their relative abundances are detailed in Tables S5 and S6. Further pathway analysis of the three proteomes (yolk sac, histotroph, and plasma) identified the biological processes that might be commonly or uniquely enriched in each dataset (enrichment p value of ≤0.05; Figure 4B; Table S7). Two functions—"*infection by RNA viruses*" and "*migration of cells*"—were consistently enriched across all three datasets. Functions shared by plasma and histotroph included "*cell movement of embryonal cell lines*," "*synthesis of fatty acid*," "*cholesterol transport*," and "*angiogenesis*." Pathways shared by histotroph and yolk sac datasets included "*embryonal tumor*," "*synthesis of protein*," and "*synthesis of reactive oxygen species*." There were no pathways identified that were shared exclusively by plasma and yolk sac but not histotroph.

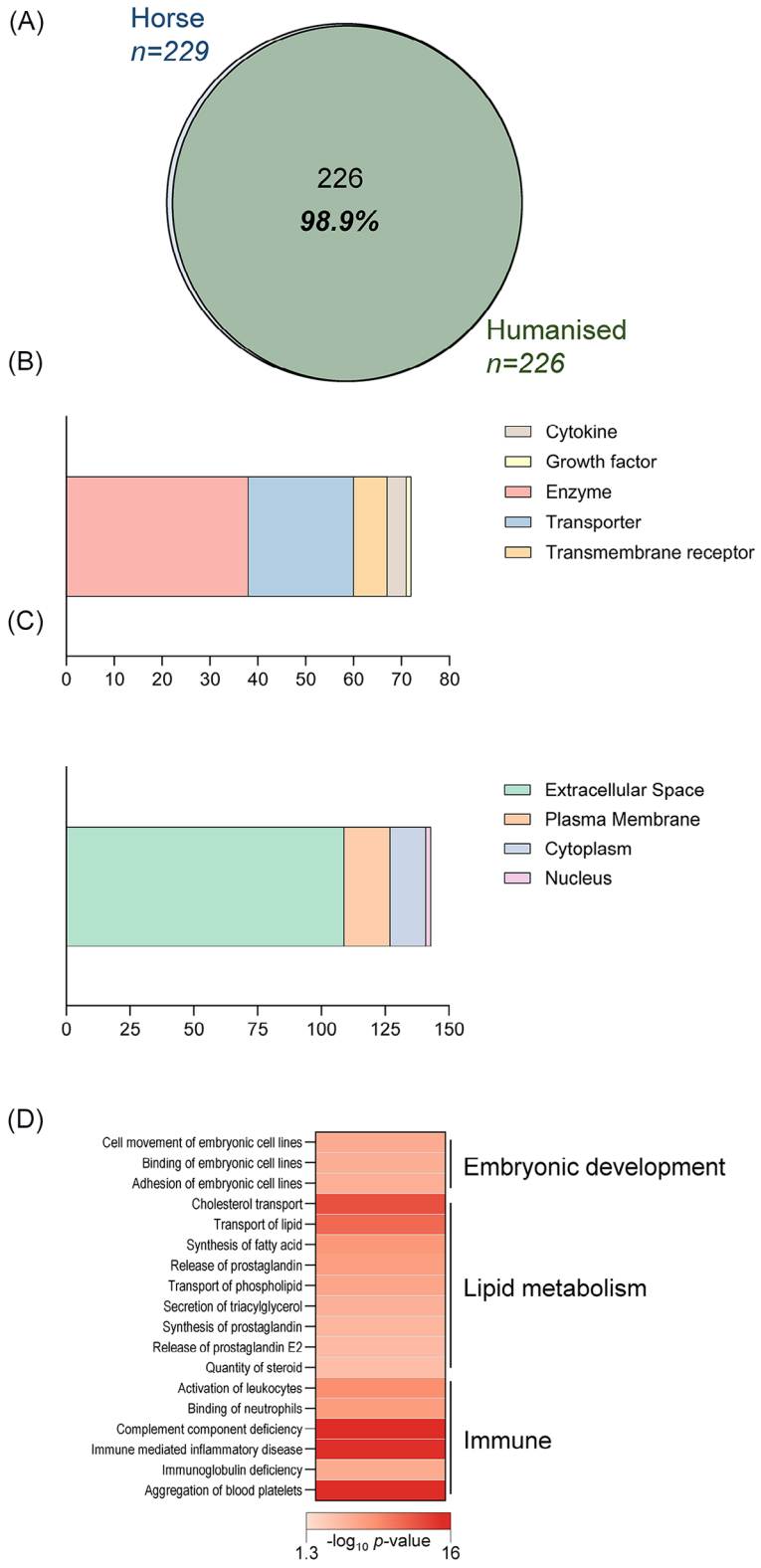

FIGURE 3 | Humanization and in silico functional analysis of pregnant mare plasma proteome. Mare plasma proteome was subjected to humanization using OmicsBox, seeking to obtain appropriate human protein orthologs with a 98.9% conversion rate (A). These humanized proteins enabled further analysis with Ingenuity Pathway Analysis (IPA), first determining (B) protein classification, and (C) cellular localization. Pathways enriched (p value of ≤ 0.05) in the proteome of mare plasma are shown in (D).

FIGURE 4 | Alignment and integration of mare plasma proteome with 14-day pregnant mare uterine fluid and conceptus proteomes. (A) Proteins shared by pregnant mare plasma, uterine fluid ("histotroph") and soluble components of the yolk sac cavity of the conceptus ("yolk sac") are depicted as a Venn Diagram. (B) Heatmaps represent the significantly enriched functions (p value of ≤0.05) across all three groups ($p \leq 0.05$) in the categories of hematological, cell signaling, immune, lipid metabolism, and embryonic development. For a full list of biological functions, please refer to Table S7.

TABLE 1 | Blood plasma proteins showing significantly different abundance in pregnant versus non-pregnant mares.

Accession	Description	Fold change (P/NP)	*p* value
B5BV12	Alpha-1-antitrypsin	4.36	0.0261
F7BQD6	Complement C1s	2.42	0.0015
A0A3Q2L2R4	Lipocalin/cytosolic fatty-acid binding domain-containing protein	2.06	0.0167
A0A3Q2I0V8	Apolipoprotein C-III	1.94	0.0305
F7DRS2	Serpin family A member 6	1.88	0.0159
A0A5F5PU06	Alpha-2-macroglobulin	1.84	0.0009
A0A5F5PI46	Interleukin 1 receptor accessory protein	1.77	0.0004
Q7M388	Serpin II (fragment)	1.71	0.0035
Q5EG14	Secretoglobin family 1A member 1 (fragment)	1.66	0.0191
F7CSL8	SERPIN domain-containing protein	1.51	0.0229
A0A5F5PUE2	Bradykinin	1.50	0.0496
F6PUW3	Maltase-glucoamylase	1.50	0.0004
H9GZQ3	Ig-like domain-containing protein	0.67	0.0151
F6WZA8	Carboxypeptidase N subunit 1	0.65	0.0006
A0A5F5PZ87	SERPIN domain-containing protein	0.64	0.0316
A0A3Q2GXU9	Ig-like domain-containing protein	0.61	0.0062
A0A0A1E550	Immunoglobulin lambda light chain variable region (fragment)	0.61	0.0130
A0A3Q2H5J1	Peptidoglycan recognition protein 2	0.59	0.0476
A0A3Q2HMJ3	Ig-like domain-containing protein	0.54	0.0055
F6UL68	Transthyretin	0.52	0.0262
A0A3Q2HBA9	Mannose binding lectin 2	0.43	0.0127
A0A5F5PGY6	Complement component C6	0.22	0.0003

The distribution of individual proteins consistently present in plasma, as well as histotroph and yolk sac fluid, is shown in Figure 5.

4 | Discussion

Efforts have been made to identify proteins secreted by early equine embryos and the endometrium [7, 11, 21–23]. The mechanism underlying MRP in horses remains elusive, with no single signaling molecule yet confirmed to mediate this process. However, recent advancements in high-throughput molecular techniques have greatly expanded our understanding of the embryo-maternal dialogue during this critical period. Notably, dynamic transcriptomic and proteomic studies have shed light on the complex interplay of molecules potentially involved in MRP using maternal and embryonic tissue [24, 25] and lipids in maternal plasma [8]. For example, the study by Vegas et al. [25] demonstrated fine-tuned regulation of mRNAs, miRNAs, and proteins in equine embryos between Days 10 and 13 of gestation, revealing pivotal molecular candidates associated with embryo growth and signaling pathways that modulate maternal immune responses and endometrial receptivity . Similarly, Smits et al. [24] integrated proteomic, transcriptomic, and miRNA analyses at the embryo-maternal interface, emphasizing the critical role of miRNAs in embryo signaling and maternal uterine response using endometrial biopsies, uterine fluid, embryonic tissues, and yolk sac fluid were collected 13 days after ovulation during pregnant and control cycles from the same mares. Recent lipidomics studies further enhance this understanding by revealing systemic biochemical adaptations critical for early pregnancy establishment. For instance, plasma lipidomic profiling has demonstrated significant alterations in lipid metabolism during early equine gestation. Key findings include the upregulation of bile acids, sphingomyelins, phosphatidylinositols, and triglycerides, particularly at Days 7 and 14 post-ovulation. These changes suggest enhanced lipid synthesis and mobilization, likely associated with the embryo's nutritional requirements and the establishment of embryo-maternal interactions [26]. These studies underscore the hypothesis that MRP in horses is mediated by a network of molecules rather than a single signal. The interplay of prostaglandins, immune-modulatory proteins, lipids, and miRNAs is particularly noteworthy, as these molecules appear to regulate key processes such as CL maintenance, uterine quiescence, and trophoblast-endometrial interaction during early pregnancy. Despite these advancements, the identity of a definitive embryo-derived signal remains unconfirmed, highlighting the need for further integrative studies that combine

TABLE 2 | Reactome pathway enrichment of proteins up- or down-regulated in pregnant mare plasma.

Upregulated in pregnant mare plasma		
Pathway name	**# Proteins**	**Enrichment *p* value**
Platelet degranulation	4	<0.001
Response to elevated platelet cytosolic Ca2+	4	<0.001
Platelet activation, signaling, and aggregation	4	<0.001
Intrinsic pathway of fibrin clot formation	2	<0.001
Plasma lipoprotein assembly	2	<0.001
Formation of fibrin clot (clotting cascade)	2	<0.001
Hemostasis	4	0.001
Plasma lipoprotein assembly, remodeling, and clearance	2	0.002
Post-translational protein phosphorylation	2	0.002
Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)	2	0.003
Neutrophil degranulation	3	0.003
Innate Immune System	4	0.008
Downregulated in pregnant mare plasma		
Pathway name	**# Proteins**	**Enrichment *p* value**
Innate immune system	6	<0.001
Complement cascade	3	<0.001
Immune system	6	0.001
Regulation of complement cascade	2	0.003
Neutrophil degranulation	2	0.030

transcriptomics, proteomics, and epigenomics, and connecting maternal tissue, blood, and embryo.

To our knowledge, there has been no comprehensive analysis of systemic proteomic changes associated with early pregnancy in the mare. This study successfully mapped the systemic changes occurring in response to early pregnancy, well before implantation and during the time of maternal endocrinological recognition of pregnancy. Further, we sought to assign putative functions and origins to these proteins by using a pathway analysis platform and by integrating our dataset with the proteomes of uterine and yolk sac fluids, at the corresponding timepoint of 14 days post-ovulation.

A key question of this study was how pregnancy affects the systemic proteome and what pathways are engaged in the mare's response to pregnancy at Day 14. This study focused on mated mares to explore physiological changes associated with conceptus presence and early pregnancy. While we categorized mares as pregnant (P) or non-pregnant (NP) based on pregnancy confirmation on Days 12–14, unmated mares were not included in the study. This limitation arose from the commercial farm setting, where unmated mares within the target age group and reproductive schedule were unavailable. Including unmated mares as a comparison group could provide valuable additional insights. This approach would help to further differentiate general post-ovulation physiological changes from those specific to pregnancy, enhancing biomarker discovery efforts. Future studies incorporating such parallel groups could further refine the understanding of early pregnancy and improve diagnostic marker development.

Proteins altered in pregnant animals included complement C1s, immunoglobulin lambda light chain variable region, serpins, and alpha-1-antitrypsin (Figure 2C and Table 1). While only a small number of proteins were consistently altered in the pregnant state, our pathway enrichment analysis of pregnancy-associated changes identified 12 pathways enriched in upregulated proteins, and five pathways enriched in downregulated proteins (Table 2). All downregulated pathways pertained to immune system function and the complement cascade. Meanwhile, pathways enriched in the cohort of upregulated proteins pertained to platelets/clotting/hemostasis, lipoprotein assembly, immune system functions, protein phosphorylation, and regulation of insulin-like growth factor (IGF) transport and uptake. These findings indicate that an immunological response to early pregnancy is well underway by Day 14. Further analysis of earlier timepoints would reveal whether this process precedes the hormonal recognition of pregnancy.

Complement proteins and immunoglobulin components are likely altered as part of the modulation of the immune system to facilitate tolerance of the embryo. Complement proteins (e.g.,

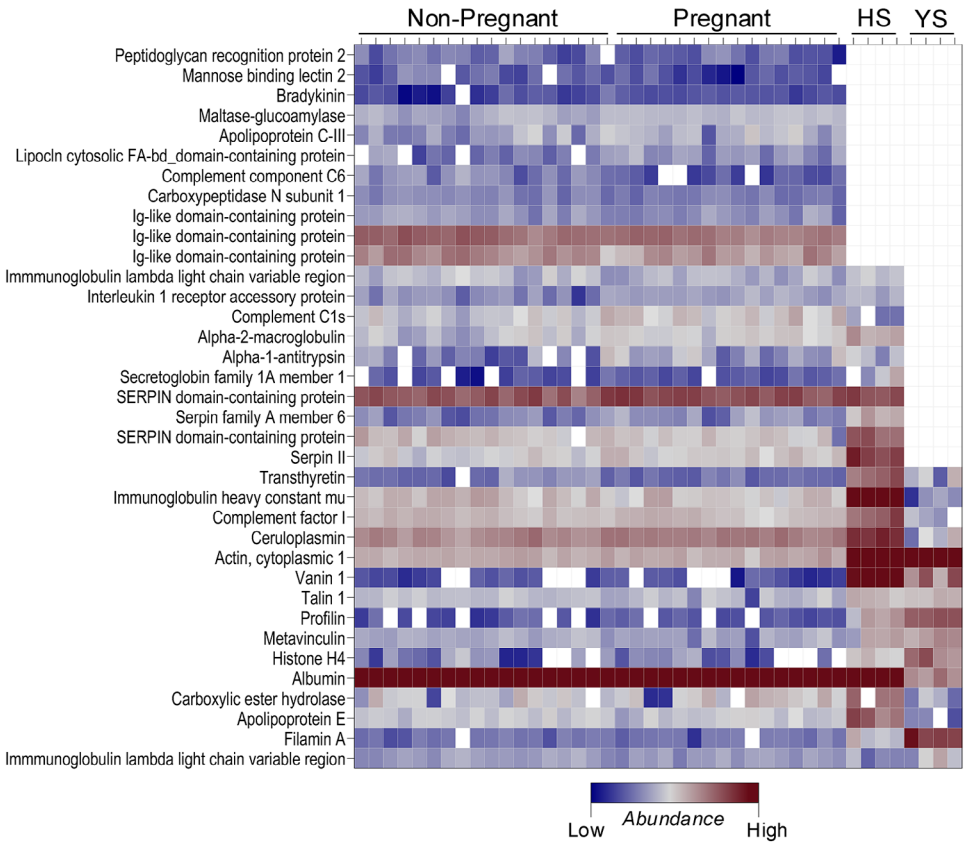

FIGURE 5 | Individual protein abundance across mare plasma, histotroph, and yolk sac proteomes. Relative abundances of proteins identified repeatedly across two or more compartments are displayed in a heatmap. Each row represents a unique protein, while each column corresponds to an individual sample from one of the three biological sources. The color intensity within the heatmap denotes the level of protein abundance, with red indicating higher abundance and blue indicating lower abundance.

C6, C1s, and factor I) are part of the immune system, which needs to be carefully regulated during pregnancy to protect the developing fetus [27]. Immunoglobulins and immunoglobulin-like proteins are part of the adaptive immune response and could be involved in protecting the pregnancy from pathogens, or inducing immune tolerance mechanisms to prevent an attack by the maternal system. Little is known about the immune response at this early stage of pregnancy, but alterations in these proteins at Day 14 suggest that immune recognition of pregnancy is already taking place well before implantation. Serpins, including alpha-1-antitrypsin, are protease inhibitors and have wide-ranging roles. Of relevance here are their documented roles in early pregnancy, including immunomodulatory functions and transplacental transport [28, 29]. Together, the pregnancy-associated plasma changes identified in this study point to a response to early pregnancy that encompasses enhanced immune protection, tissue remodeling, and metabolic adaptations to support early embryonic development and protection.

To gain an overview of functions represented by the pregnant mare plasma proteome, the full complement of proteins identified in pregnant mare plasma was subjected to IPA analysis, focusing on the disease and function nodes. The most significantly enriched functions were those related to hematological, cell signaling, immunology, lipid metabolism, and embryonic development (Figure 3C and Table S3). These outputs are largely expected and reflect the activity of cell types (along with their secreted components) and clotting factors typically found in the circulation. In an attempt to better understand the significance, functions and possible origins of plasma proteins, we then compared the plasma datasets with proteomes generated from a different cohort of 14-day pregnant Warmblood mares uterine luminal fluid, and from the yolk sac fluid of 14-day embryos (Figures 4 and 5). The histotroph is the nutritive secretion from the endometrium during early pregnancy and is solely responsible for supporting the embryo before placental formation. Proteins highly expressed in this fluid might be crucial for nutrient transfer, immune modulation, or signaling between mother and embryo. Proteins with common expression in pregnant mare plasma and in the histotroph are expected to be either ubiquitous or involved in pregnancy establishment and/or maternal support for the embryo. We note that the histotroph and yolk sac fluid samples came from a different cohort of mares to the blood plasma samples, and acknowledge this is a limitation of the study, as differences in genetics, nutrition, and season could all contribute to protein expression. Nevertheless, an analysis of the pathways common to or uniquely enriched across the three fluids should identify processes that are important in cross-talk and pregnancy establishment, and reveal the continuity of signaling pathways from embryo to dam and vice versa, even where specific individual proteins are not conserved between compartments.

We found that 132 plasma proteins were also detectable in the uterine lumen (Figure 4A). These may be involved in the

nutritional support of the developing embryo, or could comprise non-selective transudate from plasma into the uterine fluid. Only 19 proteins were shared between mare plasma and the yolk sac fluid, despite a total of 1358 proteins detected in the latter. Only 17 proteins were common to all three datasets; of these, none were significantly upregulated in pregnant versus non-pregnant mare plasma. Many of these are common structural or ubiquitous proteins (e.g., actin, filamin, albumin) or immunoglobulins. It, therefore, seems unlikely that any pregnancy-associated plasma proteins are directly derived from the embryo itself, but are probably rather the result of signaling cascades initiated by the embryo and mediated by the endometrium.

As seen in Figure 4B, pathways enriched in the uterine luminal fluid of pregnant mares were often also enriched in either the yolk sac fluid or the blood plasma, but rarely both. This is consistent with the histotroph's role as a conduit for messaging and nutrient shuttling between the mare and the conceptus, and vice versa. The categories conserved among all three components were "*infection by RNA virus*" and "*migration of cells*," likely alluding to the wide range of molecular processes encompassed by both of these classifications, including transcription, translation, protein processing, and protein transport. Viral production and functions related to viruses and RNA displayed pronounced enrichment in the yolk sac fluid, surpassing levels observed in histotroph and plasma. This enrichment was the highest score derived from IPA analysis. Whilst growth and, therefore, protein synthesis and processing are evidently the major activities of a developing embryo, overrepresentation of the "*production/replication of virus*"—related categories in the yolk sac fluid proteins may also point to the role of virus-like processes at the embryo-maternal interface and the phenomenon of retrovirus-derived genes co-opted by the trophoblast for implantation. While typically associated with invasive placentation types, endogenous retroviral envelope genes have been mapped in the horse and found to be preferentially expressed in placental tissue, pointing to a yet-uncharacterized but possibly significant role in placentation [30]. In other species—those with invasive placentae—such genes have given rise to syncytins, proteins of viral evolutionary origin with key roles in the invasive function of the syncytiotrophoblast, and possibly in the induction of maternal immune tolerance [31]. The prominence of these categories in our analysis suggests that conserved virus-like molecular processes, as in other species, may be used by the equine embryo to initiate implantation and placentation and induce immune tolerance, despite a non-invasive (epitheliochorial) placentation type. Further investigation of the proteins comprising these categories is worthwhile and may shed light on these poorly characterized aspects of early pregnancy in the horse. Other pathways identified were processes relating to lipid synthesis and transport (overrepresented in plasma) and those relating to protein synthesis (overrepresented in the conceptus). These patterns suggest lipid production is predominantly maternal and lipids are shuttled via the histotroph toward the conceptus, forming the primary energy source for protein synthesis in the embryo to be used for growth and development, and likely signaling.

When considering the individual proteins associated with pregnant mare plasma and also found in the histotroph and/or conceptus, we did not identify any proteins that were consistently upregulated in pregnant mare plasma and simultaneously abundant in both histotroph and yolk sac proteomes (Figure 5). However, one protein that was consistently downregulated in pregnant mare plasma was also detected in both histotroph and yolk sac: this was transthyretin (TTR), a protein responsible for the transport of thyroxine and retinol. Both of these hormones are critical for early embryo development, meanwhile, TTR dysregulation has been associated (in humans) with severe early-onset pre-eclampsia, intrauterine growth restriction, and early pregnancy loss [32, 33]. Emerging evidence suggests TTR's significant involvement in modulating immune responses, particularly within the uterine environment during early pregnancy. Its dual role as a transporter and a cryptic protease underscores its biological importance. Notably, TTR has been shown to cleave apolipoprotein A1 (ApoA1), leading to the inhibition of interleukin-1β (IL-1β), a key pro-inflammatory cytokine involved in immune signaling [34]. The distribution of TTR in our datasets may indicate an active sequestration of this transporter to the uterine lumen for the purpose of delivering these factors to the embryo, or reduced hepatic production of TTR in response to pregnancy. In the context of equine pregnancy, the observed upregulation of TTR in uterine fluid from infected mares treated with cortisone suggests that its role may be associated with maternal immune responses during abnormal conditions, such as infection or inflammation [34]. Previous studies on endometrial fluid proteomics have highlighted TTR's involvement in tissue defense and inflammatory regulation, particularly under stress or pathological conditions. In contrast, our findings of decreased TTR in normal pregnant mare plasma align with the notion that lower TTR levels may be indicative of a healthy, unperturbed pregnancy. Our findings of decreased plasma TTR in pregnant mares align with a recent finding in mice and women, whereby serum TTR decreased dramatically in early gestation [35]. Here, transgenic overexpression of human TTR in mice interfered with normal pregnancy (causing reduced implantation units, lower fetal weights, and smaller litter sizes). Thus, TTR regulation appears to be critical to a healthy pregnancy, and may be similarly important in the horse, warranting further investigation of both its functional role and use as a diagnostic biomarker. This supports the hypothesis that TTR regulation is context-dependent, increasing during abnormal conditions but decreasing in normal pregnancy to facilitate a balanced uterine environment conducive to embryo development and implantation. These results emphasize the potential of TTR as a biomarker, not only for pregnancy but also for distinguishing between normal and abnormal reproductive states.

A handful of proteins were upregulated in pregnant mare plasma and abundant in histotroph, but not detected in the yolk sac fluid. These were interleukin 1 receptor accessory protein, complement C1s, alpha-2-macroglobulin (A2 M), secretoglobin 1A1, and five different serpins or serpin-domain containing proteins (including alpha-1-antitrypsin and serpin A6 or corticosteroid-binding protein; CBG). Of these, interleukin accessory proteins, A2 M, secretoglobin 1A1 (SCGB1A1; uteroglobin) and serpins are known to be produced by the endometrium and secreted into the uterine lumen, in other species or indeed in the horse [36–39]. Whilst our study cannot directly compare these proteins quantitatively across compartments, their abundance in histotrophs alongside an increase in plasma of pregnant animals points to an origin in uterine secretions that impacts systemic levels in the circulation. This provides the first evidence in this

species that uterine-derived proteins are elevated in plasma at Day 14 post-ovulation. Of particular interest is SCGB1A1, or uteroglobin. This protein is known to be secreted in the uterus [40] and present in the oviduct [10], but has not previously been shown to be elevated systemically in early equine pregnancy. As Ellenberger et al. [40] highlighted, uteroglobin may act as an anti-inflammatory and anti-chemotactic agent, protecting trophoblastic cells from immune rejection by binding to antigenic determinants and impeding the migration of immune cells. Uteroglobin has been shown to inhibit cyclooxygenase-2 (COX2) expression [41], and is an inhibitor of phospholipase A2, which is the rate-limiting enzyme for the synthesis of prostaglandins. Both of these processes—reduction of COX2 expression and inhibition of endometrial PGF2α production—are central components of MRP. Uteroglobin, detected in yolk-sac fluid at Day 15.5 but absent in later stages, indicates its transient presence during early pregnancy. Its transfer into the yolk-sac cavity prior to capsule modification suggests a potential role in supporting early embryonic development and regulating immune responses during this critical pre-fixation phase. The absence of uteroglobin in non-pregnant mares further reinforces its pregnancy-specific significance, likely associated with preparing the uterine environment and supporting early embryonic development [42]. In light of the systemic increase of uteroglobin in pregnant mares in this study, these observations position uteroglobin as potentially a more important player in early pregnancy establishment and MRP than was previously thought.

In conclusion, this study has, for the first time, identified consistent systemic changes in the pregnant mare proteome at 14 days post-ovulation. These changes point to a profound and tightly orchestrated response to early pregnancy by the immune system, which likely parallels that of the endocrine system. We tentatively conclude that an "immune recognition of pregnancy" occurs well before implantation in this species, despite the absence of any structural integration of conceptus with maternal tissue at this stage of gestation. Based on the overlap of viral infection-related pathways and responses across the conceptus, uterine fluid, and maternal plasma proteomes, we suspect that the conceptus uses a strategy of immune system evasion not dissimilar to that deployed by viruses, and consider that this hypothesis deserves further investigation. Through the integration of blood plasma, embryo, and uterine fluid proteomes, this study also identified TTR and uteroglobin (SCGB1A1) as proteins of likely major functional significance in the establishment of early pregnancy in the mare. We anticipate that further functional investigation of these hypotheses will lead to novel diagnostic and treatment approaches to enhance mare fertility and reduce the incidence of early pregnancy loss in this species.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Mass spectrometry proteomics data were deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [43] with the dataset identifier PXD051312 at https://doi.org/10.6019/PXD051312.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.