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Investigation of relationships between serum lactate, acute phase proteins, pro/antiinflammatory cytokine levels, and metritis formation in Holstein dairy heifers

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Abstract: The main purpose of this investigation was to identify and suggest suitable early screening inflammatory markers for the early diagnosis of uterine infections such as acute septic metritis (ASM) in the Holstein heifers. In addition, to achieve the above -mentioned aim, the objectives of the present study were tried to determine the relationship between blood serum lactate, acute phase proteins (APPs), pro/anti inflammatory cytokine levels, and metritis formation in Holstein heifers. For this purpose, 250 Holstein heifers which were 11–13 months old, were chosen as the study material. After heifers were conceived, blood serum samples were collected at the 8th and 4th weeks before the parturition. In the postpartum period, animals with ASM (n = 15) and healthy animals (n = 15) were identified, and blood serum samples were taken. Lactate, serum amyloid-A (SAA), haptoglobin (Hp), Interleukin-1beta (IL-1 β), IL-6, IL-8, IL-10, and tumor necrosis factor-alpha (TNF - α) levels were measured from these collected blood serum samples. Acute-phase proteins, lactate, and pro and anti inflammatory cytokine levels of healthy and animals with ASM were statistically compared. Heifers with metritis had a significantly greater concentration of IL-6 and SAA versus healthy heifers in the prepartum 8th week. In addition, it was determined that IL-6, IL-1β, IL-10, TNF-α, and SAA levels were significantly higher in the 4th week before parturition in the ASM group than those of the healthy group. When all these findings are evaluated together, this study suggested that IL-6, and IL-10, can be used as early biomarkers in the prepartum preliminary diagnosis of postpartum important inflammatory processes such as ASM.

Key words: Heifer, metritis, cytokines, acute phase proteins, lactate

1. Introduction

Inflammation is the body's specific and nonspecific response to injury and damage in the tissues of humans and animals and the invasions of foreign organisms. When inflammation occurs in the uterus of the cattle, as a clinical result of this situation, the pregnancy rate in the first insemination decreases and the time between calving and conception becomes longer, in short, infertility occurs. Uterine infections not only lead to a decrease in reproductive efficiency, but also a decrease in feed consumption and consequently milk production, and an increase in the costs of treatment and involuntary culling. In addition, acute septic metritis (ASM) formed in the postpartum period can have life-threatening results [1]. Nonspecific uterine infections seen in cows can be classified as acute septic metritis, clinical metritis, clinical endometritis, pyometra, and subclinical endometritis. ASM is a uterine infection that in the first 21 days of postpartum, specific findings such as abundant red-brown, foul-smelling, watery, uterine contents with necrotic debris and vaginal discharge, an abnormally enlarged, thin-walled, and toneless uterus. Also, systemic findings such as loss of appetite, stillness, an increase in body temperature (≥39.5 °C), and a decrease in milk production are observed [2–5]. The incidence in a herd range from 2.2% to 37.3% [5,6].

Uterine infections are largely eliminated by a cellular defense mechanism consisting of cells that phagocytize and kill bacteria such as macrophages, monocytes, and neutrophils, and also by humoral defense mechanisms including immune globulins [1,7,8]. Cellular and humoral mechanisms associated with local nonspecific and specific immunity have a vital role in eliminating uterine infections. Pathogens are recognized through toll-like receptors on endometrial cells and macrophages and also molecular patterns associated with pathogens. Then, proinflammatory cytokines (IL-1β, IL-6, TNF-α) and chemokines such as IL-8 are secreted and released by activated immune cells

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[9]. These proinflammatory cytokines and chemokines stimulate the extravasation of neutrophils and monocytes into the uterine lumen (diapedesis), their chemoattraction to the inflammation site, and encourage phagocytosis [7,10–12]. Proinflammatory cytokines also stimulate the production of acute phase proteins. Acute phase proteins protect tissues against pathological damage, aid homeostasis, regulate the stages of inflammation, and assist in the elimination of infection through the stimulation of phagocytosis [13–16].

Significant differences were reported in the serum levels of proinflammatory (IL-10) and anti-inflammatory (IL-6, TNF-α) cytokines, acute phase proteins such as serum amyloid - A (SAA) between cows with metritis and healthy cows [17,18].

In some studies, at certain stages of a period from the 8th or 4th week before calving until the 4th or 8th weeks after parturition, the serum concentrations of some inflammatory indicators such as SAA, TNF-α, IL-1β, IL-6, IL-10 have been shown to be greater in cows with metritis than those of healthy ones [17,19].

As a result of the literature review, no study was found that investigated the relationship between blood serum concentrations of some inflammatory reactants and acute septic metritis formation and tried to identify early screening inflammatory markers for early diagnosis of acute septic metritis in the Holstein dairy heifers.

The main purpose of this investigation was to identify and suggest suitable early screening inflammatory markers for early diagnosis of acute septic metritis from between some inflammatory reactants for Holstein dairy heifers such as in dairy cows.

The other objectives of this study were to evaluate the blood serum levels of acute phase proteins, pro/antiinflammatory cytokines, and lactate throughout a period from the prepartum 8th week to postpartum disease diagnosis in the Holstein dairy heifers, and also to investigate whether there is a relationship between ASM formation and inflammatory reactants.

2. Material and method

2.1. Animal material, housing, and feeding

This study, which was deemed appropriate by the meeting of the Bursa Uludağ University Ethics Committee dated 30.04.2019 and the decision numbered 2019-05/07, was carried out on a farm with a disease-free certificate established within the borders of Karacabey district of Bursa province. In the first stage, a total of 250 Holstein heifers, 11–13 months old, live weight between 298 and 356 kg and mean live weight 323 ± 27.61 kg, nonpregnant, never inseminated and healthy, and had no abnormality related to reproductive organs were used as the study material.

Heifers were kept in an open barn with shade and a soil-floored yard where animals roam freely, without changing the farm's routine maintenance, housing, and feeding conditions. All animals were fed a TMR (consisting of alfalfa, straw, maize silage, and concentrates) once daily with ad libitum access to water.

2.2. Experimental processes

2.2.1. Artificial insemination (AI) and pregnancy diagnosis

The heifers underwent AI in spontaneous estrus and in estruses induced by $PGF_2\alpha$. The pregnancies were determined by transrectal ultrasonography (SIUI CTS-800 Handheld Veterinary Ultrasound; Linear Rectal Probe; L7FVC, 50mm, 6.5–9 MHz–smaller footprint, higher frequency) on the 30th and 60th days following AI.

2.2.2. Blood collection, separation of serum, and storage of samples before parturition

Two tubes of blood per animal were collected from the tail vein (vena coccygea) at 8 and 4 weeks before the estimated calving time from all pregnant heifers. Blood samples were rotated in a refrigerated centrifuge at +4 °C at 2500 rpm for 10 min and the separated serum samples were transferred in 2 mL eppendorf tubes. Tubes were stored at – 80 °C until for assay of lactate, a carbohydrate metabolism metabolite, and of some inflammatory reactants such as IL-1β, L-6, IL-8, IL-10, TNF-α, SAA, and Hp.

2.2.3. Diagnosis of metritis cases and identification of healthy animals and collection of blood serum between the 2nd and 15th postpartum days

Clinical examinations were performed as described in the "Clinical examinations, definitions and diagnostic criteria (2.2.4)" section between the 2nd and 15th days of postpartum. Heifers with acute septic metritis were diagnosed by considering the criteria in section "2.2.4". Likewise, healthy animals without metritis were determined. Serum samples were collected and stored from heifers with healthy and ASM as previously mentioned in section 2.2.2.

2.2.3.1. Experimental groups

Two hundred and fifty heifers were divided into 2 groups according to health state as those with ASM and healthy.

Group ASM (n: 15): The group consisted of 15 heifers that were diagnosed with ASM after parturition.

Control group (n: 15): The group consisted of 15 healthy heifers without metritis after parturition.

2.2.4. Clinical examinations and definitions, diagnostic criteria

During the postpartum period starting from the first day until the 15th day, all animals were examined every day clinically and by vaginoscopy, and rectal palpation.

The body temperature of all animals was measured rectally prior to morning milking every day between 2–15

after parturition and recorded. Animals were observed daily for appetite and other behavioral signs. Also, animals were concurrently examined in terms of laminitis, mastitis, displacement of the abomasum, and other infectious and metabolic diseases, and animals with such problems were excluded from the study.

Within 2–15 days after parturition, cows that had an abnormally enlarged uterus, and watery, a fetid smelling reddish-brown vaginal discharge, additional with systemic signs such as decreased milk yield, loss of appetite, stillness, fever (>39.5°C) diagnosed with acute septic metritis [3,4,20,21].

Animals with no purulent, mucopurulent, or fetid odor vaginal discharge detected by vaginoscopic examination, whose uterus completed the normal involution process, whose cervix diameter was normal (diameter < 7.5 cm), no systemic disease symptoms were detected, and had no infectious and metabolic diseases (laminitis, mastitis, displacement of the abomasum, ketosis, etc.) were considered as healthy animals without metritis, that is, as the control group [3,4].

2.2.5. Determination of serum concentrations of lactate, cytokines, and acute phase proteins

Serum concentrations of SAA, Hp, TNF-α, IL-1β, IL-6, IL-8, and IL-10 were measured by the Multiskan FC ELISA Reader (Thermo Scientific, Waltham, Massachusetts, USA) device using commercially available Bovine ELISA kit (USCN Life Science Inc., Houston, TX, USA). All measurements were made according to the manufacturer's user manual and the procedure specified in the literature [22]. Lactate concentration was determined by using the commercially produced ELISA kit (Lactate assay kit, Cayman Chemical, item no. 700510 MI-USA), and according to the manufacturer's instructions and also to a method described previously in the literature [18,23]. In the measurement of serum lactate concentration, the Fluoroskan Ascent FL device was used (Thermo Scientific, Waltham, Massachusetts, USA).

2.2.6. Statistical analysis

The conformity of the data to the normal distribution was tested with the Shapiro-Wilk test. Parametric tests were used if the data showed normal distribution and nonparametric tests were used if they did not show normal distribution. In the comparison of repeated measures over time, analysis of variance (repeated ANOVA) and Friedman's test were used for repeated measures. After pairwise comparisons, the results were interpreted according to Bonferroni's correction. T-test and Mann-Whitney U test were used for comparisons between groups. In the case of parametric tests, descriptive values were given as mean and standard deviation, and in the case of nonparametric tests, descriptive values were given as the median (min-max) value. Statistical analyzes were performed with SPSS v25. The statistical significance level was taken as $p < 0.05$.

3. Results

3.1. Serum levels of acute phase proteins

There was no significant difference between ASM and control groups in terms of mean serum Hp concentrations at the 8th and 4th weeks before parturition ($p = 0.512$; $p = 0.29$)(Table 1,2), whereas the mean serum Hp values of animals with ASM at the time of diagnosis was noticeably greater than those in healthy heifers ($p < 0.001$). The serum SAA level in the 8th and 4th weeks prior to parturition and at the time of diagnosis was significantly higher in heifers with ASM than that in healthy heifers ($p = 0.001$, p

Table 1. The comparison of serum concentrations of cytokines, APPs, and lactate measured at the 8th weeks prior to calving in dairy heifers with and without ASM.

Parameters	8 weeks before parturition		
	ASM	Control	p-value
IL-1β (pg/mL)	307.29 ± 9.38	312.92 ± 9.46	0.113
IL-6 (pg/mL)	83.02 ± 22.01	19.09 ± 2.90	<0.001
IL-8 (pg/mL)	21.21 ± 4.77	20.70 ± 3.76	0.746
IL-10 (pg/mL)	58.19 ± 3.84	55.09 ± 4.81	0.06
TNF-α (pg/mL)	605.00 ± 89.44	555.77 ± 59.95	0.08
SAA (µg/mL)	7770.91 ± 475.65	7176.62 ± 445.27	0.001
Hp (µg/mL)	280.29 (235.26: 357.37)	273.86 (215.08: 362.78)	0.512
Lactate (µmol/L)	2064.91 ± 101.58	2000.81 ± 117.57	0.121

APPs: Acute Phase Proteins, ASM: Acute Septic Metritis, IL: Interleukin, TNF-α: Tumor Necrosis Factor Alpha, SAA: Serum Amyloid A, Hp: Haptoglobin

< 0.001, p = 0.04). In particular, the serum SAA value of heifers with ASM in the 4th week before parturition was very noticeably greater than those of healthy heifers (p < 0.001) (Table 2).

3.2. Serum cytokine levels

There was no statistically significant difference in serum IL-1β concentrations measured at prepartum 8th week and at the time of diagnosis between ASM and control groups (p = 0.113, p = 0.116). The mean IL-1β serum concentration at the prepartum 4th weeks in the healthy animals was noticeably higher than those in heifers suffering from ASM (p < 0.001). The mean serum concentration of IL-1β in heifers with ASM was lower than that of the healthy animal unlike in the measurement results of other cytokines, SAA, and lactate at all measurement times.

Serum IL-6 concentrations measured in the 8th and 4th weeks before parturition and at the time of diagnosis were found to be very noticeably higher in the group with ASM than those in the healthy group (p < 0.001).

The serum IL-8 concentrations in the 8th and 4th weeks prior to calving were not significant variations between groups (p = 0.746, p = 0.66). The serum IL-8 concentration measured at the time of diagnosis of the ASM group was found to be significantly higher than the control group (p < 0.001) (Table 3).

Serum IL-10 and TNF-α concentrations measured in the 8th week before parturition are not significantly different between heifers with ASM and healthy (p = 0.06, p = 0.08). Serum IL-10 and TNF-α concentrations measured at the prepartum 4th week and at the time of diagnosis were significantly different between groups (p < 0.001, p < 0.001) (Table 2,3).

3.3. Carbohydrate metabolite; lactate

When the serum lactate concentrations that were measured in the prepartum 8th, and 4th weeks and at the time of diagnosis of the ASM were compared with those of the control group, no significant differences were detected between the groups (p = 0.121, p = 0.219, p = 0.098).

4. Discussion

In this study, it was assumed that, just as in dairy cows, dairy heifers that are likely to develop ASM postpartum will have greater serum levels of some acute phase proteins, cytokines, and lactate at the prepartum 8th and 4th weeks than those in healthy heifers. In addition, it was hypothesized that some inflammatory mediators and lactate could be used as prepartum early screening biomarkers in the predicting of heifers likely to develop ASM after parturition.

TNF-α stimulates the expression of IL-8, and cell adhesion molecules [9]. In addition, proinflammatory cytokines such as IL-6 and TNF-α provide a positive feedback loop to further increase the mobilization of immune cells [19]. In a previous study conducted by [18], serum TNF-α concentrations measured at the 4th week of prepartum and at the time of diagnosis of the disease were compared between metritis and healthy cows, and the results of the study have been shown that TNF-α levels of cows with metritis were significantly higher than those of healthy cows. However, in the prepartum 8th week, TNF-α levels were higher in cows with metritis than in healthy, but the difference was not significant. When the same researchers evaluated the findings of their study, they concluded that TNF-α level measured 4 weeks before parturition has the potential to be used as an early screening biomarker to predict some postpartum inflammatory processes such as metritis. These findings are in full agreement with our study results, which revealed that TNF-α concentrations at prepartum 4th week and at the time of diagnosis were significantly higher in ASM heifers than healthy heifers. Also in the present study, as

Table 2. The evaluation of serum levels of IL-1, IL-6, IL-8, IL–10, and TNF-α, SAA, Hp, Lactate established at the 4th weeks prior to calving.

Parameters	4 weeks before parturition		
	ASM	Control	p-value
IL-1β (pg/mL)	290.94 ± 3.83	319.50 ± 1.90	<0.001
IL-6 (pg/mL)	149.42 ± 19.50	47.49 ± 10.39	<0.001
IL-8 (pg/mL)	15.26 ± 2.48	14.84 ± 2.72	0.66
IL-10 (pg/mL)	73.78 ± 4.64	62.32 ± 3.42	<0.001
TNF-α (pg/mL)	765.24 ± 178.53	304.74 ± 38.46	<0.001
SAA (μg/mL)	5109.19 ± 187.66	3885.78 ± 165.77	<0.001
Hp (μg/mL)	153.88 ± 9.07	150.13 ± 9.98	0.29
Lactate (μmol/L)	2673.50 ± 144.23	2569.40 ± 283.88	0.219

ASM: Acute Septic Metritis, IL: Interleukin, TNF-α: Tumor Necrosis Factor Alpha, SAA: Serum Amyloid A, Hp: Haptoglobin

Table 3. The valuation of serum concentrations of some parameters determined at the time of diagnosis.

Parameters	Diagnosis week		
	ASM	**Control**	**p-value**
IL-1β (pg/mL)	273.71 (269.15: 295.10)	277.71 (271.84: 284.17)	0.116
IL-6 (pg/mL)	102.00 ± 14.11	24.53 ± 6.02	<0.001
IL-8 (pg/mL)	137.71 ± 12.88	101.97 ± 7.50	<0.001
IL-10 (pg/mL)	95.36 ± 8.67	83.03 ± 4.38	<0.001
TNF-α (pg/mL)	661.52 ± 71.61	66.02 ± 10.29	<0.001
SAA (μg/mL)	9931.94 ± 560.54	9409.13 ± 756.72	0.04
Hp (μg/mL)	291.57 ± 28.96	112.70 ± 9.48	<0.001
Lactate (μmol/L)	2368.28 ± 135.10	2267.02 ± 47.81	0.098

ASM: Acute Septic Metritis, IL: Interleukin, TNF-α: Tumor Necrosis Factor Alpha, SAA: Serum Amyloid A, Hp: Haptoglobin

in the study of [19], the difference in the 8th week before birth was not significant (Table 1). The only difference between the studies was the animal material, in our study heifers were used.

The changes in the IL-1β levels during our study are shown in Tables 1, 2, and 3. IL-1β concentrations in healthy group heifers were greater than ASM group heifers at all three different sampling times. But the difference between groups was significant ($p < 0.001$) in only the prepartum 4th week. The findings obtained from a similar study showed that [18] serum IL-1 concentrations had a tendency to be lower in cows with metritis in both prepartum 8th week and diagnosis time compared to healthy cows. The same study revealed that IL-1 concentration in healthy cows in 4th week before calving was significantly higher than that in cows suffering from metritis ($p < 0.01$). In contrast to our study findings, in another study [24], higher serum IL-1β concentrations were measured in cows with postpartum metritis than in healthy cows, and BCS in cows with metritis was lower. In a study by [25], it was found that cows suffering from acute Puerperal Metritis (APM) have lower IL-1β expression than healthy cows. Similarly, it has been reported that peripheral blood mononuclear cells have low IL-1β expression levels in cows diagnosed with APM, and this situation is associated with inflammatory responses, and it has also been expressed that this process may contribute to the deterioration of inflammatory responses and the development of the disease. When the findings of the present study and the above-mentioned literature results were evaluated together, it was concluded that IL-1β cannot be used as an early screening biomarker in the diagnosis of animals with ASM. But considering the significantly lower 4th-week prenatal IL-1β concentration in healthy heifers compared to ASM heifers, IL-1β may be an early prepartum indicator to identify healthy heifers.

IL-6 is a cytokine produced by T cells. T helper-2 cells (Th2 cells) stimulate antibody formation by producing cytokines such as IL-6 and IL-10 [26]. In this study, mean blood serum levels of IL-6, a proinflammatory cytokine, both at the time of diagnosis and in the 8th and 4th weeks before birth were compared in heifers with ASM and healthy heifers. The mean IL-6 concentration was determined to be significantly higher in heifers with ASM than that of healthy heifers at three different sampling times ($p < 0.001$) (Table 1,2,3). This result agreed with a previously reported by [27], that prepartum IL-6 levels were higher in cows that had clinical endometritis after parturition than those in healthy cows. As it is known, clinical endometritis is an inflammation of the uterus that does not show any signs of systemic disease, presenting with purulent vaginal discharge on the 21st and following postpartum days or mucopurulent vaginal discharge after the 26th day [3–5]. In contrast, ASM is a uterine infection that has systemic manifestations and infects all uterine layers [2–5]. In the study of [27], similar results were obtained to our study, although cows with clinical endometritis, which is an infection localized in the uterus and do not cause general findings. Even animals suffering from endometritis which is a nonsystemic infection confined to the endometrium, view to be significantly elevated prepartum serum IL-6 levels [27]. Our results are well supported by the study findings of [18], which showed that the mean serum concentration of IL-6 in cows affected by metritis was higher than that in healthy prior to calving 8th and 4th weeks and ($p < 0.05$, $p < 0.02$). Study results of [18] indicated that IL-6 might be used as an early prognostic marker of inflammatory processes such as metritis in transition dairy cows. Similarly, it has been demonstrated that IL-6 concentration was high levels in dairy cows that were likely to develop endometritis, and in

addition, it was shown that the determination of changes in IL-6 concentrations during the prepartum period can be used useful tool for the prediction of postpartum reproductive diseases [27]. When all these findings are considered together, it might be concluded that serum IL-6 concentration can be used as early screening inflammatory markers in the early diagnosis of inflammatory processes such as acute septic metritis in dairy heifers. However, since ASM cases can be easily diagnosed considering the clinical findings, it was concluded that the significant difference between the serum IL-6 levels of the groups at the time of diagnosis did not have much importance in the diagnosis of the disease. When findings of the this study and the result of the studies mentioned above are evaluated together, it can be claimed that prepartum serum IL-6 concentration can be used as an effective early biomarker in the prediction of cows and heifers likely to develop clinical endometritis or ASM. In addition, based on the literature, it can be said that its effectiveness is good even in the preliminary diagnosis of low-severity infections such as clinical endometritis [27].

IL-8 is synthesized by mononuclear cells, fibroblasts, endothelial cells, and keratinocytes. At the same time, IL-8 production can be stimulated by IL-1 and TNF-α. IL-8 is an important mediator (chemokine) that provides chemotaxis of neutrophils to inflammatory sites. [28]. IL-8 increases phagocytosis and bacterial clearance [29,30]. In the present study, blood serum IL-8 levels of Holstein heifers in the group with ASM and the healthy group on the 8th and 4th weeks before birth and at the time of diagnosis were compared. It was determined that at the time of diagnosis IL-8 concentration was significantly higher in the heifers developing metritis than those in healthy. When the literature was reviewed, no study was found comparing blood serum IL-8 levels of cattle with metritis and healthy in the prenatal period and at the time of diagnosis. On the other hand, in only one study, gene expressions of proinflammatory cytokines were determined in leukocytes in the peripheral blood of healthy and metritis cows at prenatal day 7, at birth (day 0), and postpartum 7, and 30 days. It was stated that the IL-8 gene expression of animals with metritis was found to be higher than that of healthy animals [19].

IL-10 is the most important antiinflammatory cytokine produced by monocytes, macrophages, dendritic cells, and various subsets of T cells [31]. Its main biological function is to inhibit various cytokines and factors that play an important role in the inflammatory process, such as monocyte and macrophage-derived TNF-α, IL-1, IL-6, IL-8, IL-12, and granulocyte colony-stimulating factor [32]. It is stated that the IL-10 response reflects the strength and source of the preformed inflammatory response and is an important factor in the resolution of the fire at the time of IL-10 release. It is remarked that antiinflammatory cytokines may have an important role in the resolution of uterine infections [17]. Given our study results, it is seen that the blood serum IL-10 concentration is significantly higher in the ASM group compared to the healthy group at the 4th prenatal week and at the time of diagnosis ($p < 0.001$). On the other hand, IL-10 measured at prepartum 8th week showed a tendency to be greater in heifers developed ASM, but the difference was not significant ($p < 0.06$). In our literature review, we did not find any study comparing at prenatal period and the moment of diagnosis, blood serum IL-10 levels of ASM, and healthy animals. In a study on IL-10, serum concentrations of prepartum (15th day) and postpartum (calving days, 15th and 30th days) of cows with and without clinical metritis (CM) were compared. IL-10 concentration was significantly greater in cows suffering CM than those in healthy cows at prepartum 15 days and the IL-10 concentrations remained significantly higher for all postpartum sampling days [17]. The findings regarding IL-10 of our study are consistent with the postnatal data of [17]. But our prepartum IL-10 results are not in agreement with the study findings of [17] who found significantly greater prepartum IL-10 concentration in cows diagnosed with CM than those in healthy cows. In the present study, prepartum samples were collected much earlier than in the study in reference [17]. This inconsistency in prepartum 4th- week findings of studies may be due to significant differences in parameters such as study material, prenatal sampling weeks, and severity of infections in the two studies.

Cytokines activate the production of APPS [16]. APPs regulate the effects of some other immune proteins or help eliminate infection by stimulating phagocytosis. Enzymes formed during the immune response can cause damage to organs. APPs also perform an important function such as protecting tissues and organs against the harmful effects of enzymes. APPs are mainly produced in liver hepatocytes. APPs are also expressed by some extrahepatic tissues such as adipose tissue, mammary gland, intestinal epithelial cells, macrophages, etc. Their blood serum concentrations in postpartum cows increase during the first few weeks after parturition in response to uterine infections caused by microorganisms [13, 16, 33].

Hp, one of the acute phase proteins, binds to hemoglobin and thus inhibits bacterial growth by reducing the availability of iron. Although there are many different acute phase proteins, great importance has been attached to the measurement of serum Hp levels to detect inflammation in cattle because they are found in sufficient levels in the serum of healthy animals [34,35]. In the study conducted to determine an early screening biomarker in the prediagnosis of cows with metritis, at prepartum 8th and 4th weeks, no statistically significant

difference could be determined between the blood serum Hp levels of cows with ASM and healthy cows. However, at the time of diagnosis, serum Hp levels of cows with ASM were significantly higher than those of healthy have been determined [18]. These findings are in largely agree with our study results (Table 1,2). When we evaluated the results of our research, it was concluded that serum Hp levels measured in the prepartum period cannot be used in the prediction of animals with ASM. However, since ASM cases can be easily diagnosed considering the clinical findings, it was concluded that the significant difference between the serum Hp levels of the groups at the time of diagnosis did not have much importance in the diagnosis of the disease. It was stated that serum Hp levels measured before birth are not important in the prediction of cows with severe metritis [36]. This finding is consistent with the results of our study. On the other hand, in the same study [36], because the acute phase response is formed before the symptoms of metritis occur, it has been stated that Hp measurements in the early postpartum period can be to help in the early diagnosis of metritis and provide an opportunity for early treatment of and prevention of metritis. This finding was not agreed with our result. In the postpartum stage of research [36] collected blood serum and measured Hp levels starting from calving day (0th Day) to the postpartum 21st day with 3-day intervals. A connection was established between metritis formation and serum Hp levels, and additionally, to determine a cut-off for Hp during the postpartum period. However, in our study, the Hp level only at ASM diagnosis time at the postpartum period was measured. For this reason, it should be considered usual that there is a disagreement in evaluation difference between the two studies in terms of the importance of postpartum ASM levels in the prediction of metritis. For this reason, it should be considered usual that there is a disagreement in evaluation difference between the two studies in terms of the importance of postpartum ASM levels in the prediction of metritis.

In the present study, at the 8th and 4th weeks before parturition and the diagnosis time, results obtained in sick heifers were compared with those in healthy, and it was detected levels of SAA in those suffering from ASM were significantly greater. The difference between the groups was very noticeable at the postpartum 8th and 4th weeks ($p = 0.001$, $p < 0.001$, respectively) (Table 1,2). In the study conducted by [18], it was showed that the blood serum SAA concentrations of cows with metritis were significantly higher in the prepartum 8th and 4th weeks than in healthy cows, and this result is consistent with our research findings. On the other hand, in the same study, no difference was found between the serum SAA levels of ASM and healthy animals at the time of diagnosis, which is inconsistent with our research findings. However, the difference between the groups had a tendency to be significant (0.05). In the study of [18], it is stated that the increase in the postpartum period SAA and Hp concentrations in cows that will develop metritis after birth is a new finding and new research should be done on the source of the inflammatory process that starts in the prepartum period and continues by clinical symptoms after parturition. In addition, it was emphasized that SAA could be used as screening biomarkers for early prepartum prediction of cows likely to develop metritis after calving. Although our study was conducted in heifers, the findings of our study are supported by almost all the results of the study in reference [18].

Lactate is produced by various tissues. Lactate is a metabolite that takes part in metabolic processes such as glycolysis and oxidative phosphorylation in the organism. These processes are related to ATP, in short, energy production. Additional energy is needed in inflammatory processes. Especially, the energy needs of vital organs such as the liver and kidneys increase. In this case, lactate is used to meet the increased energy needs. When liver dysfunction occurs, lactate cannot be metabolized and blood lactate concentration increases [37]. TNF and IL-1 lead to an increase in glycolysis, but a decrease in oxidation in the tricarboxylic acid cycle increases blood lactate level [38]. It has been shown that the transition period blood serum levels of lactate in cows with laminitis [39] and retained placenta [40] were greater than those of healthy. Based on these results, it has been stated that serum lactate, IL-6, TNF, and SAA can be used as early screening biomarkers to distinguish cows with laminitis and retained placenta from healthy ones during the transition period [40,41]. In another study, the blood serum lactate levels in the 8th and 4th weeks of prepartum of Holstein cows with metritis were significantly higher than in healthy cows was determined. Additionally, it was emphasized lactate concentrations in the transition period can be used as a very early diagnostic indicator in the detection of inflammatory processes [18]. On the other hand, when the lactate results of our study are compared with the findings of the study of [18], it was seen that there was a complete inconsistency between the results of the two studies. In short, in the present study, unlike the studies of [18], there was no significant difference between serum lactate levels of ASM and healthy animals at the 8th and 4th weeks prior to parturition. In our study, unlike the study of [18], the use of heifers as study material may have caused inconsistency between the results. Considering the fact that the heifers have never given birth before their first calving, have not undergone lactation, have not been fed intensively, have not been under metabolic stress, and have not encountered the above - mentioned diseases in the present study, we can state that it is normal that lactate levels do not show a significant increase in ASM heifers compared to healthy animals.

When all the findings in this study are evaluated together, was observed that IL-6 and SAA in the prepartum 8th week and TNF-α, IL-1β, IL-6, IL-10, and SAA in the 4th week before parturition have the potential to be used as early screening biomarkers in the prepartum prediction of ASM in Holstein heifers.

In conclusion, in this study, it was seen that prenatal IL-6 and SAA in Holstein heifers have the potential to be used as very early screening biomarkers for preliminary diagnosis of important postnatal infections such as ASM, in line with results of previous studies in Holstein cows.

In addition, it was concluded that it would be appropriate to test the efficacy of IL-6 and SAA as a biomarker in the early diagnosis of important uterine infections such as ASM in larger animal populations and to determine a cut-off for these two parameters.

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Conflict of interest

The authors declare no conflict of interest.

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