Journal of Equine Veterinary Science 99 (2021) 103425

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com

Alterations of Circulating Biomarkers During Late Term Pregnancy Complications in the Horse Part I: Cytokines

C.E. Fedorka[a], B.A. Ball[a,*], O.F. Walker[b], M.E. McCormick[c,d], K.E. Scoggin[a], L.A. Kennedy[a], E.L. Squires[a], M.H.T. Troedsson[a]

[a] University of Kentucky, Department of Veterinary Sciences, Lexington, KY
[b] Lincoln Memorial University, College of Veterinary Medicine, Harrogate, TN
[c] Rhode Island College, College of Nursing. Providence, RI
[d] Rhode Island Department of Health. Providence, RI

ARTICLE INFO

Article history:
Received 8 December 2020
Received in revised form 1 February 2021
Accepted 12 February 2021
Available online 18 February 2021

Keywords:
Placentitis
Cytokine
Biomarker
Abortion
Equine

ABSTRACT

Equine abortions are attributed to both infectious and noninfectious causes. Clinical extrapolations are often made from the experimental model for ascending placentitis towards other causes of fetal compromise, including various markers of inflammation, including the cytokines IL-2, 5, IL-6, IL-10, IFNγ, and TNF. It is unknown if these cytokine changes are noted under field conditions, or if they increase preceding other pregnancy related complications. To assess this, Thoroughbred mares (n = 702) had weekly blood obtained beginning in December 2013 and continuing until parturition. Fetal membranes were submitted to the UKVDL for complete gross and pathologic assessment and classified as either ascending placentitis (n = 6), focal mucoid placentitis (n = 6), idiopathic abortion (n = 6) or control (n = 20). Weekly serum samples were analyzed via immunoassay for concentrations of IL-2, IL-5, IL-6, IL-10, IFNγ, and TNF. For both focal mucoid placentitis and ascending placentitis, an increase ($P < .05$) in the concentrations of IL-2, IL-5, IL-6, IL-10, IFNγ, and TNF was noted preceding parturition in comparison to controls. Cytokine profiles preceding idiopathic abortion did not differ from controls. In conclusion, serum cytokines may be considered potential biomarkers for the prediction of placental infection, while no changes in cytokine profiles were noted when noninfectious causes of abortion occurred. Additionally, this is the first study to report an increase in cytokines during the disease process of focal mucoid placentitis, the etiology of which includes Nocardioform placentitis.

1. Introduction

In central Kentucky, placental infection is the leading cause of abortion in the horse [1]. The detection of pathogens by the feto-placental unit is broadly referred to as placentitis [2], and can present in various pathologies, including ascending, hematogenous, and idiopathic [3]. The experimental induction of ascending placentitis has advanced our understandings of the pathophysiology of this disease, in addition to providing prospective circulatory biomarkers for disease prediction. Unfortunately, no experimental model exists for the induction of focal mucoid placentitis [4] or abortion caused by multiple noninfectious abnormalities, including umbilical torsion or premature placental separation. Therefore, the majority of biomarker detection has been extrapolated from the experimental induction of ascending placentitis towards other pregnancy-related complications [2,5–11], although few have been critically evaluated in spontaneous disease in clinical cases.

Currently, ascending placentitis is diagnosed based on clinical alterations, including vaginal discharge, premature mammary gland development and lactation, and an increase in placental thickness at the caudal pole of the cervix as noted by transrectal ultrasonography [12]. Similar clinical modalities are utilized to diagnose focal mucoid placentitis, the etiology of which includes Nocardioform placentitis. As the placental lesion indicative of Nocardioform placentitis tends to occur at the ventral aspect of the body of the uterus, clinical examination requires transabdominal ultrasonography, and vaginal discharge is generally not observed [13]. Unfortunately, physiologic changes such as lactation and vaginal discharge are believed to occur late in the disease process, allowing for limited therapeutic intervention. Therefore, focus has shifted towards

Conflicts of interest: None.

Ethical statement: All animal procedures were completed in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Kentucky under the guidelines of the approved protocol #2013-1190.

* Corresponding author at: Barry Ball. 1400 Nicholasville Rd, Lexington KY, 40511.
E-mail address: B.A.Ball@uky.edu (B.A. Ball).

https://doi.org/10.1016/j.jevs.2021.103425
0737-0806/

an understanding of the immune response to these infectious diseases for use as biomarkers of subclinical disease.

It is now understood that the maternal immune response to ascending placentitis is primarily pro-inflammatory, initiated by the recognition of pathogens by toll-like receptors (TLR), leading to an increase in chemotactic cytokines such as interleukin (IL)-1β and IL-8, and eventual recruitment of various immune cell types, including monocytes, neutrophils, and macrophages [2,14–16]. In contrast, the feto-placental unit was found to respond to this induction of disease in an anti-inflammatory and immunomodulatory manner, with an increase in the expression of anti-inflammatory IL-10 and pleiotropic IL-6 within fetal tissues and fluids [2]. The pleiotropic activity of IL-6 allows this cytokine to function as both pro- and anti-inflammatory [17]. Recent work from our laboratory found IL-6 to be functioning primarily as anti-inflammatory during the disease process of a subacute ascending placentitis, activating the classical signaling pathway and leading to an activation of antiapoptotic and prosurvival markers [18]. However, these changes were noted primarily in tissues within the fetoplacental unit, providing limited insight into the value of potential biomarkers in maternal blood.

Consequently, considerable work has gone into utilizing the immune response to ascending placentitis to improve the early detection of pregnancy-related complications, with a focus on noninvasive sampling procedures to avoid the risk of iatrogenic abortion. Circulatory alterations among immune mediators have included an increase in positive acute phase proteins such as serum amyloid A (SAA) and haptoglobin [7], in addition to various pro-inflammatory (IL-2, IFNγ), pleiotropic (IL-6, TNF), and anti-inflammatory cytokines (IL-5, IL-10) [18,19]. An increase in circulating serum pro-inflammatory cytokines is believed to activate the effector functions of the adaptive immune response, while impeding on the regulatory aspect of the immune system [19], potentially leading to the rejection of the semi-allogeneic fetoplacental unit. In contrast, the increase in anti-inflammatory or immunomodulatory cytokines may impede these signals, and allow for the pregnancy to carry to term.

While the experimental model for ascending placentitis offers considerable inferences into the pathophysiology of the specific disease, the etiology of focal mucoid placentitis and noninfectious pregnancy-related complications are poorly understood, and extrapolations made from the experimental induction of an ascending infection need to be evaluated critically under clinical conditions. Therefore, interpretation of naturally occurring chronic disease noted in the field requires assessment utilizing clinical modalities. The objectives of this study were to (1) assess cytokine concentrations in a naturally occurring disease process, and (2) compare cytokine alterations following various pregnancy-related complications.

2. Materials and Methods

2.1. Study Design

The study was performed as a prospective enrollment of random mares before the retrospective analysis on the selected mares at the week prior to parturition/abortion (0–7 days prior to parturition/abortion) in addition to weekly, at −1 (8–14 days prior to parturition/abortion), −2 (15–21 days prior to parturition/abortion), −3 (22–28 days prior to parturition/abortion), and −4 weeks (29–35 days prior to parturition/abortion). Mares were placed into designated groups following postpartum evaluation of fetal membranes and before analysis of serum cytokines to ensure unbiased interpretation of cytokine results. As all ascending and focal mucoid placentitis mares delivered a viable neonate at term, time points are shown as weeks prior to parturition, with data from control mares also shown as weeks prior to parturition. The idiopathic abortion group underwent abortion prior to full term gestational length, and samples for this group were compared to control mares at a similar gestational length that were not immediately prepartum. Mares in the control group were selected based on specific criteria including last breeding date, age, and farm of residence, and confirmed as having no disease upon postpartum diagnosis. All groups were chosen based upon postpartum diagnosis with ascending placentitis, focal mucoid placentitis, idiopathic abortion, or no disease before concentrations of IL-2, IL-5, IL-6, IL-10, IFNγ, and TNF were detected at the specific sampling points. For the group of idiopathic abortion, mares with no disease were matched at comparable gestational lengths to the sampling prior to abortion in the diseased mare category.

2.2. Animal Enrollment

2.2.1. Blood Samples

All animal procedures were completed in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Kentucky under the guidelines of the approved protocol #2013-1190. Horses (*Equus caballus*) used in this study were Thoroughbred mares (n = 702; 4–22 years of age) housed on 15 private farms located in central Kentucky, USA and samples were obtained with owner permission. Mares were bred via live cover during the natural Northern Hemisphere breeding season to various stallions. Beginning in December 2013, blood was obtained weekly via jugular venipuncture utilizing a vacutainer tube (10 mL; Monoject; VWR, USA) for serum extraction, and sampling continued until either abortion or parturition occurred. Samples were transported back to the laboratory at ambient temperature and then centrifuged at 1,800xg for 15 minutes. Serum was aliquoted and stored at −20°C until time of analysis.

2.2.2. Postpartum Evaluation of Placenta

Upon parturition or abortion, fetal membranes from the affected mare were submitted to the University of Kentucky Veterinary Diagnostic Lab (UKVDL) within 6 hours postpartum for histopathology and disease diagnosis. Additionally, fetal membranes from control mares were enrolled based on farm residence, age, and parturition date, and evaluated by the same laboratory to confirm the lack of disease. Full term pregnancy was considered a gestational length greater than 320 days, while abortion and/or preterm parturition was considered as gestational length less than 319 days. Placental membranes were assessed as previously described by Hong et al. [1], in addition to fetal tissues if abortion occurred. In brief, before gross examination was performed, chorioallantois and amnion were weighed, and the diameter of gross lesions recorded. Sections were obtained from grossly detected lesions, in addition to the body, gravid horn, and non-gravid horn of the chorioallantois as well as amnion. If abortion occurred, sections were obtained from fetal liver, lung, heart, brain, and spleen, in addition to umbilicus. Samples of chorioallantois were cultured for bacteria and fungi as previously described [20]. All sections were stained with hematoxylin and eosin, and special stains were applied when needed, including Brown and Brenn Gram stain and/or Brown and Hopps Gram stain for the determination of gram positive vs. gram negative bacteria, Gomori's methanamine silver stain for detection of fungi, and Warthin-Starry stain for detection of spirochetes). Direct fluorescent antibody tests were conducted on tissue for *Leptospira spp.*, equine arteritis virus, and equine herpesvirus, while blood and fetal fluids were collected and titrated for antibodies against *Leptospira spp.* by the microagglutination tests. Additionally, bacterial culture isolates were assessed via polymerase chain reaction (PCR) as described by Erol et al. [13]. In brief, DNA was isolated from surface swabs taken

Table 1
Clinical variables.

Clinical Variable		Control	Ascending Placentitis	Focal Mucoid Placentitis	Idiopathic Abortion
Age		10.3 ± 0.8[a]	13.0 ± 1.5[a]	10.2 ± 1.74[a]	11.2 ± 1.4[a]
Gestational Length		347.9 ± 2.7[a]	338.6 ± 5.9[a]	344.0 ± 4.9[a]	291.0 ± 16.1[b]
Viable Neonate		20/20[a]	6/6[a]	6/6[a]	0/6[b]
Treatment	Antimicrobials	0/20[a]	0/6[a]	1/6[a]	1/6[a]
	Anti-inflammatories	0/20[a]	0/6[a]	1/6[a]	0/6[a]
	Altrenogest	6/20	2/6	1/6	4/6

[a,b] indicates $P < 0.05$.

from the chorioallantois and bacterial culture isolates utilizing the MagNAPure Compact System (Roche Applied Science, Indianapolis, IN, USA) following manufacturer's instructions. PCR reactions were used to detect common nocardioform actinomycetes *Amycolatopsis spp.* and *Crossiella equi*. Clinical data, including previous pregnancy history, detection of gestational abnormalities, treatments/vaccines administered during pregnancy and foaling information were collected for each mare.

2.3. Cytokines

Cytokines concentrations of IL-2, IL-5, IL-6, IL-10, TNF, and IFNγ were analyzed in serum using an equine-specific multiple sandwich immunoassay based on flowmetric MILLIPLEX MAP technology (MILLIPORESIGMA; Burlington MA, USA) in accord with the workflow previously published [21]. Samples of serum were measured undiluted and standards were prepared with the serum matrix added to all standards and quality controls, following the guidelines of the manufacturer. The means of intra-and inter-assay coefficients of variation were 2.7% and 3.7%, respectively. The minimum detection level was defined as the signal-to-noise-ratio (limit of detection) divided by the square root of 2 (IL-5: 7.9 pg/mL; IL-6: 2.6 pg/mL; IL-2: 4 pg/mL; IFNγ: 189 pg/mL; IL-10: 34 pg/mL; TNF: 2.0 pg/mL) [22].

2.4. Statistics

Concentrations of weekly serum cytokines were analyzed using SAS 9.4. All data were assessed for normality utilizing a Bartlett's test and a Modified Levene's test for equal variances. Weekly data were analyzed using a Repeated Measures Analysis of Variance (ANOVA). Group means for clinical data were analyzed utilizing an independent group T-test to make comparisons. Significance was set to $P < .05$ and trends at $P < .1$. Data are presented as the mean ± the standard error of the mean.

3. Results

3.1. Clinical Data

Placentas obtained from six mares were identified as having infection/inflammation at the cervical pole of the placenta based on gross and histopathology reports (n = 6; ascending placentitis). Bacterial culture samples obtained from three placentae within this ascending placentitis group were positive for *Streptococcus equi ssp. zooepidemicus*, while an additional two placentae cultured positive for nonpathogenic bacteria. Placentas obtained from six mares were identified with focal mucoid lesions within the body or horns of the placenta (n = 6; focal mucoid placentitis). Two of the placentae within the focal mucoid placentitis group were PCR positive for the common nocardioform actinobacteria *Amycolatopsis ssp*, while the other four had no bacteria detected. Finally, placenta from six mares had undetermined etiology alongside poor neonatal outcome (either abortion or term stillborn/n = 6; idiopathic abortion). Of the six idiopathic abortion mares, one was diagnosed as an umbilical cord torsion, while the remainder had undetermined etiology. Placentas from an additional twenty (n = 20; control) mares had no disease noted on gross appearance and histopathology, and culture and PCR were negative for bacterial isolates. No difference was noted in mare age when comparing focal mucoid placentitis, ascending placentitis, or idiopathic abortion (10.16 ± 1.74 years, 13 ± 1.5 years, 11.2 ± 1.4 years; respectively) to control (10.3 ± 0.8; $P = .40$; Table 1). All control mares carried to term (347.9 ± 2.7 days) and produced a viable foal. All mares with ascending placentitis (6/6) and focal mucoid placentitis (6/6) mares carried to term (within the normal range of gestational length for Thoroughbred mares) [23], and gestational length was not different from that of controls (338.6 ± 5.9 days and 344 ± 4.9 days, respectively vs. 347.9± 2.7 days for controls). In contrast, mares diagnosed with idiopathic abortion had a significantly decreased gestational length in comparison to the control group in addition to the two placentitis groups (291 ± 16.1 days; $P < .05$) and had poor fetal outcome, with none of the neonates surviving (0/6). A subset of mares in each group were treated with various therapeutics, including antimicrobials, anti-inflammatories, and synthetic progestins, but treatment had no effect on the concentrations of IL-2 ($P = .78$), IL-5 ($P = .32$), IL-6 ($P = .4$), IL-10 ($P = .75$), IFNγ ($P = .37$), or TNF ($P = .27$; Table 1).

3.2. Ascending Placentitis

Gestational age had no effect on the concentrations of cytokines evaluated. An overall increase in serum concentrations of IL-2 ($P < .01$), IL-5 ($P < .01$), IL-6 ($P < .01$), IL-10 ($P < .01$), IFNγ ($P < .01$), and TNF ($P < .01$) was noted in the ascending placentitis group compared to controls (Fig. 1). When assessing individual weeks IL-6 and IL-10 were found to be significantly elevated at all time points in mares with ascending placentitis in comparison to the control group ($P < .01$). When assessing IL-2, concentrations of this cytokine were significantly increased in mares with ascending placentitis compared to controls ($P < .05$) at all time points except at 2 weeks prior to parturition. When assessing concentrations of TNF in mares with ascending placentitis, a significant increase was noted at 4, 3, and 1 weeks prior to parturition ($P < .05$), with a trend towards a significant increase at 2 weeks prior to parturition ($P < .1$) compared to the control group. Fewer sampling time points were found to be significantly elevated when assessing IL-5 and IFNγ, although a similar profile was noted for both, with an elevated concentration of the respective cytokines noted at 2 weeks prior to parturition in the ascending placentitis group in comparison to controls ($P < .05$). While this was the only time point found to be significantly increased in mares with ascending placentitis in regard to IL-5, IFNγ was determined to be elevated at 4 weeks prior to parturition as well.

3.3. Focal Mucoid Placentitis

An overall increase in concentrations of serum IL-2 ($P < .01$), IL-5 ($P < .01$), IL-6 ($P < .01$), IL-10 ($P < .01$), IFNγ ($P < .01$), and TNF ($P < .01$) was noted during the disease of focal mucoid placentitis (Fig. 2). When assessing individual weeks, IL-5 and TNF concentrations were elevated in mares with focal mucoid placentitis com-

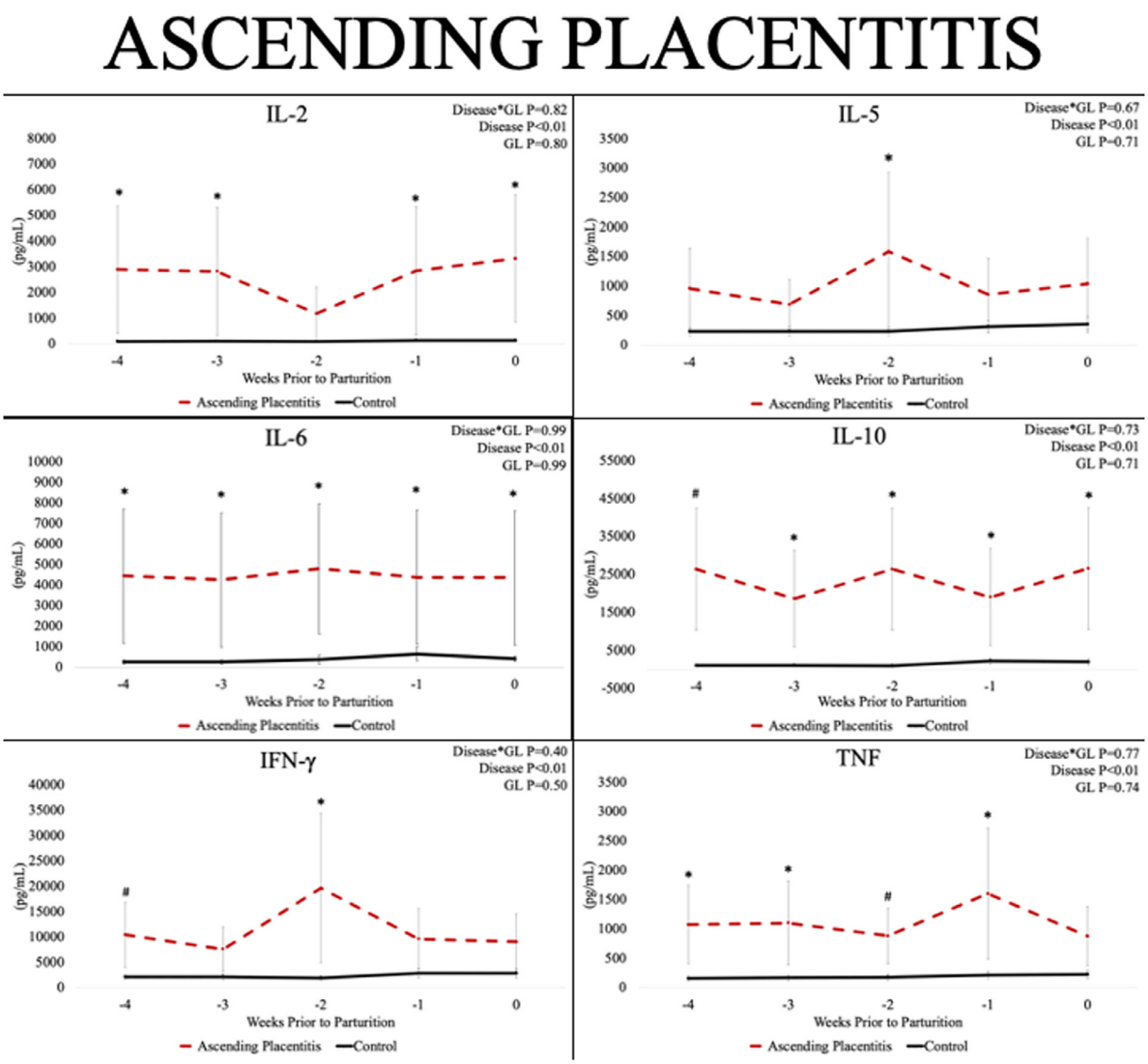

Fig. 1. Cytokine profile noted during ascending placentitis. Concentrations of IL-2, IL-5, IL-6, IL-10, IFNγ, and TNF were evaluated in mares with ascending placentitis (n = 6) compared to gestationally age-matched control mares (n = 20) at −4, −3, −2, and −1 weeks in addition to the week of parturition. All cytokines increased during the disease of ascending placentitis in comparison to controls. Data shown represent the mean ± the standard error of the mean (SEM). Asterisks (*) above data points indicates differences ($P < .05$), while pound sign (#) above the data points indicate differences ($P < .10$) within week in cytokine concentrations between the diseased and control groups. GL = gestational length.

pared to controls at all time points ($P < .05$). IL-10 concentrations were significantly elevated at all times points except 4 weeks prior to parturition in the focal mucoid placentitis group in comparison to controls ($P < .05$), but only a trend toward significance was noted at 4 weeks prior to parturition. Similarly, concentrations of IFNγ were significantly elevated at all time points ($P < .05$) prior to parturition, with a trend toward a significant increase observed at 1 week prior to parturiton when comparing focal mucoid placentitis and the control group ($P < .1$). Concentration of IL-6 was found to be significantly elevated in the focal mucoid placentitis group compared to controls at 4 and 3 weeks prior to parturition ($P < .05$), in addition to the week prior to parturition ($P < .05$), and a trend towards a significant increase was noted at 2 weeks prior to parturition ($P < .1$).

3.4. Idiopathic Abortion

Serum concentrations of the cytokines IL-2 ($P = .49$), IL-5 ($P = .33$), IL-6 ($P = .36$), IL-10 ($P = .34$), IFNγ ($P = .77$), or TNF ($P = .43$) obtained from mares experiencing idiopathic abortion were not different from controls at all time points assessed (Fig. 3).

4. Discussion

To our knowledge, this is the first study to report changes in the serum cytokine profile of mares experiencing naturally occurring pregnancy-related complications. While mares that experienced either ascending placentitis or focal mucoid placentitis experienced an increase in cytokine concentrations, no changes were noted preceding idiopathic abortion. These observed changes in affected mares included an increase in serum concentrations of IL-2, IL-5, IL-6, IL-10, IFNγ, and TNF. The changes mimic what previously has been described during experimentally induced ascending placentitis, but with no validated existing experimental model for focal mucoid placentitis (the etiology of which includes Nocardioform placentitis), these immune-mediated alterations provide insight into the pathophysiology of this elusive disease. This indicates that the disease of Nocardioform placentitis can be detected

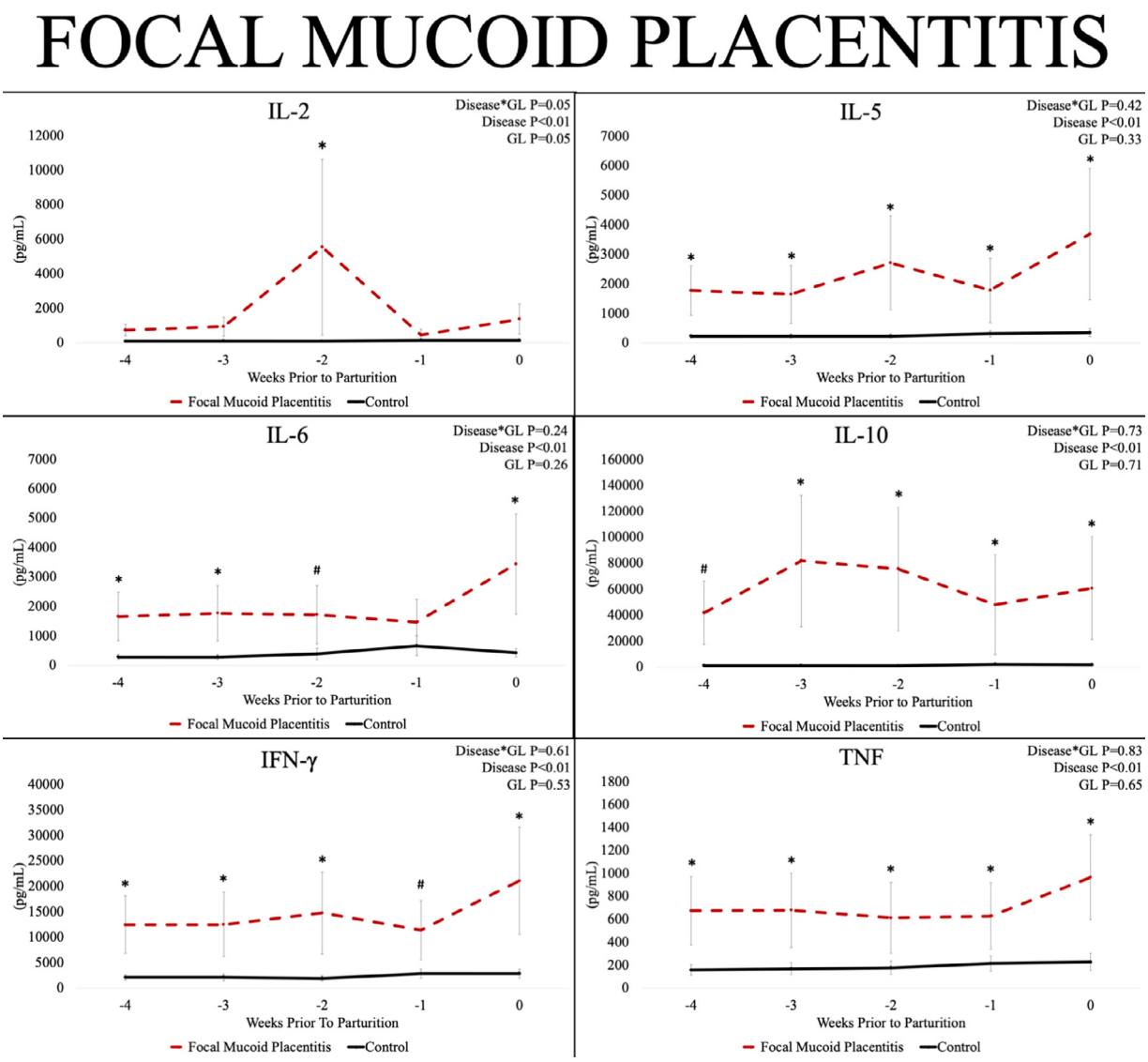

Fig. 2. Cytokine profile noted during focal mucoid placentitis. Concentrations of IL-2, IL-5, IL-6, IL-10, IFNγ, and TNF were evaluated in mares with focal mucoid placentitis (n = 6) compared to gestationally age-matched control mares (n = 20) at −4, −3, −2, and −1 weeks in addition to the week of parturition. All cytokines increased during the disease of focal mucoid placentitis in comparison to controls. Data shown represent the mean ± the standard error of the mean (SEM). Asterisks (*) above data points indicates differences ($P < .05$), while pound sign (#) above the data points indicate differences ($P < .10$) within week in cytokine concentrations between the diseased and control groups. GL = gestational length.

systemically, and therapeutic interference may be achieved prior to abortion or parturition of the dysmature neonate.

The immune system of pregnant females exists in a delicate balance between protection and recognition, as it must both tolerate the semiallogeneic fetus while still defending the body from pathogens. This is governed by both cell-mediated and humoral responses within the immune system, and the maturation and functionality of these cell-mediated responses is governed by various signaling pathways in which cytokines are key factors. Therefore, cytokines are often utilized as biomarkers due to their ability to be sensitive responders and indicators of both fetoplacental health as well as inflammation and infection [24,25]. While the cytokine profile in healthy mares during late gestation did not appear to change with time, both ascending placentitis and focal mucoid placentitis were associated with an elevated cytokine response. Similar changes have been noted during placental infection in other species, including the human [24–37]. Although considerable differences exist between species in both anatomy and physiology of the placenta, intra-amniotic infection (IAI; chorioamnionitis) in women is similar to that of ascending placentitis in horses. IAI begins with an ascending invasion of bacteria through the cervical canal, allowing for pathogens to colonize within the feto-maternal interface. This has been associated with an increase in various cytokines, including amniotic IL-6, IL-8 and IL-10 [28,38], cervical fluid IL-6, IL-8, IL-17, and IL-18 [39–41] and serum IL-6 [42,43]. The cytokine response to placental infection was recently investigated in the horse utilizing an experimental model to induce ascending placentitis, where an increase in serum concentrations of pro-inflammatory IL-2 and IFNγ, anti-inflammatory IL-5 and IL-10, in addition to pleiotropic IL-6 and TNF was noted [18,19]. Potent cell-signaling molecules, these cytokines are associated with both a rapid and transient innate immune response to the detection of pathogens, in addition to being involved in the maturation of various lymphocyte populations associated with the adaptive immune-mediated tolerance of the fetus [6,32,44,45]. While an activation of both arms of the immune system (innate and adaptive) is essential for detection, degradation, and clearance of pathogens, a persistent and overwhelmed proinflammatory re-

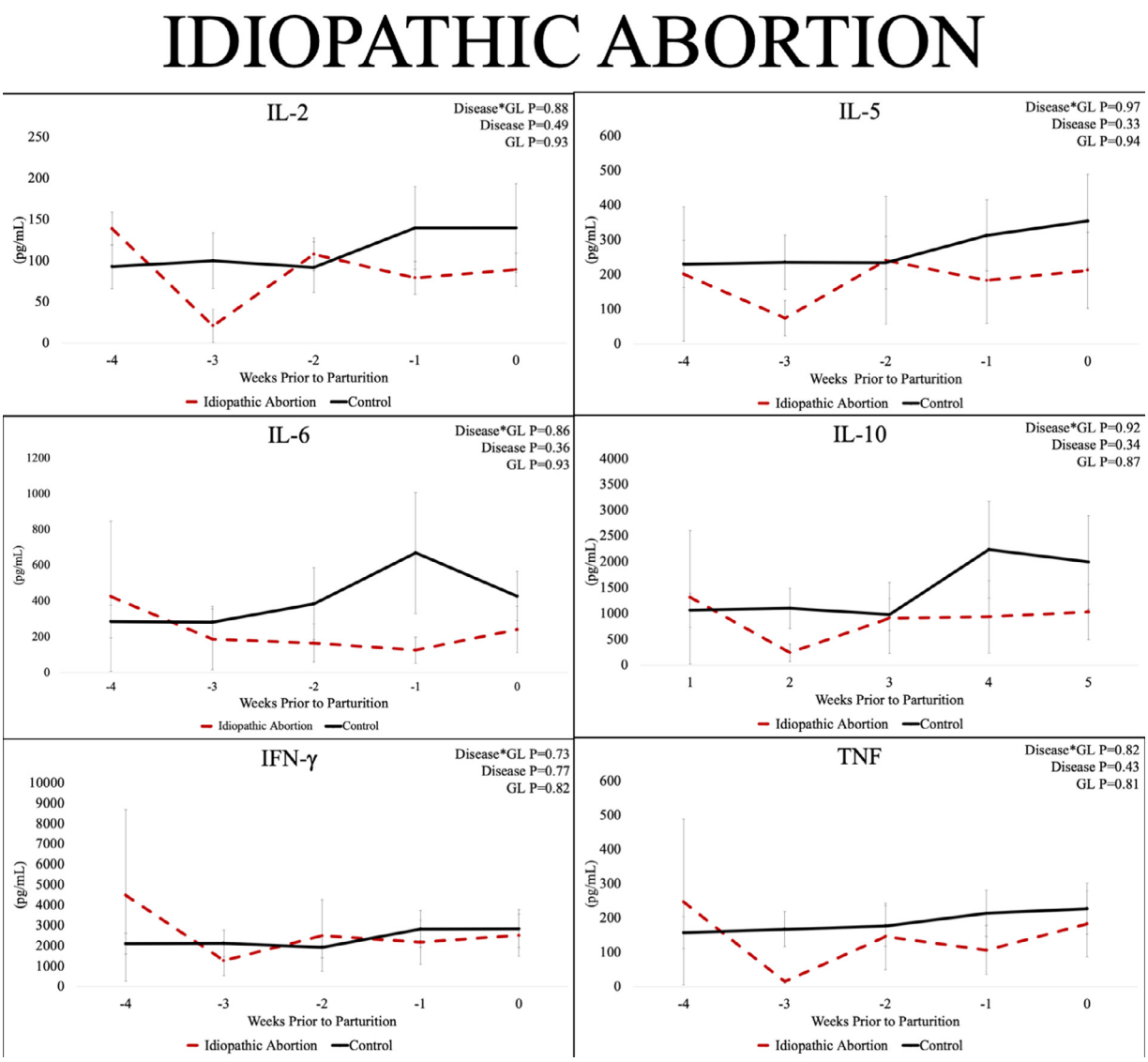

Fig. 3. Cytokine profile noted during idiopathic abortion. Concentrations of IL-2, IL-5, IL-6, IL-10, IFNγ, and TNF were evaluated in mares with idiopathic abortion (n = 6) compared to gestationally age-matched control mares (n = 20) at −4, −3, −2, and −1 weeks in addition to the week of parturition. None of the cytokines altered during idiopathic abortions in comparison to controls. Data shown represent the mean ± the standard error of the mean (SEM). Asterisks (*) above data points indicates differences ($P < .05$), while pound sign (#) above the data points indicate differences ($P < .10$) within week in cytokine concentrations between the diseased and control groups. GL = gestational length.

sponse has been shown to precede various pregnancy related complications in other species, including preterm labor, premature rupture of membranes, and fetal inflammatory syndrome [46–52]. As none of the pregnancies within the two placentitis groups resulted in abortion, it is unknown if the cytokines were elevated due to abortion signals, or in the anti-inflammatory antenatal response that has been described in the past to be necessary for pregnancy to carry to term [2,46,48,51,53]. Future research is required to expand on exact role of these mediators of inflammation in equine abortion.

In the present study, an increase in the pro-inflammatory cytokines IL-2 and IFNγ was observed in mares with both focal mucoid and ascending placentitis. In contrast, no changes in serum IL-2 and IFNγ were observed preceding idiopathic abortion. Both considered pro-inflammatory and pyrogenic signaling molecules, these Th1-related cytokines are initial responders to the detection of intracellular pathogens, in addition to being critical for recruitment of the immune cells required for the degradation of cellular adhesions between tissues, including the placenta. Elevated concentrations of IL-2 and IFNγ in serum have been noted during placental infection in humans [54], in addition to the experimental induction of ascending placentitis in the horse [19]. An elevation of these cytokines has also been found in sera of humans and mice following infection with a variety of the common pathogens responsible for ascending placentitis (*Streptococcus* [55] and *E.coli* [56,57], in addition to focal mucoid placentitis (*Actinobacteria*; [58,59]). Therefore, the cytokine increase noted during equine placental infection, regardless of etiology, is not surprising. Interestingly, no elevation of these pro-inflammatory cytokines was noted preceding noninfectious abortion. As an activation of the Th1 response is perceived to be critical for the initiation of parturition in various species, it is intriguing that no alterations of either Th1-related cytokine was noted preceding abortion. This may be due to the rapid onset of abortion disallowing for the activation of this Th1 response. Additionally, it is unknown if this prepartum Th1 signaling occurs in the horse, and may be initiated only hours prior to parturition, and therefore weekly sampling may be too infrequent to detect this elevation.

Naturally occurring ascending and focal mucoid placentitis also led to an increase in the pleiotropic cytokines IL-6 and TNF. These cytokines are able to activate both pro- and anti-inflammatory pathways, the functionality of which is dependent on the cell surface receptors through which they bind [60–62]. Although both IL-6 and TNF increase in women experiencing chorioamnionitis and preterm rupture of membranes, IL-6 is considered to be the most sensitive and specific predictor of IAI and has been evaluated in a variety of fluids including amniotic [38], vaginal [63], and cervical [64], in addition to within circulating plasma/serum in humans [65]. Following the experimental induction of equine ascending placentitis, IL-6 was found to increase in both amniotic and allantoic fluid [2,18], fetoplacental tissues [2,14], and maternal tissues [15,16], in addition to systemically in serum [18,19]. Additionally, recent research described IL-6 to be functioning as anti-inflammatory during the process of a subacute ascending placentitis in mares, activating the classical signaling pathway to act as antiapoptotic, prosurvival, and potentially impeding proinflammatory abortion signals [18]. Therefore, the observed increase in IL-6 concentrations during spontaneous placentitis is not altogether surprising. In contrast, less is known regarding the expression, secretion, and function of TNF in the pathogenesis of placental infection in the horse. In human pregnancies, TNF has been found to increase in amniotic fluid [66] as well as systemically in serum/plasma in patients experiencing chorioamnionitis [54]. While expression and secretion of TNF has been investigated in equine placentitis, results are conflicting. One report found no alterations in TNF expression following the experimental induction of an acute ascending placentitis [14], while a second study determined TNF to be a key regulator of varying downstream pathways which govern the maternal response to this disease [15]. Data from our laboratory suggests that TNF concentrations increase in circulation following the experimental induction of a sub-acute ascending placentitis (unpublished data), but it is unclear if this increase in cytokine production activates the TNF receptor (TNFR)-1 or TNFR-2 to function as pro- or anti-inflammatory. Additionally, the pleiotropic nature of these cytokines may differ if the chronicity of disease altered, or if the pregnancy produced a nonviable neonate, which could not be investigated under the confines of this study.

In addition to the increase in both pro- and pleiotropic cytokines, an increase in the concentrations of anti-inflammatory IL-5 and IL-10 was also noted during spontaneous ascending and focal mucoid placentitis. Integral to the maturation of Th2 cells, placental IL-5 expression has been found to increase in mid to late equine gestation, with the anti-inflammatory effector functions of Th2 cells believed to dictate uterine quiescence to allow for fetal growth and development [67,68]. Similarly, IL-10 is vital for the heightened Treg response noted during late gestation that allows for immunotolerance and acceptance of the semi-allogeneic fetus [67,68]. While an increase in amniotic fluid IL-10 has been associated with chorioamnionitis in humans [35,38,66], a decrease in serum IL-10 concentrations has been correlated with preterm birth [69] and pregnancy-related complications [70], indicating that the IL-10 profile may be associated with neonatal outcome. Few have reported an increase in IL-5 during pregnancy-related complications, and IL-5 was not found to increase during chorioamnionitis in women [38], although one study found elevated IL-5 as predicative of preterm birth [71]. IL-5 is poorly understood in the pathophysiology of placental infection in horses, although an increase in Th2-related transcripts and their secretory cytokines has been described [19]. An increase in anti-inflammatory and pleiotropic cytokines is hypothesized to be in part due to an active fetal immune response to pathogens, and may explain the heightened IL-5, IL-6, IL-10, and TNF response seen in the present study [72,73]. These anti-inflammatory markers have been described to promote successful cellular interactions at the feto-maternal interface, and indicate the immune-mediated cross-talk between mare and fetus [29,74]. Additionally, an activation of anti-inflammatory and/or immunomodulatory cytokines is needed to suppress the proinflammatory response noted following pathogen recognition, and may explain the viability of the neonates produced within the present study. It is unknown if the profile of these cytokines would change preceding abortion.

Limitations of this study include the retrospective analysis of the month preceding parturition, thereby limiting both the time to intervention, as well as prohibiting the prospective prediction of disease. It should be noted that although a considerable number of abortions occur prior to 300 days of gestation, epidemiological studies have indicated that the majority of abortions in Central Kentucky occur >300 days gestation [1,20]. Therefore, alterations noted in within the final month of gestation may be clinically relevant and allow for peremptory therapeutic intervention. Conclusions from this study are also limited in that the majority of mares delivered a viable term neonate, restricting extrapolations into changes noted prior to abortion. Other restraints of this study include the small sample sizes acquired in order for appropriate comparisons between diseased and control mares. Although the differences noted amongst smaller sample sizes are reliable, when assessing decreased sample size, lower power is inferred. Therefore, the risk of a statistical type II error increases, and the lack of significant findings should be interpreted carefully. Future research is required to assess the endpoints that were identified within this study and should be done so with increased sample size in order to consider differences between term pregnancies associated with placental disease in comparison to abortions caused by placental disease.

In conclusion, an active immune response occurs during naturally occurring placental infection in the horse. This was noted in both the ascending and focal mucoid placentitis groups, although no changes were noted in the idiopathic abortion group – indicating that the cytokine response may be due to infection noted, and not indicative of impending abortion. Spontaneous ascending placentitis was found to mimic that of the experimental induction of a sub-acute ascending placentitis in the research setting, leading to an increase in serum IL-2, IL-5, IL-6, IL-10, TNF, and IFNγ. This is also the first report to demonstrate that clinical focal mucoid placentitis responds similarly to that of ascending placentitis, and results in an increase in IL-2, IL-5, IL-6, IL-10, TNF, and IFNγ. No systemic alterations were noted in the observed markers preceding idiopathic abortion, suggesting an acute nature of these pathologies, and the unpredictability of their occurrence concerning cytokine response. Future research is required to determine the efficacy of these markers in predicting pregnancy-related complications in the field, in addition to assessing their association with acute disease preceding abortion.

Acknowledgments

The authors would like to thank the numerous veterinarians, farm managers, office staff, and clinics that assisted in the collection of these samples.

Funding

This study was funded by the Kentucky Thoroughbred Owners and Breeders Association, and the Albert G. Clay Research Endowment at the University of Kentucky in addition to considerable industry support.

References

[1] Hong CB. Equine abortion and stillbirth in central Kentucky during 1988 and 1989 foaling seasons. J Vet Diagn Invest 1993;5(4):560–6.
[2] Fedorka CE. The feto-maternal immune response to equine placentitis. Am J Reprod Immunol 2019 e13179.
[3] Donahue JM, Williams NM. Emergent causes of placentitis and abortion. Vet Clin North Am Equine Pract 2000;16(3):443–56 viii.
[4] Canisso IF. Attempts to induce nocardioform placentitis (Crossiela equi) experimentally in mares. Equine Vet J 2015;47(1):91–5.
[5] Fedorka CE. Interleukin-6 pathobiology in equine placental infection. Am J Reprod Immunol 2020 e13363.
[6] Fedorka CE. The imbalance of the Th17/Treg axis following equine ascending placental infection. J Reprod Immunol 2021;144:103268.
[7] Canisso IF. Serum amyloid A and haptoglobin concentrations are increased in plasma of mares with ascending placentitis in the absence of changes in peripheral leukocyte counts or fibrinogen concentration. Am J Reprod Immunol 2014;72(4):376–85.
[8] Canisso IF. Changes in maternal androgens and oestrogens in mares with experimentally-induced ascending placentitis. Equine Vet J 2017;49(2):244–9.
[9] Canisso IF. Alpha-fetoprotein is present in the fetal fluids and is increased in plasma of mares with experimentally induced ascending placentitis. Anim Reprod Sci 2015;154:48–55.
[10] Canisso IF, Loux SC, Lima FS. Biomarkers for placental disease in mares. Theriogenology 2020.
[11] Wynn MAA. Changes in maternal pregnane concentrations in mares with experimentally-induced, ascending placentitis. Theriogenology 2018;122:130–6.
[12] Renaudin CD. Ultrasonographic evaluation of the equine placenta by transrectal and transabdominal approach in the normal pregnant mare. Theriogenology 1997;47(2):559–73.
[13] Erol E. An investigation of a recent outbreak of nocardioform placentitis caused abortions in horses. Vet Microbiol 2012;158(3-4):425–30.
[14] LeBlanc MM. Relationship between infection, inflammation and premature parturition in mares with experimentally induced placentitis. Equine Vet J Suppl 2012;41(41):8–14.
[15] El-Sheikh Ali H, Boakari YL, Loux SC, Dini P, Scoggin KE, Esteller-Vico A, et al. Transcriptomic analysis reveals the key regulators and molecular mechanisms underlying myometrial activation during equine placentitis. Biol. Reprod. 2020;102:1306–25.
[16] Fernandes CB. Uterine cervix as a fundamental part of the pathogenesis of pregnancy loss associated with ascending placentitis in mares. Theriogenology 2020;145:167–75.
[17] Scheller J, Garbers C, Rose-John S. Interleukin-6: from basic biology to selective blockade of pro-inflammatory activities. Semin Immunol 2014;26(1):2–12.
[18] Fedorka CE, Scoggin KE, El-Sheikh Ali H, Loux S, Dini P, Troedsson MHT, et al. Interleukin-6 pathobiology in equine placental infection. Am J Reprod Immunol 2020:e13363.
[19] Fedorka CE, El-Sheikh Ali H, Walker OF, Scoggin KE, Dini P, Loux SC, et al. The imbalance of the Th17/Treg axis following equine ascending placental infection. J Reprod Immunol 2021;144:103268.
[20] Hong CB. Etiology and pathology of equine placentitis. J Vet Diagn Invest 1993;5(1):56–63.
[21] Wagner B, Freer H. Development of a bead-based multiplex assay for simultaneous quantification of cytokines in horses. Vet Immunol Immunopathol 2009;127(3-4):242–8.
[22] Hornung RW, Reed LD. Estimation of average concentration in the presence of nondetectable values. Appl Occup Environ Hyg 1990;5:46–51.
[23] Davies Morel MC, Newcombe JR, Holland SJ. Factors affecting gestation length in the Thoroughbred mare. Anim Reprod Sci 2002;74(3-4):175–85.
[24] Bowen JM. Cytokines of the placenta and extra-placental membranes: biosynthesis, secretion and roles in establishment of pregnancy in women. Placenta 2002;23(4):239–56.
[25] Bowen JM. Cytokines of the placenta and extra-placental membranes: roles and regulation during human pregnancy and parturition. Placenta 2002;23(4):257–73.
[26] Chaouat G. The Th1/Th2 paradigm: still important in pregnancy? Semin Immunopathol 2007;29(2):95–113.
[27] Chaouat G. Reproductive immunology 2003: reassessing the Th1/Th2 paradigm? Immunol Lett 2004;92(3):207–14.
[28] Cobo T. Intra-amniotic inflammatory response in subgroups of women with preterm prelabor rupture of the membranes. PLoS One 2012;7(8) e43677.
[29] Cubro H. The Role of Interleukin-10 in the Pathophysiology of Preeclampsia. Curr Hypertens Rep 2018;20(4):36.
[30] Dekker GA, Sibai BM. The immunology of preeclampsia. Semin Perinatol 1999;23(1):24–33.
[31] Dudley DJ. Inflammatory cytokine mRNA in human gestational tissues: implications for term and preterm labor. J Soc Gynecol Investig 1996;3(6):328–35.
[32] Eghbal-Fard S. The imbalance of Th17/Treg axis involved in the pathogenesis of preeclampsia. J Cell Physiol 2019;234(4):5106–16.
[33] Gargano JW. Mid-pregnancy circulating cytokine levels, histologic chorioamnionitis and spontaneous preterm birth. J Reprod Immunol 2008;79(1):100–10.
[34] Gomez-Lopez N. A Role for the Inflammasome in Spontaneous Preterm Labor With Acute Histologic Chorioamnionitis. Reprod Sci 2017;24(10):1382–401.
[35] Hanna N. Evidence for interleukin-10-mediated inhibition of cyclo- oxygenase-2 expression and prostaglandin production in preterm human placenta. Am J Reprod Immunol 2006;55(1):19–27.
[36] Hill JA, Polgar K, Anderson DJ. T-helper 1-type immunity to trophoblast in women with recurrent spontaneous abortion. JAMA 1995;273(24):1933–6.
[37] Ito M. A role for IL-17 in induction of an inflammation at the fetomaternal interface in preterm labour. J Reprod Immunol 2010;84(1):75–85.
[38] Cobo T. Intra-amniotic inflammation predicts microbial invasion of the amniotic cavity but not spontaneous preterm delivery in preterm prelabor membrane rupture. Acta Obstet Gynecol Scand 2012;91(8):930–5.
[39] Holst RM. Prediction of microbial invasion of the amniotic cavity in women with preterm labour: analysis of multiple proteins in amniotic and cervical fluids. BJOG 2011;118(2):240–9.
[40] Holst RM. Prediction of spontaneous preterm delivery in women with preterm labor: analysis of multiple proteins in amniotic and cervical fluids. Obstet Gynecol 2009;114(2 Pt 1):268–77.
[41] Jacobsson B. Microbial invasion and cytokine response in amniotic fluid in a Swedish population of women in preterm labor. Acta Obstet Gynecol Scand 2003;82(2):120–8.
[42] Cobo T, Kacerovsky M, Jacobsson B. Noninvasive sampling of the intrauterine environment in women with preterm labor and intact membranes. Fetal Diagn Ther 2018;43:241–9.
[43] Cobo T. A prediction model of histological chorioamnionitis and funisitis in preterm prelabor rupture of membranes: analyses of multiple proteins in the amniotic fluid. J Matern Fetal Neonatal Med 2012;25(10):1995–2001.
[44] Figueiredo AS, Schumacher A. The T helper type 17/regulatory T cell paradigm in pregnancy. Immunology 2016;148(1):13–21.
[45] Qian J. Distinct pattern of Th17/Treg cells in pregnant women with a history of unexplained recurrent spontaneous abortion. Biosci Trends 2018;12(2):157–67.
[46] Cornelius DC. Preeclampsia: From Inflammation to Immunoregulation. Clin Med Insights Blood Disord 2018;11 1179545X17752325.
[47] Galinsky R. The consequences of chorioamnionitis: preterm birth and effects on development. J Pregnancy 2013:412831 **2013**.
[48] Kemp MW. Selective exposure of the fetal lung and skin/amnion (but not gastro-intestinal tract) to LPS elicits acute systemic inflammation in fetal sheep. PLoS One 2013;8(5) e63355.
[49] Kim CJ. Acute chorioamnionitis and funisitis: definition, pathologic features, and clinical significance. Am J Obstet Gynecol 2015;213(4 Suppl):S29–52.
[50] Kwak-Kim J, Lee SK, Gilman-Sachs A. Elevated Th1/Th2 cell ratios in a pregnant woman with a history of RSA, secondary Sjogren's syndrome and rheumatoid arthritis complicated with one fetal demise of twin pregnancy. Am J Reprod Immunol 2007;58(4):325–9.
[51] Ruschoff J, Boger A, Zwiens G. Chronic placentitis–a clinicopathological study. Arch Gynecol 1985;237(1):19–25.
[52] Tchirikov M. Mid-trimester preterm premature rupture of membranes (PPROM): etiology, diagnosis, classification, international recommendations of treatment options and outcome. J Perinat Med 2018;46(5):465–88.
[53] Romero R. Clinical chorioamnionitis at term V: umbilical cord plasma cytokine profile in the context of a systemic maternal inflammatory response. J Perinat Med 2016;44(1):53–76.
[54] Romero R. Clinical chorioamnionitis at term IV: the maternal plasma cytokine profile. J Perinat Med 2016;44(1):77–98.
[55] Danilova TA. Changed Serum Cytokine Profile in Mice in Response to Streptococcus A Culture. Bull Exp Biol Med 2015;159(1):66–9.
[56] Ronit A. Inflammation-induced changes in circulating T-cell subsets and cytokine production during human endotoxemia. J Intensive Care Med 2017;32(1):77–85.
[57] Griesinger G. Production of pro- and anti-inflammatory cytokines of human placental trophoblasts in response to pathogenic bacteria. J Soc Gynecol Investig 2001;8(6):334–40.
[58] Rovetta AI. IFNG-mediated immune responses enhance autophagy against Mycobacterium tuberculosis antigens in patients with active tuberculosis. Autophagy 2014;10(12):2109–21.
[59] Goyal N, Kashyap B, Kaur IR. Significance of IFN-/IL-2 ratio as a circulating diagnostic biomarker in extrapulmonary tuberculosis. Scand J Immunol 2016;83(5):338–44.
[60] Augustyniak D. [The role of IL-6/sIL-6R complex and its natural inhibitor sgp130 in modulation of inflammatory process]. Postepy Biochem 2006;52(2):194–203.
[61] Baran P. The balance of interleukin (IL)-6, IL-6.soluble IL-6 receptor (sIL-6R), and IL-6.sIL-6R.sgp130 complexes allows simultaneous classic and trans-signaling. J Biol Chem 2018;293(18):6762–75.
[62] Kalliolias GD, Ivashkiv LB. TNF biology, pathogenic mechanisms and emerging therapeutic strategies. Nat Rev Rheumatol 2016;12(1):49–62.
[63] Musilova I. Vaginal fluid IL-6 concentrations as a point-of-care test is of value in women with preterm PROM. Am J Obstet Gynecol 2016.
[64] Kacerovsky M. Cervical fluid IL-6 and IL-8 levels in pregnancies complicated by preterm prelabor rupture of membranes. J Matern Fetal Neonatal Med 2015;28(2):134–40.
[65] Park JW. Immune biomarkers in maternal plasma to identify histologic chorioamnionitis in women with preterm labor. Arch Gynecol Obstet 2019;299(3):725–32.
[66] Revello R. Differential amniotic fluid cytokine profile in women with chorioamnionitis with and without funisitis. J Matern Fetal Neonatal Med 2016;29(13):2161–5.
[67] Saito S. Cytokine network at the feto-maternal interface. J Reprod Immunol 2000;47(2):87–103.
[68] Saito S. Th1/Th2/Th17 and regulatory T-cell paradigm in pregnancy. Am J Reprod Immunol 2010;63(6):601–10.

[69] Pandey M, Chauhan M, Awasthi S. Interplay of cytokines in preterm birth. Indian J Med Res 2017;146(3):316–27.
[70] Azizieh FY, Raghupathy R. IL-10 and pregnancy complications. Clin Exp Obstet Gynecol 2017;44(2):252–8.
[71] Cordeiro CN. Mathematical Modeling of the Biomarker Milieu to Characterize Preterm Birth and Predict Adverse Neonatal Outcomes. Am J Reprod Immunol 2016;75(5):594–601.
[72] Perryman LE, McGuire TC, Torbeck RL. Ontogeny of lymphocyte function in the equine fetus. Am J Vet Res 1980;41(8):1197–200.
[73] Battista JM. Hematopoiesis in the equine fetal liver suggests immune preparedness. Immunogenetics 2014;66(11):635–49.
[74] Fedorka CE. The feto-maternal immune response to equine placentitis. Am J Reprod Immunol 2019;82(5) e13179.