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Original Research Article

Associations between immunological and hormonal parameters during healthy pregnancy in mares

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ABSTRACT

Associations between the immune and endocrine systems during equine pregnancy remain poorly understood. Based on the hypothesis that distinct phases of the equine healthy gestation are characterized by specific associations between circulating immunological and hormonal parameters, contributing to pregnancy maintenance, this investigation aimed to: (i) evaluate how circulating immunological and hormonal parameters change across different phases of pregnancy; (ii) investigate associations between changes in circulating immunological and hormonal parameters; and (iii) propose potential hormonal drivers of immunological modulation during pregnancy. Peripheral blood samples were prospectively collected from mares (n = 8) before ovulation and during pregnancy at 30, 90, 150, 210, 240, 270, 300, and 330 days of their healthy gestations. An immunological panel included the distribution of circulating T cell (CD3, CD4, and CD8) and B cell subpopulations, complete blood counts (CBC), and serum protein profile. Hormonal analyses included equine chorionic gonadotropin (eCG), progestogens, androgens, estrogens, corticosteroids, and thyroid hormones. At 90 days, a statistically significant increase in peripheral blood CD4 T cell distribution was accompanied by a concomitant reduction in B cell distribution. This immunological modulation correlated positively with eCG, progesterone (P4), 5α-dihydroprogesterone (DHP), and estrone sulfate, and inversely with B cell levels. In contrast, at 210 days, B cell distribution peaked significantly while CD4 T cell distribution declined, concomitant with a rise in albumin levels. These changes positively correlated with cortisone and hematocrit. Mid-gestation was characterized by associations between different androgens and circulating T cell and B cell distributions. Consistent negative associations were observed between progesterone, DHP, and estradiol-17β with glucocorticoid metabolites throughout gestation. Estradiol-17β and IgM concentrations showed a positive correlation in late gestation. Immune-hormone and hormone-hormone associations were more pronounced during early and mid-gestation, while the final 100 days of pregnancy were characterized by relatively constant levels. Collectively, our findings suggest immune-hormone associations that potentially orchestrate immunomodulation, fetal development, successful pregnancy maintenance, and parturition in the mare.

1. Introduction

The state of pregnancy represents a complex challenge for the immune system of the mare: immunomodulation is critical for maternal tolerance to the presence of the fetus and appropriate fetal development, while a functional immune system is required to protect the dam and fetus against infections [1]. Maternal tolerance to the fetus involves several processes at the maternal-fetal interface and systemic circulation [2,3]. In pregnant women, hormones contribute significantly to a shift in the immune function over the three trimesters of pregnancy [4]. However, the understanding about the relationship between the immune and endocrine systems remains poorly described in the pregnant mare.

Studies describing the distribution of CD4 T cells, CD8 T cells, and B cells in peripheral blood throughout pregnancy in mares remain incomplete. Most available data have focused on late gestation [5,6]. Agrícola et al. (2008) reported a significant decline in CD3 T cells during

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the last third of pregnancy compared to early postpartum but found no changes in CD4 and CD8 T cell subpopulation distributions [5]. In contrast, B cell distribution was lower in early postpartum compared to the final third of gestation [5]. Aoki et al. (2013) evaluated immunological parameters in periparturient mares and found no significant changes in CD4 T cell, CD8 T cell, and B cell distributions during the last four weeks of pregnancy [6].

Dynamic hormonal changes take place during pregnancy in the mare. Approximately 8 days post-ovulation, progesterone levels are maintained by the primary corpus luteum (CL). The trophoblast-derived endometrial cups produce high concentrations of equine chorionic gonadotropin (eCG), which is detectable in the circulation from 40 to 120 days of pregnancy, with a peak around 70 days [7–9]. The eCG luteinizing activity promotes the formation of secondary CLs and the ovarian production of progesterone and estrogen [7,10–12]. Around days 110–120, ovarian progesterone declines as the corpora lutea regress. The feto-placental unit then assumes progestogen synthesis, marking the luteal–placental shift [12]. This transition is indicated by the 5α-reduction of progesterone in the placenta, leading to undetectable circulating progesterone and a parallel rise in 5α-dihydroprogesterone (DHP) concentrations [11,12]. Progesterone and associated progestogenic compounds are essential for pregnancy maintenance and counteract prostaglandin synthesis, which in the early stages of pregnancy could result in luteolysis of the CL, and later in pregnancy cause myometrial contractions [11].

Estrogens are produced throughout pregnancy, and their concentrations and sources vary by gestational stage. During early pregnancy, estrone sulfate and estradiol-17β are primarily synthesized by the conceptus and later by the primary and accessory CLs, stimulated by eCG [13,14]. In later stages, the equine fetal gonads contain high concentrations of dehydroepiandrosterone (DHEA), which serves as the primary androgen substrate for estrogen synthesis via aromatization in the placenta [15,16].

In addition to their roles in pregnancy establishment and maintenance, studies in women have shown that progestogens, estrogens and androgens display immunomodulatory properties for maternal-fetal tolerance [17–23]. To our knowledge, no studies have examined blood immunological changes throughout pregnancy in mares while specifically addressing their relationship with hormonal fluctuations. The objectives of this study were to: i) evaluate how circulating immunological and hormonal parameters change across different phases of pregnancy; ii) investigate associations between changes in immunological and hormonal parameters; and iii) propose potential hormonal drivers of immunological modulation during pregnancy. We hypothesized that distinct phases of the equine healthy gestation are characterized by associations between circulating immunological and hormonal parameters.

2. Material and methods

This study was carried out during the physiological breeding seasons in the Northern Hemisphere between 2021 and 2023. All animal procedures were approved by the Institutional Animal Care and Use Committee (protocol #2018-0030), and samples were obtained with the owners' consent.

2.1. Study design

Eight Thoroughbred mares (mean 9 ± 0.8, range 6 and 13 years of age) housed on private broodmare farms in Central Kentucky, USA were enrolled in this prospective study based on the following criteria: healthy status with no signs of systemic disease or reproductive disorders during the study period, as determined by repeated physical and reproductive examinations, including transrectal ultrasonography performed at the timepoints described below. Only mares with normal parturition and delivery of healthy foals were included. The mares were vaccinated against equine herpesvirus-1 (Pneumoabort-K® + 1b, Zoetis, Pasippany-Troy Hills, NJ, USA) at the third, fifth, seventh, and nineth months of pregnancy. Additional vaccinations included those against Eastern and Western Equine Encephalitis viruses, West Nile virus, rabies virus, tetanus, influenza virus, equine herpesvirus-1 and 4 (Core Equine Innovator® and Fluvac Innovator EHV-1/4, Zoetis), rotavirus (Equine Rotavirus, Zoetis), and botulism (*Clostridium botulinum* type B toxoid Neogen®Vet BotVax® B, Neogen, Lansing, MI, USA) administered 30 days prior to the expected foaling date. The day of ovulation was defined as Day 0 (zero) and all pregnancies were confirmed by transrectal ultrasonography at 14 days post-ovulation. Blood samples were collected at nine time points: immediately prior to ovulation of the estrous cycle leading to pregnancy or the preceding cycle, defined as *pre-ovulation*, and subsequently at *30*, *90*, *150*, *210*, *240*, *270*, *300*, and *330 days of gestation*.

2.2. Blood sampling and processing

Blood samples were collected via jugular venipuncture into vacutainer tubes containing sodium heparin, ethylenediaminetetraacetic acid (EDTA), and tubes without anticoagulant and were kept on ice until processing. Heparinized and serum samples were centrifugated at 700×*g* for 15 min within 2 h after collection, and the separated plasma and serum transferred to fresh tubes. Aliquoted samples and remaining vacutainer tubes (sodium heparin and EDTA) with whole blood (not centrifuged) were transported overnight to the Equine Immunology Laboratory, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA. Upon arrival, serum and plasma samples were stored at −80 °C until further analysis. The EDTA samples were immediately processed for complete blood count (CBC), whereas sodium heparin tubes were used for peripheral blood mononuclear cell (PBMC) isolation and subsequent lymphocyte phenotyping according to the methods below.

2.3. Immunological parameters

The peripheral blood lymphocyte subpopulation distribution was determined using flow cytometric analysis by the Equine Immunology Laboratory, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA, and as previously published [24–26]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from heparinized samples using Ficoll-Paque TM Premium 1077 (GE Health Care, Chicago, IL) density centrifugation (700×*g* for 15 min). Cells were treated with a 20 min blocking step using 10 % goat serum (Jackson ImmunoResearch Lab, West Grove, PA) to prevent non-specific first-stage antibody reagent binding. Next, 1×10^6 PBMCs were incubated individually with murine monoclonal antibodies to the following antigens: equine CD3 (clone F6G.3-G12, UC Davis), equine CD4 (clone HB61A, Washington State University), equine CD8 (clone HT14A, Washington State University), and human CD21 (clone B-ly4, BD Pharmingen). These reagents were validated in previous studies [26–28]. Second-stage labeling was performed using fluorescein isothiocyanate (FITC)-conjugated AffiniPure goat anti-mouse IgG (H + L) monoclonal antibody (Jackson ImmunoResearch Lab, West Grove, PA). Processed cells were acquired and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and Cell Quest software (Becton Dickinson, San Jose, CA). Percentages of positive cells were measured within the lymphocyte-gated area determined by forward- and side-scatter plots. Subpopulation lymphocyte counts were calculated by multiplying the percentage of positive cells and the CBC absolute lymphocyte count of the same sample.

Complete blood count (CBC) and serum total protein, albumin, and globulin concentrations were analyzed from EDTA-treated samples by the Clinical Pathology Laboratory at the Animal Health Diagnostic Center (AHDC), College of Veterinary Medicine, Cornell University, Ithaca, NY, USA.

All serum samples were analyzed simultaneously within the same

assay following storage to minimize analytical variation. For immunoglobulin quantification, serum IgM and IgG concentrations were measured using a commercial radial immunodiffusion assay kit (Radial immunodiffusion kit SRID, Triple J Diagnostics, Bellingham, WA). For each isotype, a standard curve was generated by graphing known concentrations (mg/dL) of immunoglobulin solutions provided by the manufacturer and their diffusion diameter (mm) at 48 h. Serum cytokines IL-4, IL-10, IL-17, interferon (IFN)-α, and IFN-γ were analyzed by the Serology Laboratory at the Cornell University AHDC using a validated equine cytokine 5-plex assay [29]. Serum tumor necrosis factor (TNF)-α concentrations were measured in duplicate using a commercially available equine-specific enzyme-linked immunosorbent assay (ELISA) kit (Equine TNF-α Duo-Set, R&D Systems, Minneapolis, MN), following the manufacturer's protocol, and as previously published by this laboratory [30]. Serum concentrations of IL-6 were determined in duplicates using a commercially available equine-specific IL-6 ELISA kit (Equine IL-6 Duo-Set, R&D Systems, Minneapolis, MN) following the manufacturer's protocol, and as previously described [31,32]. Intra- and inter-assay coefficients of variation (CVs), and minimum detection values are reported in Table S.1.

2.4. Equine chorionic gonadotropin

Serum samples collected at 30, 90 and 150 days of pregnancy were submitted to the Endocrinology Laboratory at the Cornell University AHDC for determination of equine chorionic gonadotropin (eCG) concentrations using a commercially available ELISA.

2.5. Steroid hormones profiling

Plasma samples were shipped on dry ice to the West Coast Metabolomics Center at the University of California-Davis, Davis, CA for targeted steroid quantification by mass spectrometry. The targeted steroid panel included the following analytes: progesterone (P4), pregnenolone (P5), allopregnanolone (3α-DHP), 17α-hydroxyprogesterone (17OH-progesterone), 17α-hydroxypregnenolone (17OH-pregnenolone), 20α-hydroxy-5α-dihydroprogesterone (20α-DHP), 2-methoxyestradiol, estradiol-17β, estriol, androstenedione, androsterone, androsterone glucuronide, androstenediol, epiandrosterone, testosterone, testosterone glucuronide, 5α-dihydrotestosterone (5α-DHT), 5β-dihydrotestosterone (5β-DHT), etiocholanolone, 11-deoxycortisol, cortisone, corticosterone, deoxycorticosterone, aldosterone, liothyronine (T3), thyroxine (T4), 7α-hydroxycholesterol, and 7-ketocholesterol. Plasma samples were also shipped on dry ice to the Department of Clinical Chemistry, University Hospital (CHU), University of Liège, Belgium, for quantification of 5α-dihydroprogesterone (DHP), dehydroepiandrosterone (DHEA), cortisol, estrone, and estrone-sulfate using Liquid Chromatography coupled with electrospray tandem mass spectrometry (LC-MS/MS) as previously described [33,34]. Minimum detection limits are reported in Table S1.

Plasma estradiol-17β (E2) concentrations were analyzed by the Human Nutritional Chemistry Service Laboratory, Division of Nutritional Sciences, Cornell University, Ithaca, NY, USA, using a commercial immunoassay kit (Estradiol, Siemens Healthcare Diagnostics, Tarrytown, NY, USA) and a chemiluminescent platform (Immulite® 2000, Siemens Healthcare Diagnostics, Tarrytown, NY, USA) as previously described [32,35]. Intra- and inter-assay CVs, and minimum detection limits are reported in Table S1. Cross-reactivity for this assay was non-significant for most steroid hormones. The specificity of this assay for the equine hormone was previously published [32]. The steroidogenic pathways analyzed, including progestogens, androgens, estrogens, and corticosteroids are illustrated in Fig. S1 [36,37].

2.6. Erythropoietin

Serum erythropoietin (EPO) concentrations were determined using a commercially available equine-specific EPO ELISA kit (Horse EPO ELISA kit, Abbexa LTD, Cambridge, UK) following the manufacturer's protocol.

2.7. Data analyses

All data were assessed for normality using the Shapiro-Wilk test. Non-normally distributed data were log-transformed. Linear mixed models were used to assess changes through time in immunological and hormonal parameters, with mare included as a random effect and gestational age timepoint as fixed effect. Post-hoc comparisons were performed using Tukey test, with P-values adjusted accordingly. Spearman's rank correlation coefficient was used to determine the strength and direction of the relationship between immunological and hormonal parameters. For the correlation analysis, pregnancy was divided into five gestational periods: **Period 1** (30–90 days), **Period 2** (91–150 days), **Period 3** (151–240 days), **Period 4** (241–270 days); and **Period 5** (271–330 days). For statistical analyses, when the results were below the detection limit of the assay (i.e., non-detected concentration of cytokines and hormones), a value of zero was assigned. Statistical analyses were performed using JMP Pro 16 (SAS Institute Inc., NC, USA). Statistical significance was set at P-value <0.05.

3. Results

3.1. Immunological parameters

Circulating white blood cell (WBC) and neutrophil counts increased at 330 days of pregnancy compared to 270 days ($P < 0.01$) (Fig. 1). The remaining CBC parameters are depicted in Fig. S2. There were no statistically significant differences in the analyses of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) throughout pregnancy (Fig. S2).

There were no statistically significant differences in absolute lymphocyte counts throughout the study period (Fig. 1). The analysis of peripheral lymphocyte subpopulation distributions during pregnancy revealed two dynamic changes: at 90 days of pregnancy, the CD4 T cell distribution increased ($P = 0.03$), accompanied by a concomitant decrease in B cell distribution ($P < 0.01$); and at 210 days of pregnancy, the opposite trend was detected, with a peak in B cell ($P < 0.01$) and a decrease in CD4 T cell ($P = 0.03$) distributions (Fig. 1). Serum IgM concentrations showed no statistically significant differences through time. Serum IgG concentrations peaked at 90 days of pregnancy ($P < 0.01$) compared with the pre-ovulation timepoint, and 240 and 270 days of pregnancy (Fig. 1).

Total protein concentrations did not change through time (Fig. 2). However, serum albumin concentrations showed an increase at 210 days of pregnancy ($P < 0.05$) (Fig. 2). Serum albumin concentrations followed a similar pattern to hematocrit, hemoglobin concentration, and red blood cell (RBC) counts, with an increase at 210 days of pregnancy ($P < 0.05$) (Fig. 2). Graphical comparison of the serum albumin and globulin concentration changes through time revealed an opposing trend: globulin concentrations peaked during the first half of pregnancy, while albumin concentrations increased in the second half. Additionally, albumin/globulin ratio followed albumin fluctuations (Fig. 2). There were no statistically significant differences in the analyses of serum cytokine concentrations throughout time (Fig. 3).

3.2. Equine chorionic gonadotropin

For the hormonal analyses, serum eCG concentrations peaked at 90 days of pregnancy ($P < 0.01$) compared to other timepoints (Fig. 4).

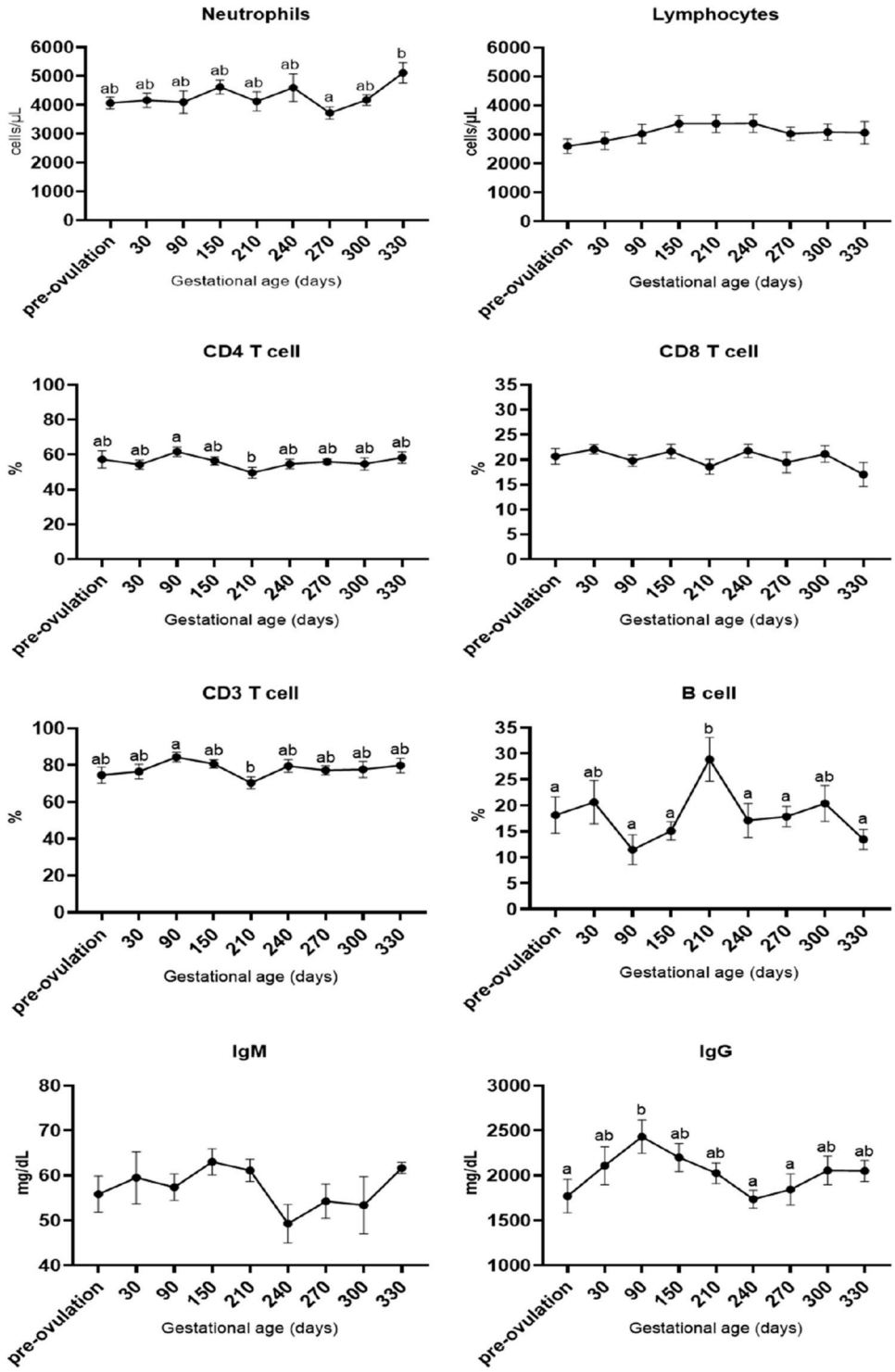

Fig. 1. Peripheral blood neutrophil and lymphocyte absolute counts, lymphocyte subpopulation distributions (CD3 T cell, CD4 T cell, CD8 T cell, and B cell), and serum IgG and IgM concentrations measured in healthy mares (n = 8) before ovulation and throughout gestation. Different letters represent statistically significant differences between timepoints ($P < 0.05$). Data are shown as mean ± SEM.

3.3. Steroid hormones profiling

Progesterone concentrations were detectable during the first half of pregnancy, peaking at 150 days ($P < 0.01$), and became undetectable thereafter (Fig. 5). In contrast, circulating DHP concentrations were present during the first half of gestation, and increased markedly after 150 days ($P < 0.01$), remaining elevated toward the 330 days of gestation (Fig. 5). Both 17OH-progesterone and 17OH-pregnanolone paralleled progesterone concentrations, with a peak ($P < 0.01$) during the first half of pregnancy and a decline after 150 days of gestation (Fig. 5). No statistically significant temporal variations were observed in allopregnanolone concentrations.

For androgens, DHEA concentration remained low until 90 days of pregnancy, began to rise at 150 days, and peaked at 210 days ($P < 0.01$) (Fig. 6). Androstenediol and androsterone glucuronide followed a similar pattern, with low plasma concentrations during the first 150

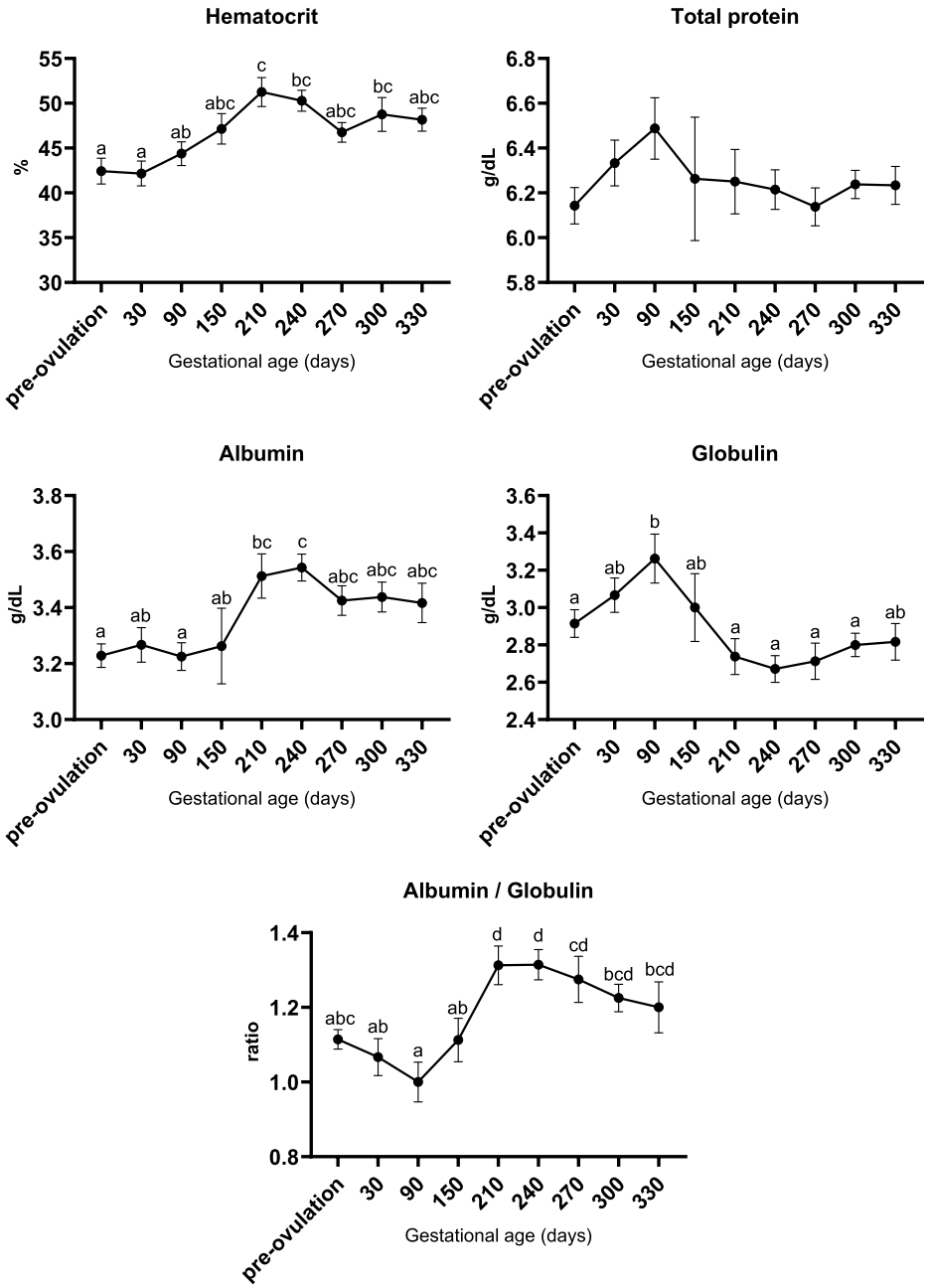

Fig. 2. Hematocrit and serum protein concentrations (total protein, albumin, globulin, and albumin to globulin ratio) measured in healthy mares (n = 8) before ovulation and throughout gestation. Different letters represent statistically significant differences between timepoints ($P < 0.05$). Data are shown as mean ± SEM.

days of pregnancy, followed by a significant increase thereafter ($P < 0.01$) (Fig. 6). In contrast, no statistically significant differences were detected in plasma androstenedione and etiocholanolone concentrations through time (Fig. 6).

Estrogens showed a similar pattern in our study, with estrone and estrone-sulfate concentrations beginning to rise in the second half of pregnancy and peaking around 150 and 210 days ($P < 0.01$) (Fig. 7). Plasma estradiol-17β concentrations were assessed using both mass spectrometry and immunoassay. Both analytical methods revealed similar dynamics throughout pregnancy, with a pattern comparable to that of estrone and estrone-sulfate. Despite differing units of measurement, the immunoassay detected detailed increases in estradiol-17β after 210 days of gestation ($P < 0.01$) (Fig. 8).

Cortisol concentrations remained relatively stable throughout pregnancy, with the exception of a distinct peak observed at 150 days of gestation ($P < 0.01$), followed by a noticeable decline by 210 days (Fig. 9). Plasma corticosterone concentrations peaked at 150 days of pregnancy compared to pre-ovulation timepoint, 30 and 300 days of pregnancy ($P = 0.01$) (Fig. 9). In contrast, no statistically significant differences were detected in plasma 11-deoxycortisol, cortisone, aldosterone, and thyroxine concentrations (Fig. 9).

Plasma concentrations of cholesterol metabolites 7α-hydroxycholesterol and 7-ketocholesterol were elevated at the pre-ovulation timepoint and remained high in the first 150 days of pregnancy ($P < 0.01$), with concentrations becoming undetectable thereafter (Fig. 10).

Mass spectrometry data for the following hormones were not provided by the Metabolomics Center at any timepoint and, consequently, results were not reported and analyzed using this assay: 20α-DHP,

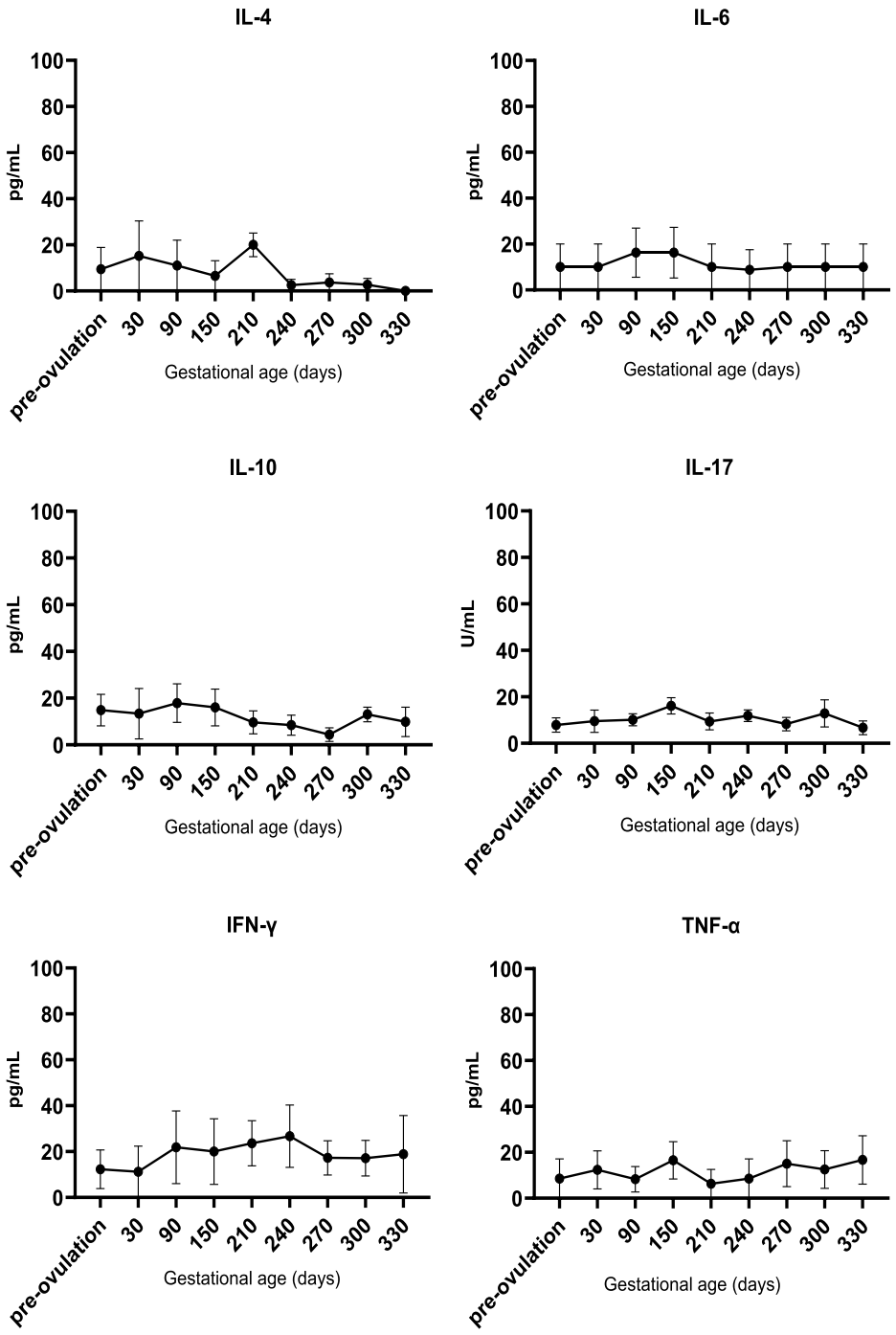

Fig. 3. Serum cytokine concentrations (IL-4, IL-6, IL-10, IL-17, IFN-γ, and TNF-α) measured in healthy mares (n = 8) before ovulation and throughout gestation. Different letters represent statistically significant differences between timepoints ($P < 0.05$). Data are shown as mean ± SEM.

pregnanolone, androsterone, epiandrosterone, testosterone, testosterone glucuronide, 5α-DHT, 5β-DHT, estriol, 2-methoxyestradiol, deoxycorticosterone, and T3.

3.4. Erythropoietin

No statistically significant differences were detected in the analyses of serum erythropoietin concentrations through time (Fig. S3).

3.5. Correlation results

Immune-hormone and hormone-hormone parameter correlation analyses were performed in the 5 gestational periods described above and only statistically significant correlations between immune-hormone and hormone-hormone parameters are presented. Correlations observed during the gestational period 1 (30–90 days of pregnancy) are summarized in Table 1. Positive correlations were observed between eCG concentration and CD3 T cell and CD4 T cell distributions. In contrast, eCG concentration showed a strong negative correlation with B cell distribution. A similar pattern was observed for estrone-sulfate concentration, which was positively correlated with CD3 T cell and negatively correlated with B cell distributions. Progesterone and DHP concentrations were negatively correlated with B cell distribution. Globulin concentrations were positively correlated with IgG ($r = 0.64$, p

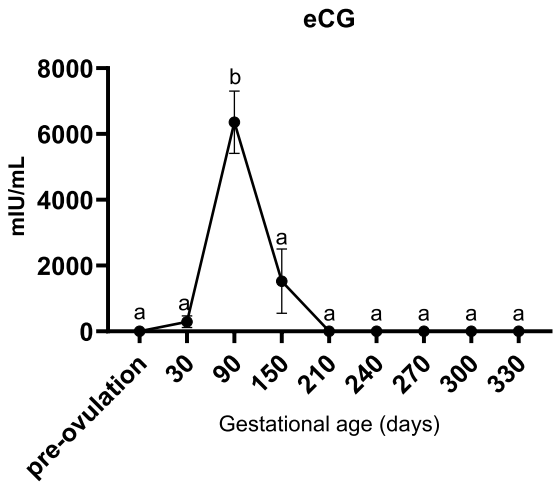

Fig. 4. Plasma equine chorionic gonadotropin (eCG) concentrations measured in healthy mares (n = 8) before ovulation and throughout gestation. Different letters represent statistically significant differences between timepoints ($P < 0.05$). Data are shown as mean ± SEM.

= 0.01) but not with IgM concentrations, and this pattern was consistent across all gestational periods. The eCG was positively correlated with progesterone, DHP, and estrone-sulfate concentrations. Estrone-sulfate was also positively correlated with progesterone and DHP concentrations ($r = 0.66$, $p < 0.01$). Additionally, eCG was positively correlated with androstenediol, while progesterone and DHP showed negative correlations with cortisone concentrations.

Correlations during gestational period 2 (91–150 days of pregnancy) are summarized in Table 2. Regarding immune-hormone correlations, androsterone glucuronide and DHEA concentrations were negatively correlated with CD4 T cell distribution. The DHEA concentration showed a positive correlation with B cell distribution. Additionally, progesterone concentration maintained its negative correlation with B cell distribution, although the association was weaker compared to gestational period 1. A negative correlation was observed between IgG and IgM concentrations ($r = -0.58$, $p = 0.01$). Although eCG maintained positive correlation with progesterone, it was negatively correlated with DHP and estrone-sulfate concentrations. Progesterone maintained its negative correlation with cortisone and showed additional negative correlations with DHP and estrone-sulfate concentrations. The eCG also showed negative correlations with cortisone, estradiol-17β, and DHEA concentrations. Progesterone was negatively correlated with DHEA and androsterone glucuronide, and positively correlated with androstenedione, whereas estradiol-17β displayed the opposite pattern. Furthermore, estradiol-17β was negatively correlated with eCG, progesterone, and cortisone concentrations. Positive correlations were observed between estrone-sulfate and both 11-deoxycortisol and corticosterone concentrations.

Immune-hormone and hormone-hormone correlations identified during the gestational period 3 (151–240 days of pregnancy) are presented in Table 3. Both androstenediol and DHEA concentrations were negatively correlated with CD3 T cell distribution and positively correlated with B cell distribution. Additionally, androstenediol was negatively correlated with IgG concentrations. Cortisone concentration was positively associated with hematocrit ($r = 0.48$, $p = 0.03$). Furthermore, albumin concentration was positively correlated with hematocrit ($r = 0.76$, $p < 0.01$), hemoglobin concentration ($r = 0.78$, $p < 0.01$), and RBC count ($r = 0.46$, $p = 0.03$). Progesterone was negatively correlated with both DHP and estradiol-17β concentrations. Additionally, DHP and estradiol-17β showed negative correlations with glucocorticoid metabolism: DHP with cortisol and corticosterone, and estradiol-17β with corticosterone and cortisone concentrations. Estrone-sulfate showed positive correlations with both cortisol and corticosterone concentrations. Additionally, DHEA was positively correlated with both estradiol-17β and androstenediol concentrations (Table 3).

Estrogen and androgen metabolism characterized the gestational period 4 (241–270 days of pregnancy (Table 4). Estradiol-17β was positively correlated with both IgM and DHEA, which also showed a positive correlation with estrone-sulfate concentration. Androstenediol was positively correlated with androsterone glucuronide concentration. Additionally, the significant positive correlations observed in gestational period 3 among hematocrit, hemoglobin concentration, RBC count, albumin, and cortisone were maintained during gestational period 4.

Correlations identified during gestational period 5 (271–330 days of pregnancy) are presented in Table 5. Estradiol-17β concentration was negatively correlated with CD3 T cell and CD4 T cell distributions, as well as with cortisone concentration. Estrone-sulfate was positively correlated with estradiol-17β, DHEA, cortisone, and 11-deoxycortisol, and negatively correlated with DHP concentrations (Table 5).

Fig. 11 depicts proposed relationships of circulating immune-hormone changes during pregnancy in healthy mares.

4. Discussion

This study revealed dynamic immune-hormone and hormone-hormone associations at different gestational periods, suggesting their potential roles in the immunomodulation of the maternal immune system for pregnancy establishment and maintenance, fetal development, and successful parturition in mares. The eCG, secreted by trophoblast-derived endometrial cups during early gestation, is a unique feature of equine pregnancy. In this study, eCG levels were positively correlated with CD4 T cell distribution, with both peaking at 90 days of gestation. While studies investigating the effects of eCG on T cell responses in horses are scarce, insights from human chorionic gonadotropin (hCG)-related immunomodulation may offer relevant parallels, given their shared biological roles and functional similarities [38]. Several of these studies showed that hCG upregulates FoxP3 expression and promotes the expansion of regulatory T cells (Tregs), which play a critical role in maintaining immune tolerance during pregnancy [39,40]. Progesterone contributes synergistically with hCG to maternal-fetal tolerance by expanding Treg cells and suppressing pro-inflammatory cytokine production [22,41]. Immunological tolerance is essential for successful pregnancy, with Treg cells implicated in this process in cows, mice, and humans [2,3,42]. In the horse, the in vitro study by Flaminio and Antczak (2005) demonstrated that trophoblast cells or their conditioned medium, but not fibroblasts, decreased phytohemagglutinin-induced lymphocyte proliferation and cytokine expression, supporting a role for trophoblast-derived factors in modulating lymphocyte responses but not explaining the peak of circulating CD4 T cells [43].

In the horse, de Mestre et al. (2010) reported no differences in peripheral blood lymphocyte CD4 and CD8 T cell distributions between pregnant and non-pregnant mares within 30 and 46 days of gestation, perhaps missing the peak observed around 90 days [44]. This same study observed a mild trend toward an increase in FoxP3- and a modest increase in IL-4-expressing circulating lymphocytes, suggesting that a subset of CD4 T cells were Tregs and/or had a Th2 bias during early pregnancy [44]. In the present study, the distribution of Tregs was not measured; however, there were no statistically significant differences in serum IL-4 concentrations or any other cytokines between timepoints throughout pregnancy, suggesting a low impact of systemic cytokines on the immune system during pregnancy. In the uterus, there is ambiguous evidence for immunomodulation during the early equine pregnancy at the endometrial cup site, with the surrounding presence of both FoxP3+ CD4 T cells and IFN-γ+ lymphocytes [44]. Despite these observations, it is still to be determined how immunomodulation is characterized and controlled systemically and locally in early gestation.

Notably, during the first half of pregnancy, there was a significant

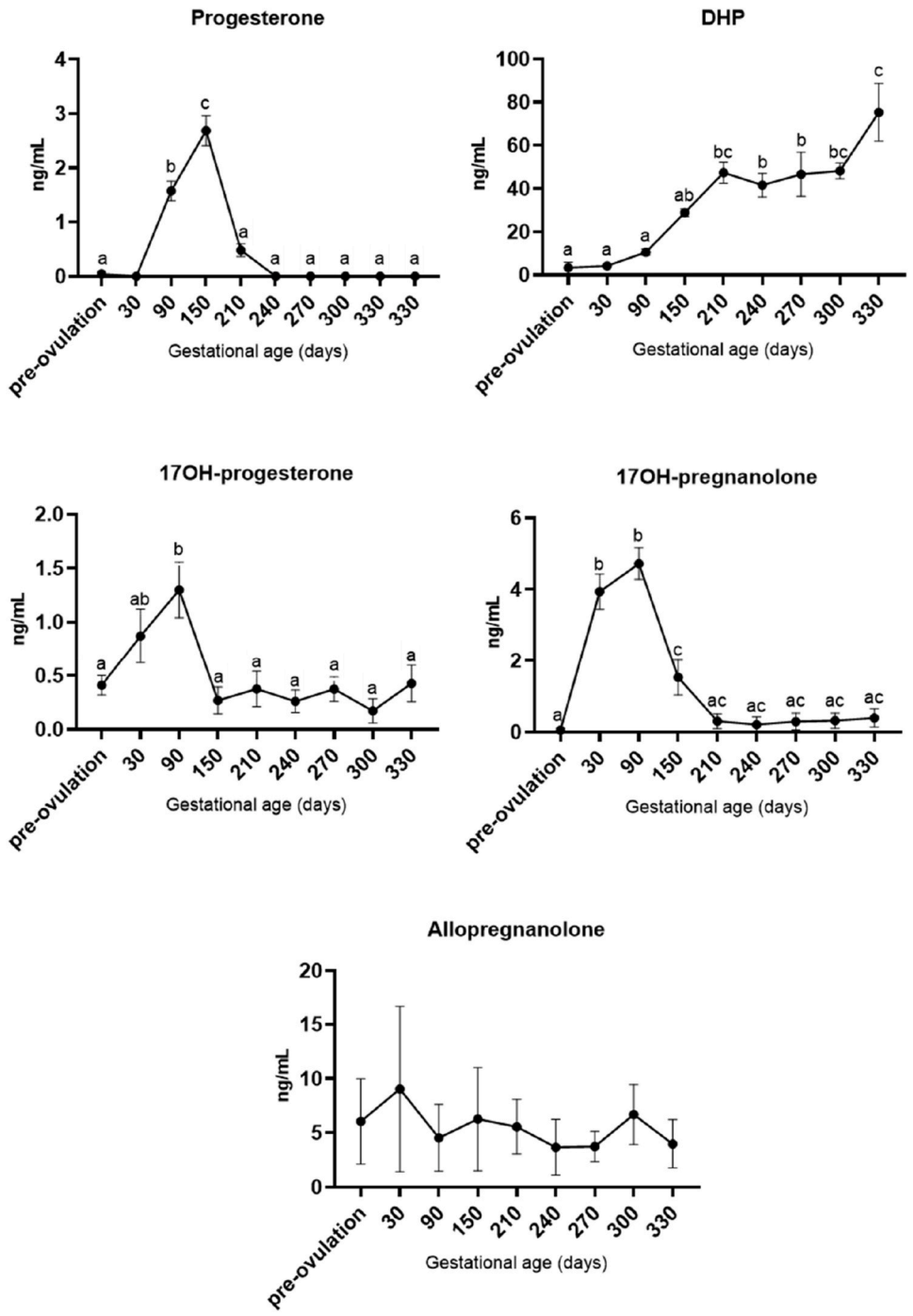

Fig. 5. Plasma progestogen concentrations (17-OHprogesterone, 17-OHpregnanolone, progesterone, 5α-dihydroprogesterone - DHP, and allopregnanolone) measured in healthy mares (n = 8) before ovulation and throughout gestation. Different letters represent statistically significant differences between timepoints ($P < 0.05$). Data are shown as mean ± SEM.

negative association between progesterone and cortisone. Progesterone, which shows structural similarities to glucocorticoids, may take on an immunomodulatory role in pregnancy through mechanisms used by glucocorticoids. *In vitro* studies in mice suggested that progesterone can use the glucocorticoid receptor (GR), in addition to the conventional nuclear progesterone receptor (PR), to modulate maternal immune response, including increasing Tregs frequencies and favoring maternal immune tolerance to the fetus [20,45]. Indeed, the progesterone production in this phase of pregnancy is highly dependent on the effects of eCG produced by the endometrial cups, which resist the paternal alloantigen-specific antibodies and the surrounding accumulation of maternal lymphocytes [43,44].

Plasma estrone-sulfate concentration showed positive association with T cell distribution. As an inactive estrogen metabolite, estrone-sulfate may serve as a hormonal reserve, rapidly converting into bioactive estrone; however, its role in pregnancy remains poorly characterized [46]. Estrogens promote both stimulatory and inhibitory effects on T cells, depending on the context [47–50]. Although estrone-sulfate cannot bind to nuclear estrogen receptors (ER-α and ER-β) due to its inactive nature, it has been reported that estrogens may impact immune cells via non-genomic signaling pathways, potentially including estrone-sulfate [51]. The positive correlation between

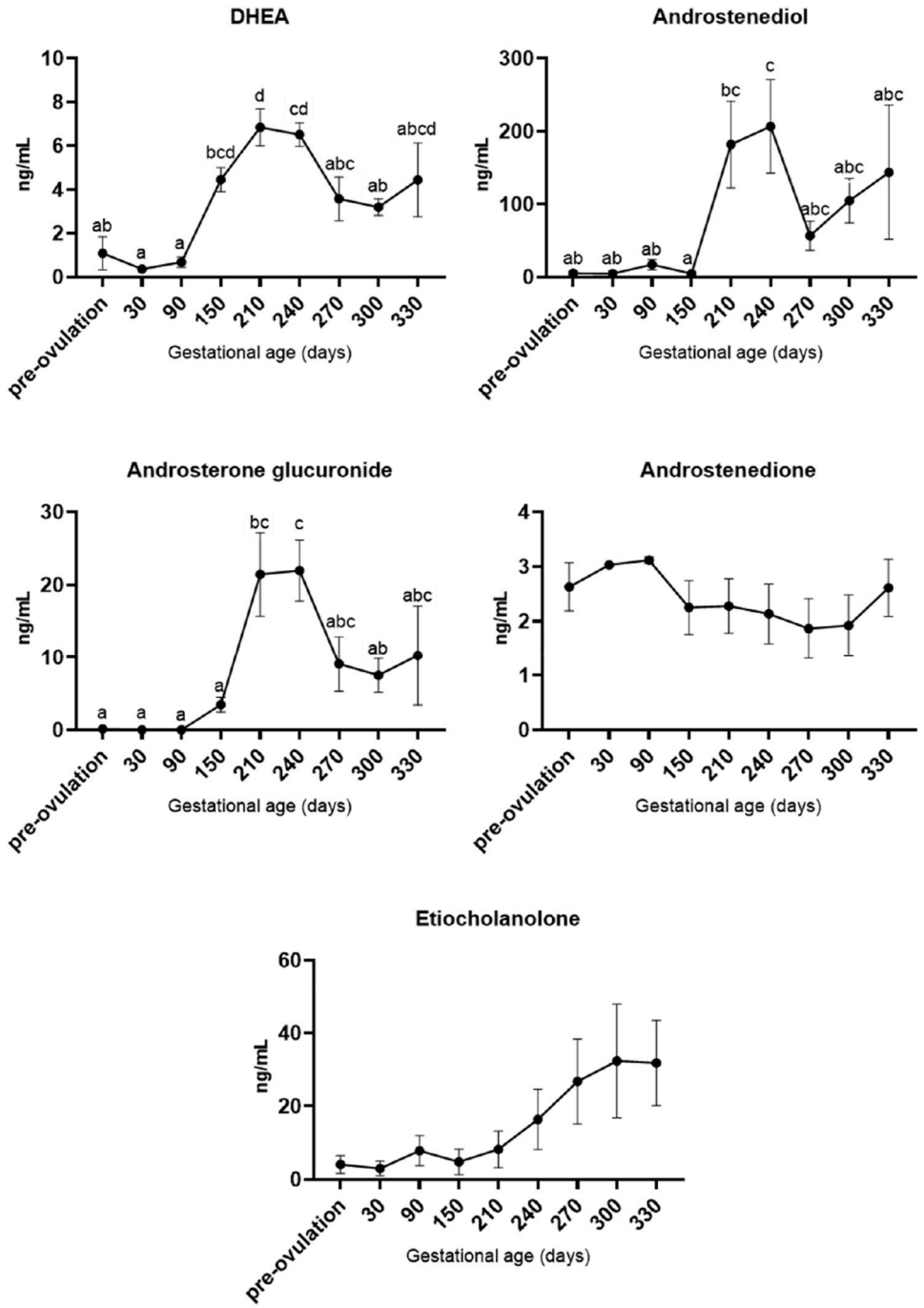

Fig. 6. Plasma androgen concentrations (dehydroepiandrosterone - DHEA, androstenedione, androstenediol, androsterone glucuronide, and etiocholanolone) measured in healthy mares (n = 8) before ovulation and throughout gestation. Different letters represent statistically significant differences between timepoints ($P < 0.05$). Data are shown as mean ± SEM.

estrone-sulfate and T cells warrants further research to better understand the relationship between them and consequent impact on the immune system.

Also, during early pregnancy, the results from this study showed negative correlations between eCG or progesterone concentrations and B cell distributions. Similar findings have been reported in mice and humans. Studies in pregnant mice suggest that progesterone plays a protective and regulatory role in the immune response by down-regulating B cell activating factor (BAFF), potentially reducing B cell maturation [52,53]. Additionally, a recent study showed that hCG can inhibit the differentiation of murine splenic B cells into plasma cells, suggesting a regulatory effect on antibody production [54]. Similarly, estrone-sulfate concentration showed a negative association with B cell distribution. Studies in women and murine models have reported that estrogens can partially suppress the B cell compartment during normal pregnancy [55–57]. Although the full biological significance of this suppression remains unclear, it may contribute to maternal–fetal immune tolerance [58].

In contrast to reduced B cell distribution, there was a peak of globulin concentration at 90 days of pregnancy, driven by serum IgG concentration. The days preceding this gestational period (i.e., between 45 and 60 days) are characterized not only by the endometrial cup formation but also by the high expression of allogeneic (paternal) major histocompatibility (MHC) molecules in the trophoblast girdle [59]. Paternal

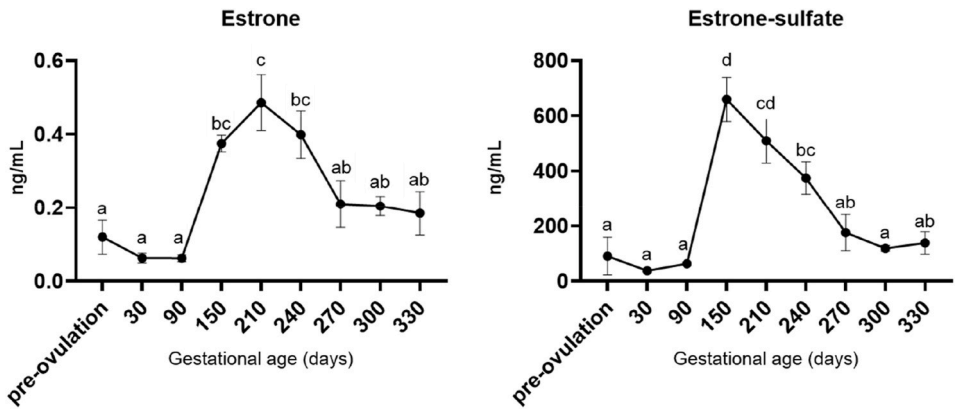

Fig. 7. Plasma estrone and estrone-sulfate concentrations measured in healthy mares (n = 8) before ovulation and throughout gestation. Different letters represent statistically significant differences between timepoints ($P < 0.05$). Data are shown as mean ± SEM.

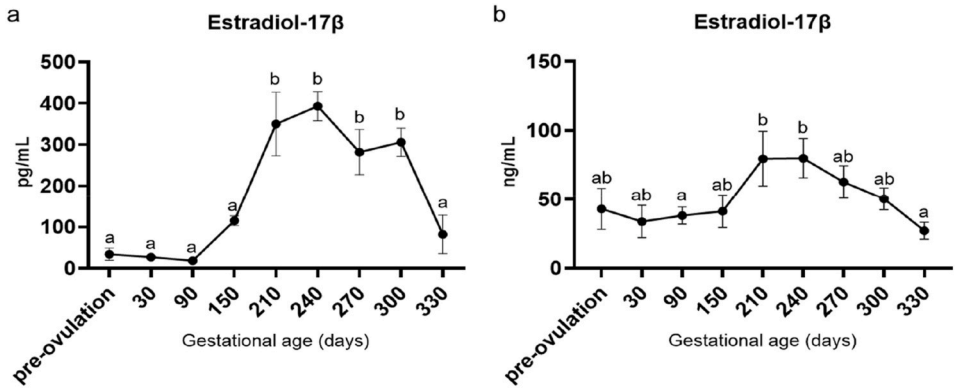

Fig. 8. Plasma estradiol-17β concentrations measured in healthy mares (n = 8) before ovulation and throughout gestation using (a) an immunoassay (results reported in pg/mL) and (b) mass spectrometry (results reported in ng/mL). Different letters represent statistically significant differences between timepoints ($P < 0.05$). Data are shown as mean ± SEM.

MHC antigens induce a strong maternal cytotoxic antibody response that is detectable by day 60 in primiparous and by day 41 in multiparous mares, persisting for more than 160 days of pregnancy [60,61]. Therefore, humoral response does not seem modulated during this phase of pregnancy despite a decrease in B cell distribution. This observation supports another possibility that the decreased B cell distribution is secondary or relative to a primary increase in CD4 T cell distribution during the same period. Nonetheless, it is unclear if the increase in serum IgG concentration during this period was a non-specific, general humoral response induced by pregnancy factors or timely and quantitatively proportional to a specific humoral response to the paternal antigens. The pregnant mares in this study received immunization soon after this timepoint, discarding the influence of vaccination as the antigenic stimulation.

Cortisol concentrations throughout pregnancy remained within the normal reference range for pregnant mares [62]. Notably, a peak in cortisol levels was observed at 150 days of gestation, followed by a decline to concentrations comparable to those seen in early pregnancy, as also previously reported [62]. The increase in cortisol at this stage coincides with the luteo-placental shift in horses. A similar pattern has been described in pregnant women; however, unlike mares, cortisol levels in women continue to rise from mid-to late-gestation [63–65]. No associations were observed between glucocorticoids and immunological parameters, despite their known immunosuppressive effects. Significant correlations with other hormones were, however, evident, suggesting a potential role for glucocorticoids in broader hormonal regulatory networks. For instance, glucocorticoids were negatively correlated with estradiol-17β and positively associated with estrone-sulfate through gestational periods.

The negative correlation between estradiol-17β and multiple glucocorticoids, including cortisol, cortisone, and corticosterone, suggests an important hormone-hormone association. While cortisol is essential for many maternal and fetal biological functions, including the regulation of fetal metabolism and growth in late gestation, fetal exposure to high cortisol concentrations can be detrimental [66,67]. To protect the fetus from cortisol overexposure, the placenta expresses high levels of the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2), which converts biologically active cortisol to inactive cortisone [67]. Reduced 11βHSD2 activity is related to unfavorable pregnancy outcomes in women [68]. Therefore, this consistent pattern of negative correlation between estradiol-17β and multiple glucocorticoids through gestational periods suggests a regulated hormonal balance, which may serve as a protective mechanism to reduce fetal exposure to glucocorticoids. Further research is needed to explore maternal peripheral glucocorticoid metabolism and its relationship with estradiol-17β concentration in pregnant mares. Additionally, the positive association between estrone-sulfate and glucocorticoids through different gestational periods may be linked to the enzymatic conversion of this estrogen. The GR activation by glucocorticoids has been shown to upregulate the expression and activity of estrogen sulfotransferase (SULT1E1), a key enzyme involved in estrone sulfonation [69]. Our findings consistently demonstrated this positive association during distinct gestational periods, suggesting a relevant regulatory mechanism during equine pregnancy.

From 151 to 240 days of gestation, a decrease in serum IgG concentration was correlated with an increase in androstenediol, an androgen steroid hormone known to suppress immunoglobulin production [18,23]. At 210 days of pregnancy, a peak of B cell distribution

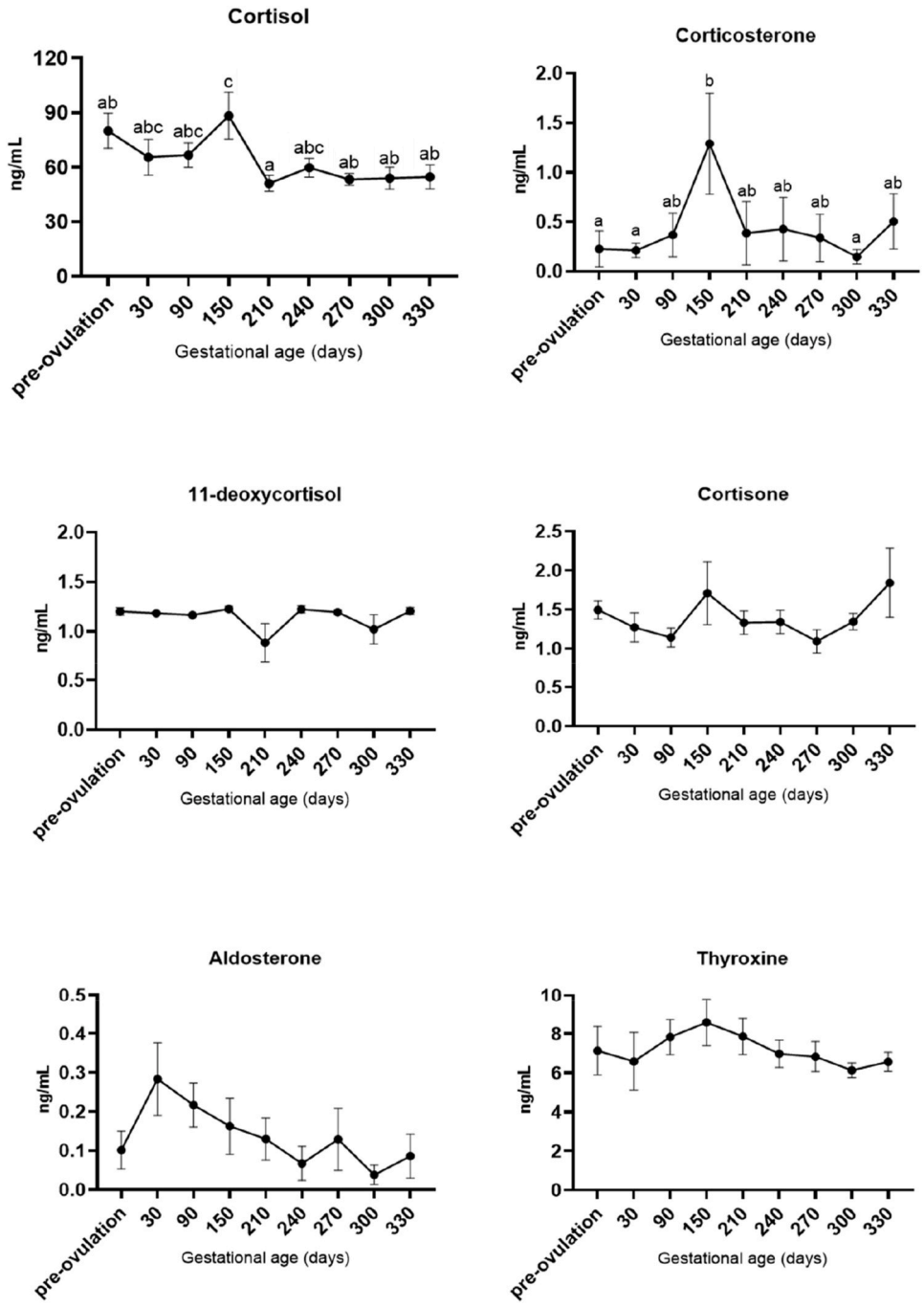

Fig. 9. Plasma steroid hormone concentrations (cortisol, 11-deoxycortisol, cortisone, corticosterone, aldosterone, and thyroxine) measured in healthy mares (n = 8) before ovulation and throughout gestation. Different letters represent statistically significant differences between timepoints ($P < 0.05$). Data are shown as mean ± SEM.

coincided with a peak of plasma estradiol-17β, estrone, and estrone-sulfate levels, alongside a decrease in CD4 T cell distribution. Although no statistically significant correlation between estradiol-17β and B-cell distribution was detected in the present study, previous research in humans and mice have shown that estrogens may increase the development of memory B cells, regulatory B (Breg) cells, and plasma cells, in addition to augmenting the humoral immune response, although the underlying mechanisms in pregnancy remain unclear [21,54,70,71]. The change in B cell distribution observation warrants further studies characterizing the phenotype and function of B cells during this period of pregnancy, including under the influence of estrogens.

Beyond 240 days of gestation, an increase in serum IgM concentration was correlated with an increase in estradiol-17β, which has been shown to enhance immunoglobulin production in humans and mice [17,19,70]. Yet, no changes in serum IgG and IgM concentrations were detected in the late gestation period, consistent with some previous studies but in contrast with others that showed a decline in maternal serum IgG levels attributed to colostrum production in pregnant mares, goats, sows, cows, and ewes [5,6,72–74].

Although total protein concentrations did not change during gestation, albumin levels showed a peak at 210 days of gestation, with a positive correlation with cortisone and hematocrit during 151 and 270 days of gestation [75–78]. Albumin binds with low affinity to all classes

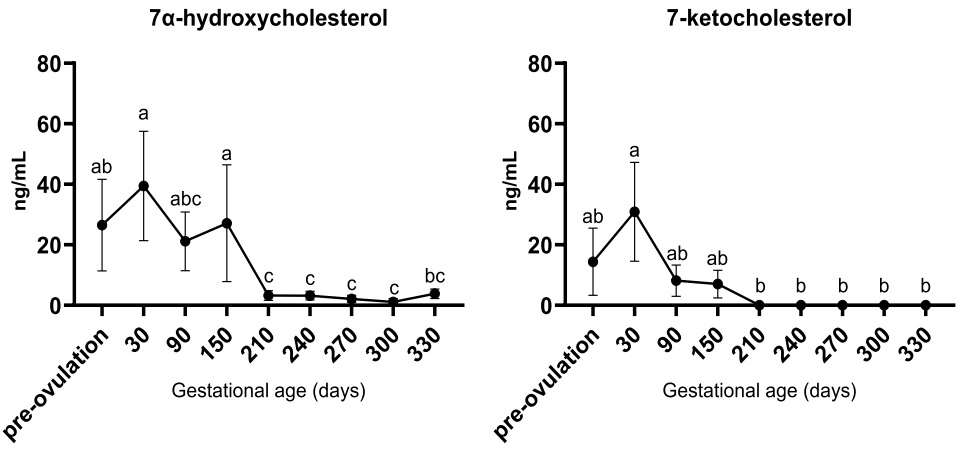

Fig. 10. Plasma cholesterol metabolite concentrations (7α-hydroxycholesterol and 7-ketocholesterol) measured in healthy mares (n = 8) before ovulation and throughout gestation. Different letters represent statistically significant differences between timepoints ($P < 0.05$). Data are shown as mean ± SEM.

Table 1
Correlation analyses of immune-hormone and hormone-hormone parameters at gestational period 1 (30–90 days of pregnancy).

	CD3 T cell	CD4 T cell	B cell	Progesterone	eCG	Cortisone	Androstenediol
eCG	0.51	0.54	**−0.73**	0.67	1.00		0.60
Progesterone			**−0.60**	1.00	**0.67**	**−0.54**	
DHP			**−0.68**	0.83	**0.79**	**−0.56**	
Estrone-sulfate	0.66		**−0.54**	0.71	**0.55**		

Spearman correlation coefficient (*r*) showing positive (normal font) and negative (bold font) associations between parameters ($p < 0.05$).

Table 2
Correlation analyses of immune-hormone and hormone-hormone parameters at gestational period 2 (91–150 days of pregnancy).

	CD4 T cell	B cell	Progesterone	Estradiol-17 β	Cortisone	DHP	Estrone-sulfate
DHEA	**−0.58**	0.58	**−0.66**	0.73		0.74	
A. glucuronide	**−0.57**		**−0.65**	0.71			
Androstenedione			0.51	**−0.55**			
Progesterone		**−0.45**	1.00	**−0.80**	**−0.55**	**−0.65**	**−0.70**
Cortisone			**−0.55**	**−0.51**	1.00		
Corticosterone							0.55
11-deoxycortisol							0.50
eCG			0.82	**−0.75**	**−0.56**	**−0.56**	**−0.55**

Spearman correlation coefficient (*r*) showing positive (normal font) and negative (bold font) associations between parameters ($p < 0.05$). A. glucuronide: androsterone glucuronide.

Table 3
Correlation analyses of immune-hormone and hormone-hormone parameters at gestational period 3 (151–240 days of pregnancy).

	CD3 T cell	B cell	IgG	DHEA	Progesterone	Cortisol	Corticosterone	Cortisone
Androstenediol	**−0.46**	0.42	**−0.41**	0.60				
DHEA	**−0.44**	0.42		1.00	**−0.41**			
DHP					**−0.51**	**−0.48**	**−0.64**	
Estrone-sulfate						0.41	0.48	0.48
Estradiol-17β				0.58	**−0.65**		**−0.53**	**−0.44**

Spearman correlation coefficient (*r*) showing positive (normal font) and negative (bold font) associations between parameters ($p < 0.05$).

Table 4
Correlation analyses of immune-hormone and hormone-hormone parameters at gestational period 4 (241–270 days of pregnancy).

	IgM	Estrone-sulfate	Estradiol-17β	Androsterone glucuronide
Estradiol-17β	0.57		1.00	
DHEA		0.72	0.64	
Androstenediol				0.68

Spearman correlation coefficient (*r*) showing positive (normal font) and negative (bold font) associations between parameters ($p < 0.05$).

of steroid hormones, buffers fluctuations of steroid levels, and regulates their distribution between other steroid-binding proteins and the free fraction in plasma [79,80]. Consequently, albumin plays a role in cortisone transport and availability. Cortisone, an inactive precursor, is converted into active cortisol by the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), which is present in the liver, adipose tissue, and placenta [81,82]. In this study, a relative hemoconcentration was characterized by increases in both albumin and hematocrit, and their association with cortisone suggests a hormonal regulation for internal homeostasis and metabolic adjustments during pregnancy. In contrast with a study that previously described changes in aldosterone

Table 5
Correlation analyses of immune-hormone and hormone-hormone parameters at gestational period 5 (271–330 days of pregnancy).

	CD3 T cell	CD4 T cell	Estrone-sulfate	Cortisone	11-deoxycortisol
Estradiol-17β	**−0.43**	**−0.61**	0.44	**−0.57**	
DHEA			0.52		
DHP			**−0.42**		
Estrone-sulfate			1.00	0.50	0.43

Spearman correlation coefficient (*r*) showing positive (normal font) and negative (bold font) associations between parameters ($p < 0.05$).

levels in Spanish mares during pregnancy, there were no significant changes through time or correlations between aldosterone concentrations and other parameters in the present study, suggesting that the transient hemoconcentration was minimally influenced by renal regulation [83]. Furthermore, the lack of changes in erythropoietin, MCV, MCH, and MCHC values throughout pregnancy do not indicate enhanced erythropoiesis. Therefore, hemoconcentration may reflect fluid systemic and cavitary distribution, and alterations in placental permeability to support the increased fetal growth and metabolic demands during mid to late pregnancy, when fetal growth accelerates and fetal fluid volume decreases [8]. In humans and mice, glucocorticoids at physiological levels have been shown to play a crucial role in placental development by regulating angiogenesis and cellular structure, whereas excessive glucocorticoid exposure leads to apoptosis and impaired vascular development in the placenta [84,85].

Reactive oxygen species-mediated oxidation of cholesterol produces 7-ketocholesterol and 7-hydroxycholesterol, which are categorized as oxidized cholesterol metabolites, also known as oxysterols [86]. In the present study, these oxysterols did not correlate with any immune or hormonal parameters but were correlated with each other. Additionally, higher plasma levels were also observed during the first 150 days of pregnancy. These oxysterols bind to liver X receptors (LXRs), which are involved in regulating important metabolic pathways, such as cholesterol homeostasis, lipogenesis, and glucose homeostasis, while also preventing the expression of proinflammatory factors, such as nuclear factor kB (NF-kB) in humans and rodents [86]. Although research on oxysterols during pregnancy in mares is lacking, one study suggested that oxysterols have a predominant effect of proinflammatory cytokine secretion in cultured placental trophoblast cells via TLR (Toll-like receptor) 4 activation of NF-kB, rather than exerting anti-inflammatory effects mediated by LXR activation and/or cholesterol modulation [87]. Further studies are needed to explore the potential roles of oxysterols in equine pregnancy.

The present study offered an innovative approach to investigating potential associations between immunological and hormonal parameters during distinct stages of pregnancy in mares. However, certain limitations should be acknowledged. The relatively small sample size of pregnant mares may have limited the ability to detect additional significant correlations and relationships between parameters. Conversely, it may also have highlighted associations that might not be supported in a larger dataset. Furthermore, comprehensive immune cell phenotyping (e.g., Tregs, Bregs) and functional assays (e.g., lymphocyte cytokine expression in the presence of hormones) may have provided greater insights about direct immunomodulatory mechanisms promoted by key hormones of pregnancy.

5. Conclusion

This study revealed dynamic associations between immunological and hormonal parameters across gestation in mares, likely reflecting adaptations for immunotolerance, fetal development, pregnancy maintenance, and parturition. Early and mid-gestation were characterized by stronger immune–hormone and hormone–hormone interactions, particularly involving T- and B-cell distributions with eCG, DHP, estrone-sulfate, and androgens, whereas the final 100 days of pregnancy showed relatively stable profiles. Importantly, immune parameters in pregnant mares remained within reference ranges for non-pregnant horses, suggesting a balance between immune tolerance and protection. These findings also point to a potential role of hormones in systemic fluid regulation during late pregnancy. Further studies are warranted to better elucidate the immunological mechanisms underlying these hormonal changes and their implications for pregnancy outcomes.

CRediT authorship contribution statement

Lorena S. Feijo: Writing – review & editing, Writing – original draft,

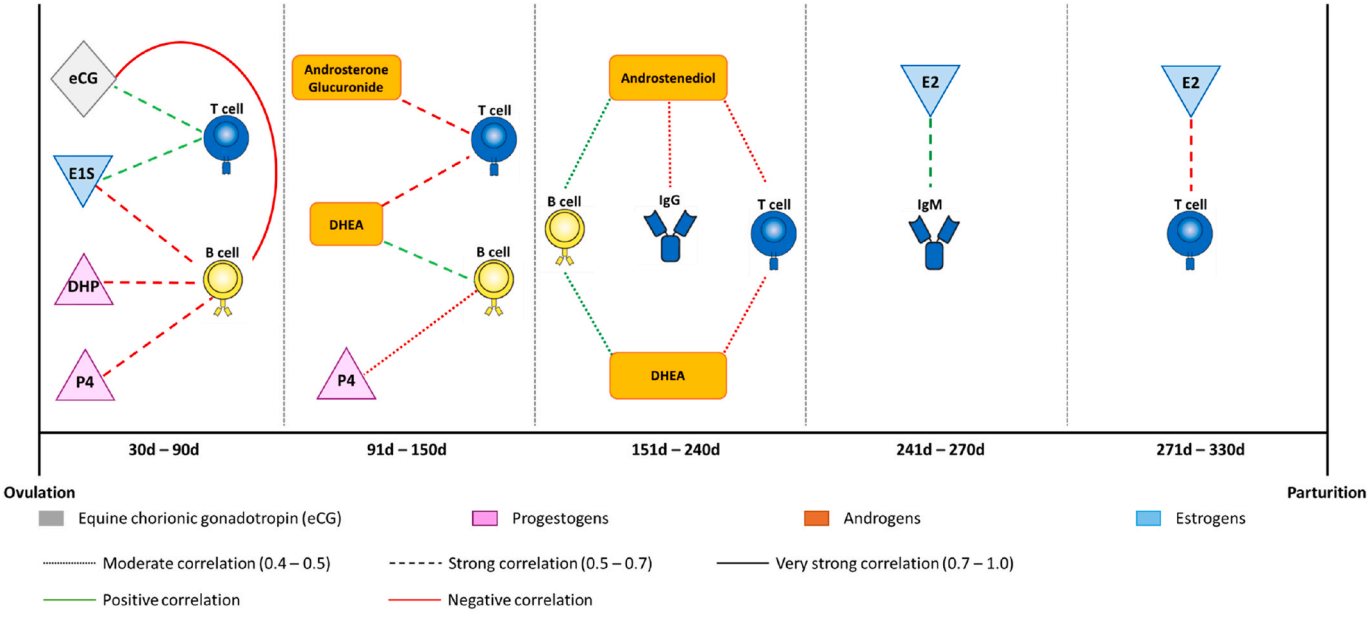

Fig. 11. Proposed relationships of circulating immune-hormone changes during pregnancy in healthy mares. E1S: estrone-sulfate; DHP: 5α-dihydroprogesterone; P4: progesterone; DHEA: dehydroepiandrosterone; E2: estradiol-17β.

Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Joy Ledeck:** Writing – review & editing, Validation, Methodology. **Karen Wolfsdorf:** Writing – review & editing, Visualization, Resources, Investigation, Funding acquisition, Conceptualization. **Jerome Ponthier:** Writing – review & editing, Validation, Methodology. **Stephen Parry:** Writing – review & editing, Validation, Methodology, Formal analysis. **M. Julia B. Felippe:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

None of the authors have any conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2025.117719.

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