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Peripartum changes in the activity and expression of neutrophils may predispose to the postpartum occurrence of metritis in dairy cows

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Abstract

Metritis is a postpartum uterine pathology that causes a huge economic loss due to increased culling risk and impaired milk yield and reproduction in cows. The present study was carried out to study the changes in the activity and expression of blood neutrophils in crossbred dairy cows with and without metritis. Collection of blood samples was done at -3, -2 and -1 weeks before calving, at calving and during the first day of metritis diagnosis in metritis group (n = 8) or at day 8-10 post calving in healthy group (n = 8). Neutrophils were studied for its percentage (microscopically), respiratory burst (nitro blue tetrazolium assay), myeloperoxidase (MPO) concentrations (sandwich ELISA) and expression of CXCR1, CXCR2, TLR2, TLR4, GRα, CD11b, CD14, CD25, CD44, CD47 and CD62L (RT-PCR). Immunocytochemistry was used to

investigate MPO concentration and CD14 activity, and western blotting was used for estimating MPO. Although most of these parameters changed in the cows that developed metritis one week before calving, MPO and CD14 got altered much earlier. Myeloperoxidase concentrations and expression of CD14 were considerably lower starting from -2 weeks before calving in cows that developed metritis compared to healthy cows. Further studies are warranted to study the possible use of MPO and CD14 to identify transition cows more vulnerable to develop metritis several weeks before disease occurrence.

Keywords: Transition dairy cow, metritis, neutrophil molecule, myeloperoxidase, CD14

1. Introduction

Metritis is a uterine pathology associated with inflammation of the wall of the uterus that mostly occurs during the first 21 days postpartum (Sheldon et al., 2006, 2009). It is one of the major reproductive diseases encountered by up to 40 % of dairy cows after calving (LeBlanc, 2008; Sheldon et al., 2009). The prevalence of metritis in Indian crossbred (Karan Fries) cows is around 22.5 % and the infected cows usually have poor reproductive performance (Gilbert et al., 2005; Kumari et al., 2016). The economic cost of a single case of metritis has been estimated to be about €292 (Drillich et al., 2001). Moreover, the annual cost of uterine disorders in the European Union is €1.411 billion and in the United States is $650 million (Sheldon et al., 2009). High producing cows are more susceptible to uterine infections and around 3.7 kg daily decrease in milk production has been reported due to metritis in multiparous cows (Dubuc et al., 2011). Therefore, early diagnosis of metritis is essential to improve animal productivity and avoid enormous economic losses to the dairy industry.

It is well known that innate immune response plays a major role in the defence mechanism of the uterus after parturition when the chance of bacterial infection is maximum. Out of all leucocytes, neutrophils are the major and first line of cellular defence that efficiently phagocytose bacteria and clear the infection (Alhussien and Dang, 2017). Many studies have correlated parturition-associated impaired neutrophil functions and higher prevalence of metritis in dairy cows (Cai et al., 1994; Hammon et al., 2006). Neutrophils from cows with uterine disorders have decreased myeloperoxidase and cytochrome c activities (Hammon et al., 2006). Identification of novel biomarkers prior to uterine infection can be useful for early diagnosis,

treatment, and prevention of huge economic losses. The use of acute phase proteins such as haptoglobin and serum amyloid A as well as inflammatory cytokines as valuable biomarkers to predict postpartum uterine infections has been reported (Chapwanya et al., 2009; Dervishi et al., 2016; Manimaran et al., 2016; Sheldon and Dobson, 2004). Although the alteration in these proteins and inflammatory cytokines is more or less connected to the response of neutrophil, there is limited knowledge about the alteration in the functions and expression of neutrophil molecules in transition dairy cows that develop metritis. Therefore, this study was formulated to investigate the changes in various neutrophil molecules in Indian crossbred dairy cows with and without metritis.

2. Materials and methods

2.1. Ethics approval

All experimental procedures were approved by the Institutional Animal Ethics Committee of Indian Council of Agricultural Research (ICAR)-National Dairy Research Institute (NDRI) and were carried out as per the guidelines of the article 13 of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) regulations, laid down by the Government of India (Reg. No. 1705/GO/ac/13/CPCSEA dated. 3/7/2013).

2.2. Animal selection and management

In the present study, 32 Karan Fries (*Bos indicus* ×*Bos taurus*) cows were followed during the transition period. All the cows were kept in Livestock Research Center (LRC) of the National Dairy Research Institute (NDRI), Karnal. Cows that had other diseases such as Foot-and-Mouth Disease, Bovine viral diarrhea (BVD), Brucellosis, mastitis, etc. were excluded to avoid the overlapping effects of other diseases. All the cows recruited in the present study had metritis only without displaying any symptoms of endometritis. Therefore, sixteen cows were finally selected and followed with eight cows in each group. The first group was of healthy cows (control), whereas the second group was of cows that developed metritis after parturition. All the selected cows had similar parity (2.9±0.6; Mean ±SEM), body condition score (3-3.5). Cows with metritis were diagnosed between days 4-8 postpartum (5 cows), and days 9-13 (3 cows). The experiment lasted for five weeks for each cow starting from −3 weeks before the expected day of calving and up to the time of metritis diagnosis which mainly occurred in the first two weeks postpartum. Cows were housed in a well-ventilated stall with an asbestos roof in which

drinking water was always available. The cows were shifted from their stall to the calving pen shortly before calving and returned to the stall after the colostrum stage. Diets were offered as a total mixed ration (TMR) which was formulated to meet the exceeding demands of nutrients for transition dairy cows as per standard practices followed at LRC, NDRI, Karnal. The energy content of TMR was 0.98 UFL/kg dry matter. However, the crude protein was 16 -17% and total neutral detergent fiber was around 33 to 35% in TMR.

The percentage of ingredients of TMR were as following: berseem fodder (20%), ground yellow maize (28.7%), groundnut cake (16%), corn silage (14.5%), rice bran (8.4%), wheat bran (6.5%), de-oiled Mustard cake (4.9%), dicalcium phosphate (0.7%) and salt (0.3%). Water was offered *ad libitum* to all the cows.

2.3. Monitoring the health status of cows

The health status (HS) of cows was observed daily starting from −3 weeks before calving and continuing up to the time of metritis diagnosis. The evaluation of HS of cows was done through observing various clinical signs of disease by a veterinary practitioner. Estimation of the expected day of the calving was made based on the available data of artificial insemination which was further supported by the data of pregnancy diagnosis. The expected day of calving was estimated by counting 280 days starting from the day of the AI. A veterinary practitioner diagnosed the occurrence of metritis as per standard operating procedures of the dairy farm of NDRI. The major symptoms used to diagnose animals with metritis were as follows; reddish brown vaginal discharge with fetid odour along with rectal temperature greater than 39.5 °C, abnormal enlargement of the uterus, decreased appetite and milk yield within the first three weeks after calving (Darvishi et al., 2016). Cows that were diagnosed with any periparturient diseases along with metritis were excluded from this study.

2.4. Blood sample collection and estimation of neutrophil percentage and type

Two tubes of blood (7 ml each tube/animal) were collected through jugular vein puncture with minimum disturbance to the cows using sterile EDTA vacutainer tubes (Vacuette® EDTA, Greiner Bio-One GmbH, Austria). The blood sampling was done from 16 cows on a weekly basis at 5-time points: −3, −2 and −1 weeks before calving, at calving (day 0), and during the first day of disease diagnosis in metritis group or at day 8-10 post calving in healthy group. Blood samples were transported immediately to the laboratory in ice for further processing. For counting the percentage of neutrophil, a blood smear was prepared and stained with 5-7 drops of

May-Grunwald stain (Sigma-Aldrich, St. Louis, MO, USA) for 2 minutes and then with 5-7 drops of Giemsa stain (Sigma-Aldrich, St. Louis, MO, USA) for 1 minute. The stained blood smear was examined under Olympus IX51 microscope (Olympus, Tokyo, Japan). About 100 cells were counted in each smear to determine the percentage of blood neutrophils. Segmented neutrophils were having divided or multilobed nucleus, whereas, band neutrophils were characterized as having a curved nucleus which was not lobar in form as described by Alhussien et al. (2015).

2.5. *Isolation of blood neutrophils and evaluation of their viability and purity*

The separation of blood neutrophils was performed by the gradient density centrifugation method using Histopaque solutions 1077 and 1119 (Sigma Aldrich, St. Louis, MO, USA) as described by Alhussien et al. (2018). The cell pellet obtained was diluted with serum-free RPMI-1640 medium (Sigma Aldrich, St. Louis, MO, USA) and the cell number and viability was determined by Haemocytometer (Reinfeld, Germany) using 0.4 % Trypan blue method (Sigma Life Sciences, St. Louis, MO, USA) as described by Dang et al. (2010). The cell viability was found to be more than 95 % within 3 hours of neutrophil processing and declined gradually afterwards. The purity of neutrophils was greater than 90 % as evaluated through May-Grunwald Giemsa staining.

2.6. Myeloperoxidase concentration and respiratory burst of neutrophils

In vitro respiratory burst of blood neutrophils was estimated using Nitro blue tetrazolium (NBT) assay. The neutrophil suspension was adjusted to 5 x 10^6 live cells/ml by culture media, RPMI-1640 in a 96-well flat-bottomed tissue culture plate (Coster, Sigma Aldrich USA). The cells were incubated with 650 µg/ml of Zymosan-A and 250 µg/ml of NBT (Sigma Aldrich, St. Louis, MO, USA) at 37°C in a humidified carbon dioxide (CO2) incubator (95% air and 5% CO2) for 3 h. Finally, optical density (OD) was taken at 540 nm using a Microplate reader (MutiSkan GO, ThermoScientific, Finland). The concentrations of myeloperoxidase (MPO) in neutrophil lysates were estimated using bovine specific sandwich ELISA kit (Uscn Life Science, Houston, USA) as prescribed by the manufacturer's protocol. To prepare lysates of neutrophils, few glass beads were mixed with the isolated neutrophils (5 x 10^6 cells/ml), and shock was given thrice for 30 seconds each time using bead beater (Unigenetics Instrument Pvt. Ltd., India). The supernatant was then obtained by centrifuging the cells at 1000 x g for 10 min. The minimum detectable dose of bovine MPO was 0.64 ng/ml, and the detection range of the assay was 1.56-

100 ng/ml. The intra and inter-assay CV were 10 % and 12 %, respectively. The optical density (OD) was measured by a Microplate reader (Multiskan Go, Thermoscientific, Finland).

2.7. RNA isolation from neutrophils

Total RNA from blood neutrophils was isolated using RNeasy Mini Kit (Qiagen, India Pvt. Ltd.) as per the manufacturer's guidelines. The contamination due to genomic DNA was removed using the RNase-Free DNase Set (Qiagen, India Pvt. Ltd.) as per manufacturer's protocol. Agarose gel (1.8%) electrophoresis was used to evaluate the integrity of RNA by observing RNA bands (28S and 18S). The purity of RNA was verified by OD absorption ratio at λ260/λ280 using Biospec-nano Spectrophotometer (Shimadzu Corp., Japan). A ratio of (1.9 to 2.0) was accepted as "pure" for RNA.

2.8. cDNA Synthesis and Real-Time Polymerase Chain Reaction (RT-PCR)

A total amount of RNA (1 µg) was reverse transcribed into cDNA using Revert Aid First Strand cDNA synthesis kit (Thermo Scientific, USA) as per the manufacturer's instructions. The real-time reaction was done at 65°C for 5 minutes, 42°C for 60 minutes, and then at 70°C for 5 minutes in a thermal cycler (Bio-Rad, USA). Gene transcripts, primer sequences and annealing temperature for housekeeping genes (GAPDH, ß-Actin), chemokine receptors (CXCR1, CXCR2), Toll-like receptors (TLR2, TLR4), glucocorticoid receptor (GRα) and cluster of designation (CD11b, CD14, CD25, CD44, CD47, CD62L) can be seen in our previous published papers (Alhussien et al., 2018, 2016; Alhussien and Dang, 2018, 2017). The RT-PCR reaction was performed by Applied Biosystems 7500 RT- PCR using template cDNA (1 µl), SYBR green (10 µl), Forward and Reverse primers (1 µl each) and total reaction volume up to 20 µl was made using nuclease-free water. The protocol of RT-PCR consisted of initial heating at 50°C for 2 min, 95°C for 10 min and then the content was amplified for 40 cycles (95°C for 30s and at the appropriate annealing temperature for 30s). GAPDH and ß-Actin were used as endogenous genes and the mRNA abundance of the week -3 of healthy group was taken as a calibrator with which relative expression of all genes during different time points prior to metritis diagnosis was calibrated. The relative quantification of all the studied genes was done by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.9. Immunocytochemistry of MPO and CD14 in blood neutrophils

The isolated neutrophils were washed thrice with Dulbecco's Phosphate-Buffered Saline (DPBS, Himedia, India Pvt. Ltd) and fixed with 4% paraformaldehyde for 10 minutes. After that,

it was washed twice with PBS-Tween (0.1% Tween-20 (Sigma Aldrich, St. Louis, MO, USA) in PBS) and then treated with PBST (0.1% Triton X-100 in PBS) for permeabilisation of the cells. The permeabilisation of the cells was done for MPO only. The cells then were washed thrice with PBS-tween. Blocking solution (1% BSA in PBST) was then used for 1 hour to block non-specific binding sites. For MPO, Rabbit Polyclonal Anti-MPO primary antibody (Cloud-Clone Corp, Houston, USA) was added (1:1000 in blocking solution) and incubated overnight at 4^{o}C on a shaker and then FITC conjugated Goat Anti-rabbit secondary antibody (Boster Biological Technology, Ltd. USA) was added and allowed to incubate at room temperature for 90 min in dark condition. For CD14, Mouse Monoclonal Anti-CD14 Primary antibody (Thermo Fisher Scientific, USA) and FITC conjugated Goat Anti-mouse secondary antibody (Thermo Fisher Scientific, USA) were used. The cells were then washed thrice with PBS-Tween in a dark room, and Hoechst stain (Sigma Aldrich, St. Louis, MO, USA) was used (1:2000 in PBS) to visualise the nucleus. Finally, the cells were examined in the dark under a fluorescent microscope (Olympus, Japan).

2.10. Determination of MPO by western blotting

Mammalian Protein Extraction Reagent (M-PER, Thermo Scientific, Rockford, IL 61105, USA) was used to lyse neutrophils, and Phenylmethylsulfonyl Fluoride (PMSF, Thermo Scientific, Rockford, IL 61105, USA) was added at the rate of 0.5 mM to the lysis buffer to prevent protein degradation. To determine the concentration of total protein in the samples, Bradford protein assay was used, and the final concentration of protein in all samples was 30 µg/ml. The SDS-PAGE was performed as per the method described by Laemmli (1970).

Stacking gel (4%, 5 ml) used in the study consists distilled water (3.05 ml), 30% Acrylamide (0.665 ml), 0.5 M Tris-HCl (pH 6.8, 1.25 ml), 10% SDS (50 µl), 10% APS (25 µl), and TEMED (5 µl). Whereas, separating gel (12%, 8 ml) consists of distilled water (2.60 ml), 30% Acrylamide (3.20 ml), 1.5 M Tris-HCl (pH 8.8, 2.00 ml), 10% SDS (80 µl), 10% APS (80 µl), and TEMED (8 µl). After electrophoresis, stacking gel was removed and the separating gel was equilibrated in transfer buffer for 30 minutes at room temperature. To transfer the proteins from the gel to membrane in a transfer unit, a sandwich of Blotting paper, PVDF, Electrophoresed gel and blotting paper was used. The voltage and current in a transfer unit were 120V and 54 mA, respectively for 3 hours for complete transfer. After that, specific primary polyclonal antibodies against MPO (Cloud-Clone Corp, Houston, USA) and the HRP-conjugated secondary antibody

(Boster Biological Technology, USA) were used. DAB (Amresco, Solon, OH, USA)-peroxidase system was used to detect the signal, and the PVDF membrane containing the target protein bands was photographed with a Sony digital camera.

2.11. Statistical analysis

All data were analysed by repeated measures two-way ANOVA using mixed procedure of SAS (Proc Mixed, SAS Institute Inc., CARY, NC, USA, version 9.1). The pairwise comparison was performed using a multiple comparison test (Tukey). The following statistical model was used to estimate the effect of group (health status), weeks of transition period and their interactions.

$Y_{ijk} = \mu + H_i + W_i + (HW)_{ij} + e_{ijk}$

Where Y_{ijk} is a dependent variable, μ is the population mean, H_i is the effect due to health status ($i = 2$), W_j is the effect due to the measurement week ($j = 5$), and HW_{ij} is the effect due to health status by measurement week interactions, and e_{ijk} is the residual error. P-values less than 0.05 were declared significant.

3. Results

3.1. Percentage of Neutrophils, band neutrophil percentage, respiratory burst and myeloperoxidase concentration

Health status, period of sampling and their interactions affected ($p \leqslant 0.001$) neutrophil percentage and their activity in term of respiratory burst and MPO levels (Table 1). Neutrophil (%) was similar in both healthy and metritis cows at -3 and -2 weeks before calving. However, it was higher ($p<0.012$) in metritis cows at -1 week before parturition and showed highest ($p \leqslant 0.001$) values at the week of disease diagnosis as compared to healthy cows (Fig. 1). The percentage of band neutrophils was higher ($p \leqslant 0.001$) at the day of calving and at the week of disease diagnosis in metritis cows as compared to healthy cows. Similarly, PA of blood neutrophils exhibited differences between the groups at -1 week before calving ($p \leqslant 0.001$), calving ($p<0.007$) and during the period of metritis diagnosis ($p \leqslant 0.001$) in which it was higher in healthy cows as compared to cows with metritis. The overall mean demonstrated that MPO concentrations were greater ($p \leqslant 0.001$) in healthy cows as compared to metritis cows. Furthermore, MPO concentration was higher ($p<0.004$) at -2 weeks before calving in healthy

cows as compared to cows diagnosed with metritis and remained higher until the end of the experiment (Fig. 1).

3.2. Relative mRNA expression of chemokine, Toll-like and glucocorticoid receptors

The fragment size of PCR amplified products was confirmed by agarose gel electrophoresis on 1.8 % agarose gel against gene rulers and DNA ladders, i.e. 50 bp and 100 bp (Fig. 2). Health status, period of sampling and their interactions had significant ($p \leqslant 0.001$) effects on the expression of chemokine and Toll-like receptors. Although the period of sampling significantly ($p \leqslant 0.001$) affected the mRNA expression of GRα, health status did not (Table 1). The overall mean value was higher ($p \leqslant 0.001$) for chemokine receptors and lower ($p \leqslant 0.001$) for Toll-like receptors in metritis cows as compared to healthy cows. All these receptors did not differ between the groups at -3 and -2 weeks prior to calving. However, the expression of CXCR1 increased ($p<0.042$) and the expression of both TLR2 and TLR4 decreased ($p<0.031$; $p \leqslant 0.001$) at -1 week before calving in cows diagnosed with metritis compared to healthy cows (Fig. 3). At the time of disease diagnosis, the expression of chemokine receptors was higher ($p \leqslant 0.001$), whereas the expression of Toll-like and glucocorticoid receptors was lower ($p \leqslant 0.001$; $p<0.015$) in metritis cows as compared to healthy cows. The maximum difference in the expression between the groups was observed for TLR4, TLR2 and CXCR2 at -1 week before calving, calving and at the time of disease diagnosis, respectively (Fig. 3).

3.3. Cluster of designation

The health status and period of sampling affected ($p \leqslant 0.001$) the mRNA expression of all the studied genes. The health status by period of sampling interactions also affected ($p<0.05$) these CD molecules (Table 1). There was no difference in the expression of these genes at -3 and -2 weeks prior to calving in both groups of cows. However, the expression of CD14 was lower ($p \leqslant 0.001$) at -2 weeks and CD25 was higher ($p \leqslant 0.001$) at -1 week before calving in cows diagnosed with metritis compared to healthy cows (Fig. 4). Although the expression of CD11b did not differ between the groups throughout the study period, it increased significantly ($p \leqslant 0.001$) in cows diagnosed with metritis after calving as compared to healthy cows. The expression of CD25, CD44 and CD47 was greater, whereas CD14 and CD62L tended to be lower at the time of calving in cows that developed metritis later as compared to healthy cows. Out of all CD molecules, CD14 showed a maximum difference in the mRNA expression

between groups starting from -2 weeks before calving until the time of metritis diagnosis (Fig. 4).

3.4. Immunocytochemistry of MPO and CD14 in neutrophils

Immunocytochemistry studies revealed positive and comparatively stronger signals of MPO and CD14 in blood neutrophils obtained from healthy cows at -2 weeks before calving as compared to cows that developed metritis later (Fig. 5).

3.5. Immunoblotting of MPO in neutrophils

Like Immunocytochemistry, western blot analysis of the protein fraction of blood neutrophil showed a gradual decrease in the MPO signal starting from -2 weeks before calving in cows that developed metritis later compared to healthy cows (Fig. 6).

3.6. Timeline alteration in neutrophils activity and expression prior to metritis occurrence

Decreased myeloperoxidase concentrations and expression of CD14 in blood neutrophils starting from -2 week before calving were associated with a higher chance of metritis prevalence post-calving (Fig. 7). Also decreased respiratory burst and expression of Toll-like receptors and increased neutrophil count and expression of CXCR1 and CD25 at -1 week before parturition were associated with the postpartum prevalence of metritis. Increased expression of CXCR2, CD44 and CD47, as well as decreased expression of GRα and CD62L at calving, were associated with metritis occurrence. All these parameters were associated with a higher risk of metritis occurrence within 21 days postpartum.

4. Discussion

The infection of the uterus during the first few weeks after parturition is a common phenomenon in dairy animals which causes enormous economic loss. Cows with metritis are not only at great risk of having endometritis and mastitis; they also experience lower reproductive efficiency (Hossein-Zadeh and Ardalan, 2011; Sheldon et al., 2009). The present investigation was an effort to identify transition cows susceptible to metritis by targeting blood neutrophil as it is an essential cell of innate immunity and plays a primary role in preventing bacterial infections around the transition period. Several studies reported that neutrophil activity declines 3–5 weeks before parturition, reach a minimum level between calving and the first week postpartum and returns to its normal activity 2–4 weeks postpartum (Crookenden et al., 2016; Kehrli et al., 1989; Kimura et al., 1999). The present study was aimed to study the changes in the activity and expression of blood neutrophils between cows with and without metritis. We observed higher

percentage of neutrophils in cows that developed metritis later as compared to healthy cows starting from -1 week before parturition. A cutoff point of neutrophils proportion can be used to diagnose cytological endometritis at the time of artificial insemination (Pascottini et al., 2017). In our study, the observed elevation in the percentage of blood neutrophils in cows that developed metritis later reflects higher inflammatory response which could stimulate the bone marrow to release more neutrophils to be recruited to the uterus. We observed more immature, band-shaped neutrophils in the blood samples of cows that developed metritis as compared to healthy cows. This may be due to the fact that inflammatory mediators instigate the bone marrow to release more immune cells at high rate which leads to more immature band cells being released in the circulation. Respiratory burst, one of the major steps of phagocytosis, is critical mechanism by which neutrophils defend the uterus against invading pathogens (Cai et al., 1994; Kim et al., 2005). We observed increased percentage of total neutrophils and band neutrophils with decreased respiratory burst in metritis cows which could be a main predisposing factor for disease occurrence in them. Similarly, several *in vitro* and *in vivo* studies have reported decreased capacity of neutrophil phagocytosis and bactericidal activity in cows experiencing uterine disorders (Kim et al., 2005; Zerbe et al., 1996, 2001). Moreover, glycogen content, which is the main oxidative fuels in neutrophils, decreased on the day of calving in cows developing metritis compared to healthy cows (Galvão et al., 2010).

The enzyme MPO is stored in the azurophilic granules of neutrophils and plays a critical role in the transformation of hydrogen peroxide into hypochlorous acid which is highly efficient in killing the invading microorganisms (Borregaard and Cowland, 1997; Cooray et al., 1993). Since it is secreted abundantly during infectious and inflammatory diseases, several researchers tried to develop diagnosis techniques for various diseases in bovine using MPO enzyme. More than two decades ago, Cooray (1994) had developed a specific enzyme immunoassay to quantify MPO in milk for the diagnosis of intramammary infections in dairy cows. Recently, an innovative flow cytometric method was developed to assess MPO in blood leukocytes which can be useful for early diagnosis of dairy cows with a higher risk of developing inflammatory diseases around the transition period (Depreester et al., 2017). Interestingly, we observed significantly lower MPO concentrations in neutrophils lysate isolated from cows developing metritis compared to healthy cows starting from -2 weeks before calving and up to the time of metritis diagnosis. Although many studies have associated decreased concentrations of MPO

with metritis, this is the first study to report the alterations in neutrophils MPO as early as −3 weeks before parturition. Similarly, a significant decrease in MPO activity and cytochrome c of blood neutrophils at -1 week before calving in cows that developed metritis later compared to healthy cows has been reported (Hammon et al., 2006). However, a decline in the percentage of neutrophils and MPO activity only after calving, but not before, in cows that developed metritis later compared to healthy cows have also been reported (Cai et al., 1994).

Bovine neutrophils express glucocorticoids receptor (GRα) by which cortisol mediates its immunosuppressant effects on these cells (Burton et al., 2005; Preisler et al., 2000). Although plasma cortisol was not estimated in the present study, we have reported earlier an inverse relationship between plasma cortisol levels and the expression of GRα in cows around calving and during mammary infection with different pathogens (Alhussien and Dang, 2017, 2016). The expression of GRα decreased at calving and at the time of disease diagnosis in metritis cows compared to healthy cows. This might be due to the higher level of stress, i.e. plasma cortisol in cows that developed metritis later and can explain the lower respiratory burst and MPO activities in these cows. Similarly, Pathak et al. (2015) reported that higher cortisol concentrations was associated with impaired functions of blood neutrophil in cows developing retention of fetal membranes.

The chemokine receptors have been proved to play a critical role in both homeostasis and inflammatory conditions by controlling the activation, migration, and survival of leukocytes (Kufareva et al., 2015; Rambeaud and Pighetti, 2005). We observed increased mRNA expression of the chemokine receptors starting from -1 week before calving in cows that developed metritis later compared to healthy cows. The interaction between chemokine receptors (CXCR1 and CXCR2) and IL-8 stimulates conformational changes in blood neutrophils that permit their transmigration to the site of infection (Olson and Ley, 2002). Higher IL-8 expression in the endometrial stromal cells of metritis cows infected with bovine herpesvirus 4 has been reported (Donofrio et al., 2010). The up-regulated expression of these receptors in our study could be to mediate neutrophil migration in response to cell stimulation which were reflected by higher neutrophil percentage during the same period in the cows that developed metritis later. Similarly, we also found higher expression of chemokine receptors on blood neutrophils around calving and milk neutrophils of cows susceptible to mastitis (Alhussien and Dang, 2017, 2016).

Bovine endometrium expresses various Toll-like receptors that detect a variety of microbial components and initiate an inflammatory response and subsequent neutrophils recruitment to the site of the infection (Davies et al., 2008; Pinedo et al., 2013). Bovine neutrophils also express both TLR2 and TLR4 which help in identifying both gram-positive and gram-negative bacterial components, respectively (Alhussien and Dang, 2017; Swain et al., 2014). TLR4 recognizes lipopolysaccharides (LPS) of different bacterial species including *Escherichia coli* which are the major bacteria responsible for the postpartum infection of the uterus in bovine (Dohmen et al., 2000). The transcription profile of all TLRs was investigated in postpartum endometrium, and it was found that only TLR4 was upregulated in cows with uterine disease compared to healthy cows (Herath et al., 2009). We noticed lower expression of both TLR2 and TLR4 starting from -1 week before calving in blood neutrophils isolated from cows that would develop metritis as compared to healthy cows. This is because the cows were still not in disease state and indicate the suppressed ability of neutrophils to detect and phagocytose bacteria which might be a major reason for the establishment of uterine infection in these cows.

The recruitment process of circulating neutrophils and their subsequent activity at the infected site is mediated through specific adhesion molecules present on them. The initial step of neutrophils binding to the endothelial cells is mediated through selectins (CD62L), whereas integrins (CD11b) cause these cells to decelerate from rolling state to a stable arrest (Alhussien et al., 2016; Diez-Fraile et al., 2002). During the interactions of neutrophils with the endothelium, neutrophils receive signals from selectins and integrins which can activate different pathways in these cells leading them to transmigrate through the endothelium and initiate respiratory burst and degranulation to kill the pathogens (Zarbock and Ley, 2008). In the present study, no difference was observed in the expression of integrin until the day of metritis occurrence where it was higher in cows with metritis indicating higher sensitivity for endothelial binding and subsequent transmigration of neutrophils to the uterus. However, selectins showed lower expression in cows diagnosed with metritis starting from calving day. The opposite modulation of these two molecules is essential for an effective response to an inflammatory process which requires a rapid shedding of CD62L and upregulation of CD11b on blood neutrophils. Similarly, Diez-Fraille et al. (2004) reported a progressive decline of CD62L expression and continuous increase of CD11b on both blood and milk neutrophils after mastitis induction in cows using LPS. CD47, also known as Integrin-associated protein, has been

reported in bovine neutrophils (Lamote et al., 2006) and found to be essential for neutrophil migration in human (Seiffert et al., 1999). We observed increased expression of CD47 at calving and remained high in cows that developed metritis later which might indicate a similar function for this molecule in bovine neutrophil.

CD14 is an essential co-receptor required for the efficient recognition of microbial components by TLR2 and TLR4, and its absence significantly hampers cellular response to infectious agents (Ibeagha-Awemu et al., 2008; LeBouder et al., 2003). The expression of CD14 was reported in bovine neutrophils and is capable of translocating to the surface of the cell (Paape et al., 1996). The binding between CD14 and LPS-protein complexes stimulate the synthesis and secretion of tumor necrosis factor-α which up-regulates neutrophil adherence, chemotaxis, phagocytosis and release of reactive oxygen metabolites (Yang et al., 1994). Interestingly, the expression of CD14 in our study was lower starting from -2 weeks before parturition in blood neutrophils isolated from cows that developed metritis later. This reflects that their neutrophils had lower activity which might facilitate pathogenic invasion and inflammation establishment in these cows. CD14 can also be found in a soluble form (sCD14) which originates during CD14 shedding from monocytes and neutrophils as a result of cell activation and can play a major role in regulating different bacterial infections primarily those caused by gram-negative bacteria (Lee et al., 2003; Paape et al., 1996). Although plasma sCD14 was not estimated in the present study, future evaluation and correlation of sCD14 with metritis occurrence might also help in early diagnosis of this disease in cattle.

CD25 is the main ligand of IL-2 in neutrophils and mediates its signaling which causes initiation of an innate immune response (Yamanaka et al., 2003; Zoldan et al., 2014). In the present study, the expression of CD25 was higher at -1 week before parturition in cows that developed metritis later indicating initiation of an immune response in these cows. Similarly, higher expression of CD25 in bovine neutrophils isolated from cows with uterine and udder infections has been reported (Zoldan et al., 2014). CD44 is not only contributing to neutrophil migration but also stands as a phagocytic receptor that helps neutrophils to identify microbial components and perform phagocytosis (Gonen et al., 2008; Vachon et al., 2006). In our study, the expression of CD44 increased gradually starting from -1 week before calving and attained a maximum level at the time of disease diagnosis in metritis cows compared to healthy cows. The increased expression of CD44 in metritis cows may be associated with increased percentage of

neutrophils and decreased respiratory burst. Similarly, the expression of CD44 on blood neutrophil increased at calving and in mastitis cows which was also accompanied with higher percentage of neutrophils and impaired activity (Crookenden et al., 2016; Gonen et al., 2008).

5. Conclusions

Our results showed that the activity and expression of blood neutrophils got impaired in cows that developed metritis. The concentration of MPO and the mRNA expression of MPO and CD14 declined in the cows that developed metritis later several weeks before the occurrence of the disease as compared to healthy cows. These findings highlight the necessity of future studies to target neutrophil molecules as novel biomarkers for early detection of uterine infections in transition dairy cows. Although our study was limited to metritis, neutrophil molecules might be used as early indicators of other diseases associated with parturition in dairy cows since the innate immune response is general and not specific to the inflammatory agents.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Figure captions

Fig. 1. Neutrophil percentage (A), band neutrophil percentage (B), phagocytic activity (C), and myeloperoxidase (D), concentrations in neutrophil lysate of periparturient dairy cows without (—;n=8) or with (----; n = 8) metritis (Hs = health status; Ts = time of sampling; Hs × Ts = health status by time of sampling interaction).

Fig. 2. Agarose gel electrophoresis of Real-time PCR amplified products of target genes products on 1.8 % agarose gel.

Fig. 3. Relative mRNA expression of CXCR1 (A), CXCR2 (B), TLR2 (C), TLR4 (D), and GRα (E) in blood neutrophils of periparturient dairy cows without (—;n=8) or with (----; n = 8) metritis (Hs = health status; Ts = time of sampling; Hs × Ts = health status by time of sampling interaction).

Fig. 4. Relative mRNA expression of CD11b (A), CD14 (B), CD25 (C), CD44 (D), CD47 (E), and CD62L (F) in blood neutrophils of periparturient dairy cows without (—;n=8) or with (----; n = 8) metritis (Hs = health status; Ts = time of sampling; Hs × Ts = health status by time of sampling interaction).

Fig. 5. Immunostaining of myeloperoxidase (MPO) and CD14 in blood neutrophils at two weeks prior to calving in healthy cows and cows that developed metritis later (Hoescht-Nuclear stain & FITC-Target stain).

Fig. 6. Western blot analysis of myeloperoxidase (MPO) in blood neutrophils of healthy cows (n = 3) and cows that developed metritis (n = 3).

Fig. 7. Timeline alterations in the activity and expression of blood neutrophils in cows that developed metritis compared to healthy cows.

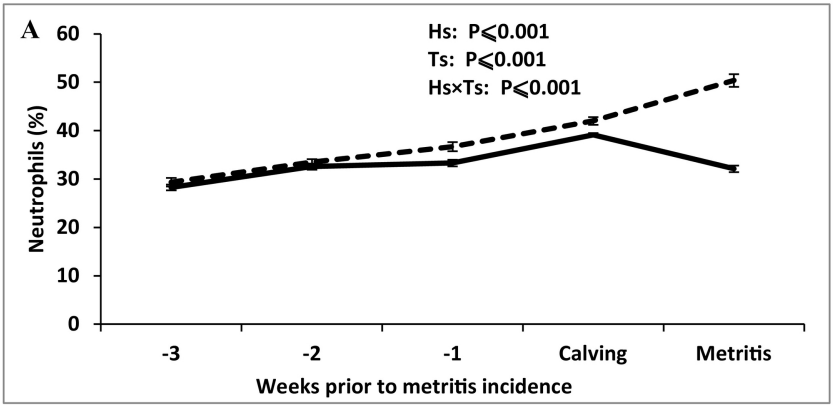
Table 1. The overall mean of blood neutrophil percentage and activity as well as relative mRNA expression of various receptors and adhesion molecules in neutrophils of dairy cows with (n = 8) and without (n = 8) metritis during the transition period.

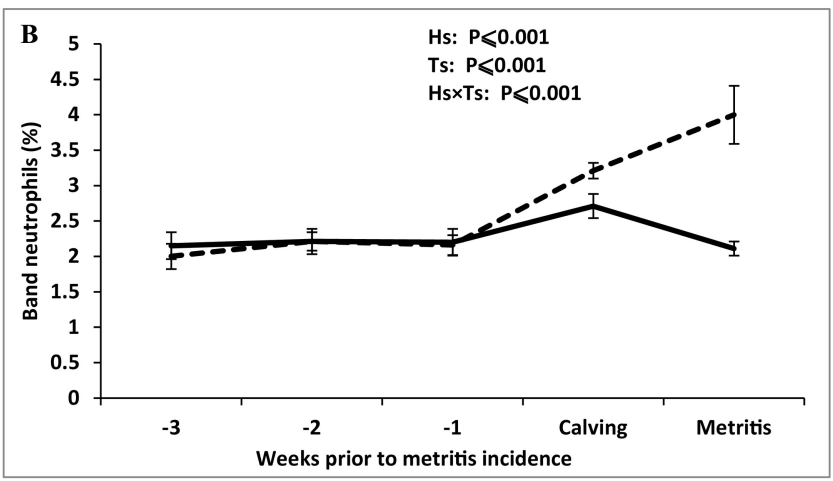
	Group			P-value		
Parameter	Control	Metritis	SEM[1]	Hs[2]	Ts[3]	Hs × Ts[4]
Neutrophils (%)	33.07	38.38	0.74	⩽0.001	⩽0.001	⩽0.001
Band neutrophils (%)	2.28	2.72	0.18	⩽0.001	⩽0.001	⩽0.001
Respiratory burst	0.66	0.52	0.02	⩽0.001	⩽0.001	⩽0.001
[5]MPO (ng/ml)	4.05	2.90	0.15	⩽0.001	⩽0.001	0.032
Relative mRNA expression of blood neutrophils						
CXCR1	1.37	2.20	0.15	⩽0.001	⩽0.001	⩽0.001
CXCR2	1.32	2.03	0.14	⩽0.001	⩽0.001	⩽0.001
TLR2	1.42	0.86	0.15	⩽0.001	0.005	⩽0.001
TLR4	1.26	0.83	0.12	⩽0.001	⩽0.001	⩽0.001
GRα	0.92	0.81	0.10	ns	⩽0.001	0.03
CD11b	1.06	1.52	0.13	⩽0.001	⩽0.001	0.034
CD14	1.02	0.87	0.07	⩽0.001	⩽0.001	⩽0.001
CD25	1.39	2.27	0.12	⩽0.001	⩽0.001	⩽0.001
CD44	1.18	1.50	0.14	⩽0.001	⩽0.001	0.013
CD47	1.18	1.46	0.12	0.022	⩽0.001	⩽0.001
CD62L	0.89	0.67	0.08	⩽0.001	⩽0.001	0.039

[1]SEM: standard error of the mean; [2]Hs: health status; [3]Ts: time of sampling; [4]Hs × Ts: health status by time of sampling interaction, [5]MPO: myeloperoxidase.

Highlights

1. Changes in the activity and expression of neutrophils during the transition period studied.
2. Poor functions of neutrophils observed in cows experiencing uterine infections.
3. Myeloperoxidase enzyme and CD14 expression were lower two weeks before calving in cows that developed clinical metritis.
4. Neutrophil molecules can be targeted for early identification of increased susceptibility to metritis.


A
Hs: P⩽0.001
Ts: P⩽0.001
Hs×Ts: P⩽0.001
Neutrophils (%)
60
50
40
30
20
10
0
-3
-2
-1
Calving
Metritis
Weeks prior to metritis incidence


B
Hs: P⩽0.001
Ts: P⩽0.001
Hs×Ts: P⩽0.001
Band neutrophils (%)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
-3
-2
-1
Calving
Metritis
Weeks prior to metritis incidence

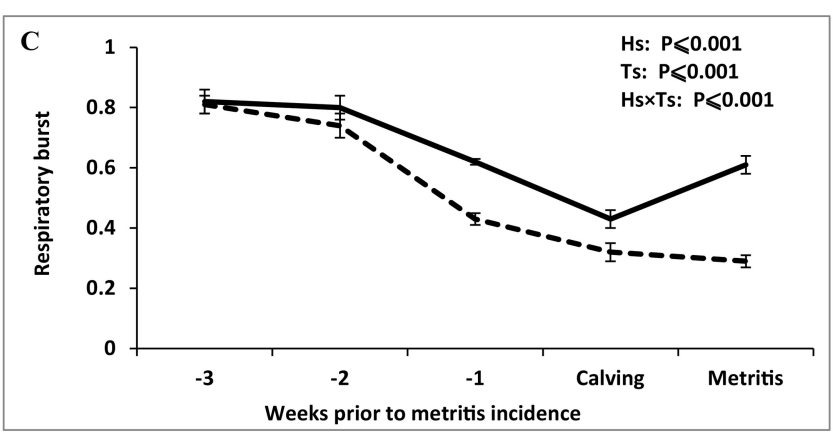

C
Hs: P⩽0.001
Ts: P⩽0.001
Hs×Ts: P⩽0.001
Respiratory burst
1
0.8
0.6
0.4
0.2
0
-3
-2
-1
Calving
Metritis
Weeks prior to metritis incidence

Figure 1A

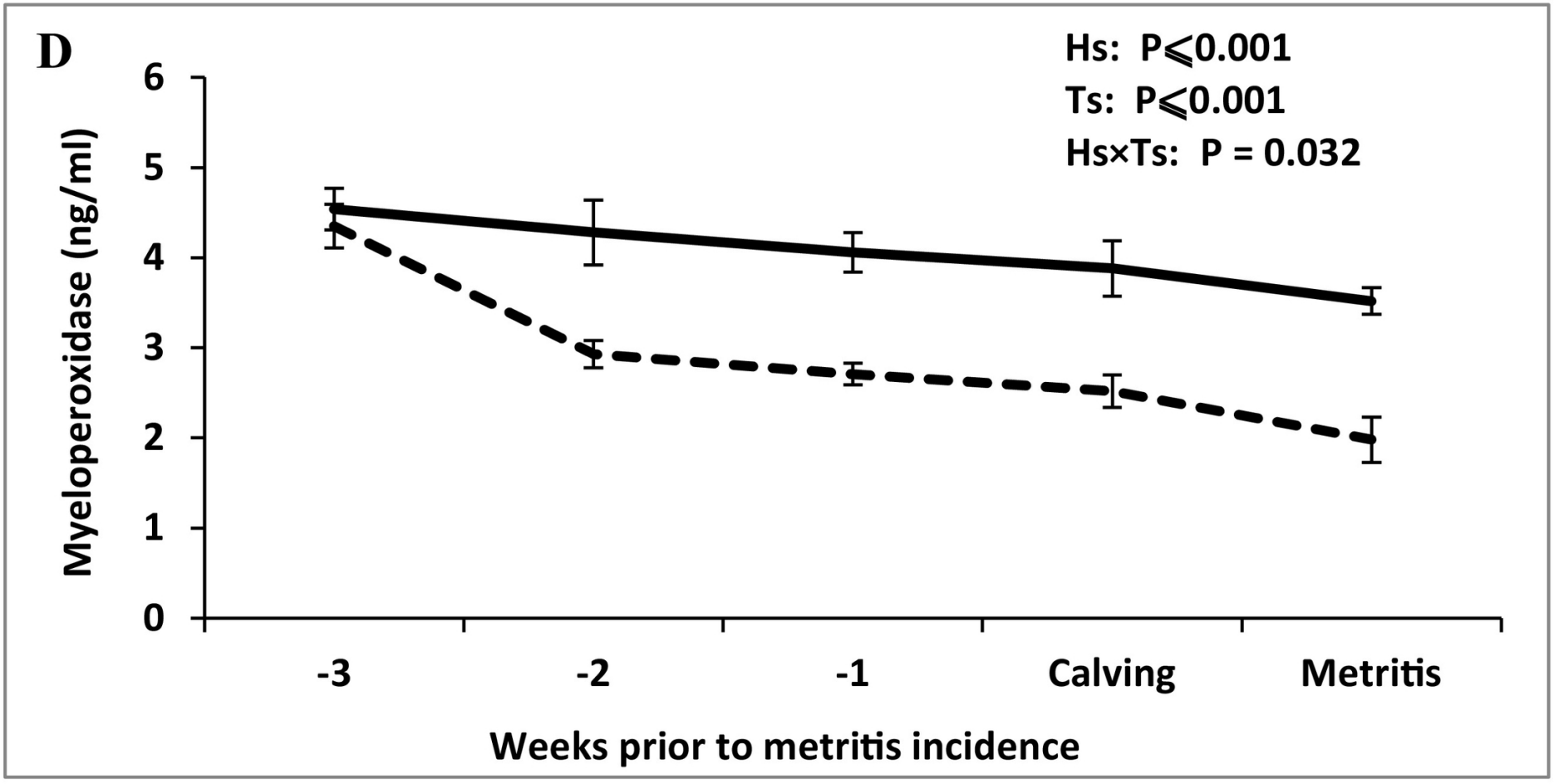
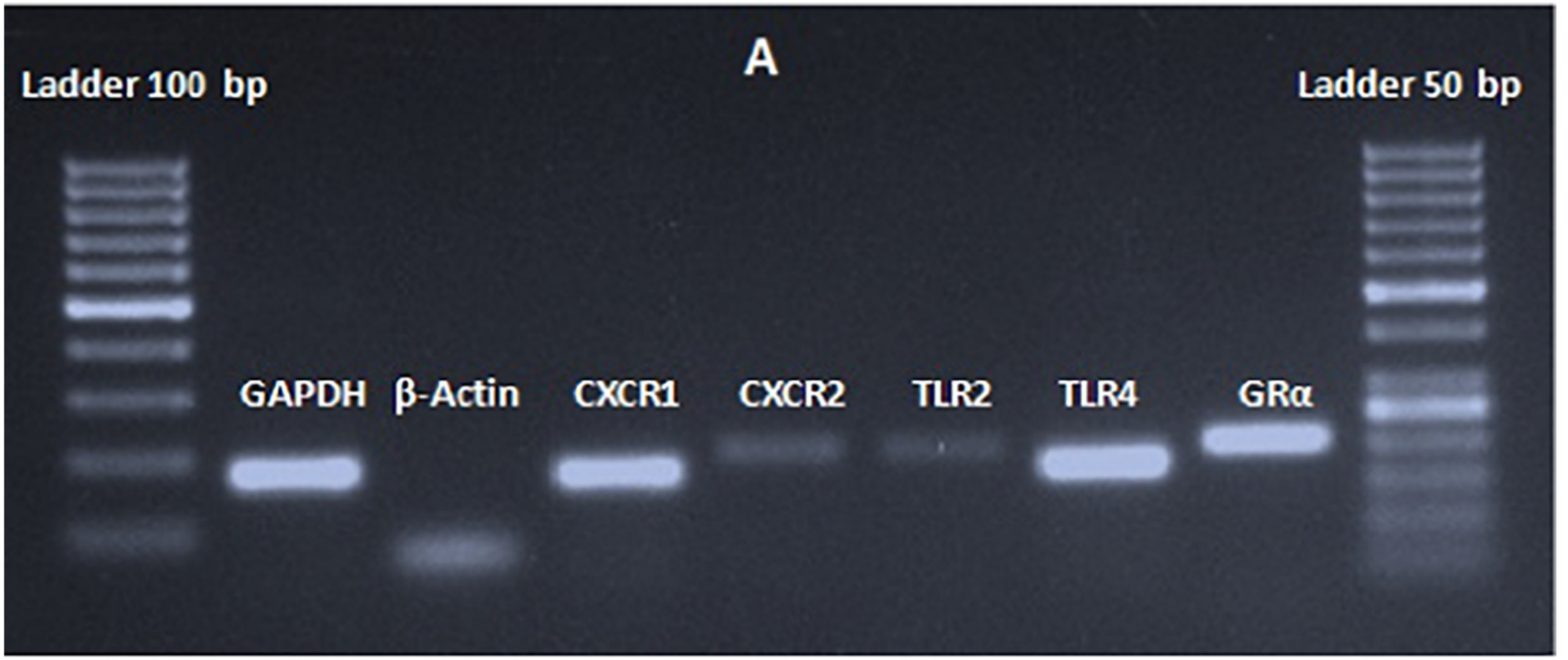

D
Hs: P⩽0.001
Ts: P⩽0.001
Hs×Ts: P = 0.032
Myeloperoxidase (ng/ml)
0
1
2
3
4
5
6
-3
-2
-1
Calving
Metritis
Weeks prior to metritis incidence

Figure 1B


A
Ladder 100 bp
Ladder 50 bp
GAPDH
β-Actin
CXCR1
CXCR2
TLR2
TLR4
GRα

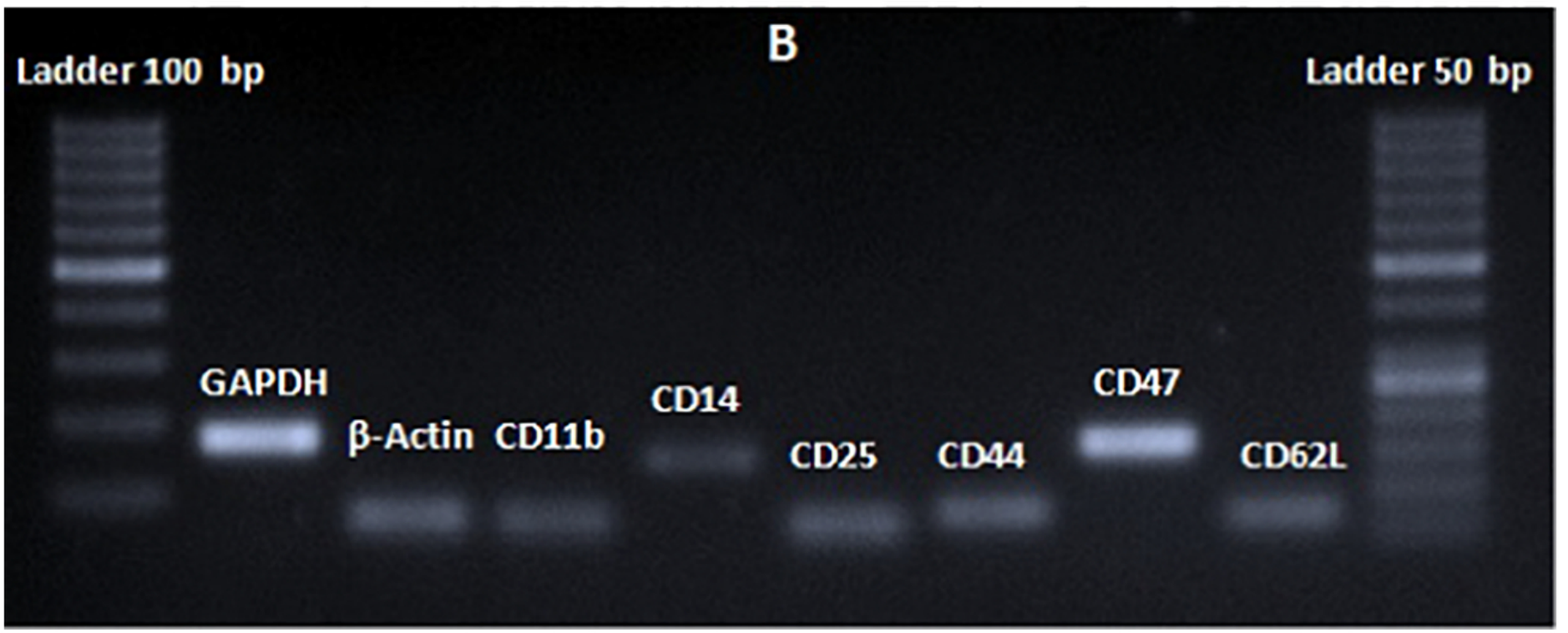
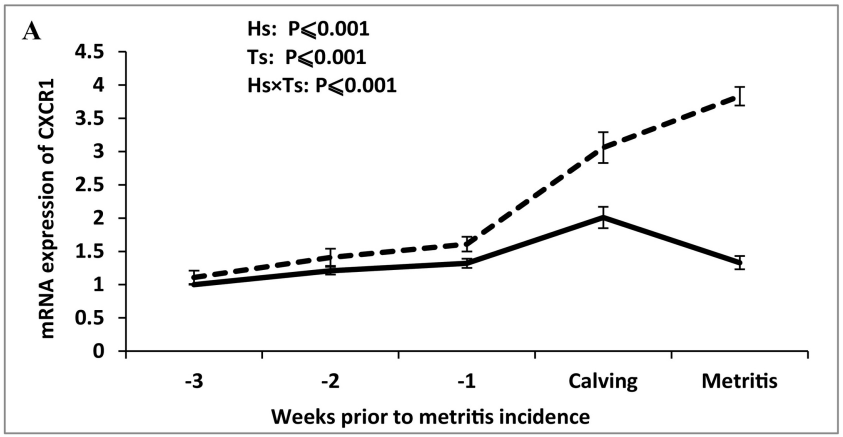
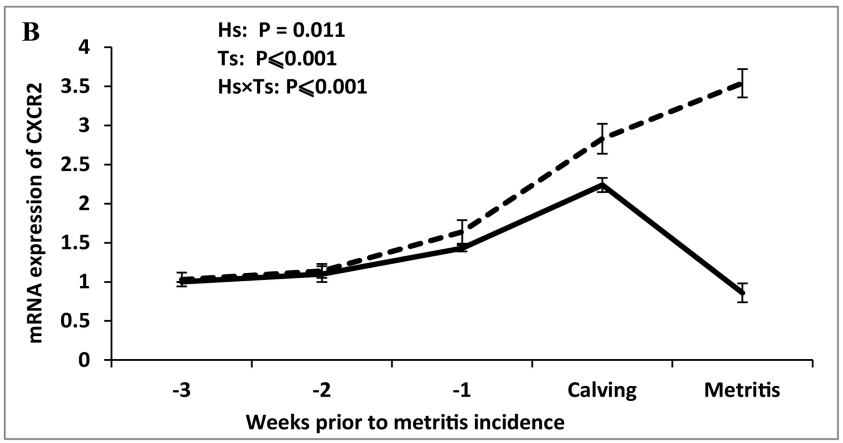

B
Ladder 100 bp
Ladder 50 bp
GAPDH
β-Actin
CD11b
CD14
CD25
CD44
CD47
CD62L

Figure 2


A
Hs: P⩽0.001
Ts: P⩽0.001
Hs×Ts: P⩽0.001
mRNA expression of CXCR1
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
-3
-2
-1
Calving
Metritis
Weeks prior to metritis incidence


B
Hs: P = 0.011
Ts: P⩽0.001
Hs×Ts: P⩽0.001
mRNA expression of CXCR2
4
3.5
3
2.5
2
1.5
1
0.5
0
-3
-2
-1
Calving
Metritis
Weeks prior to metritis incidence

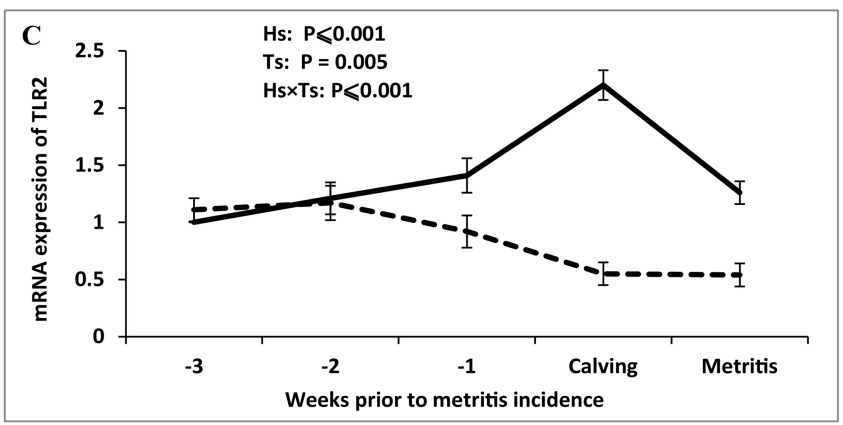
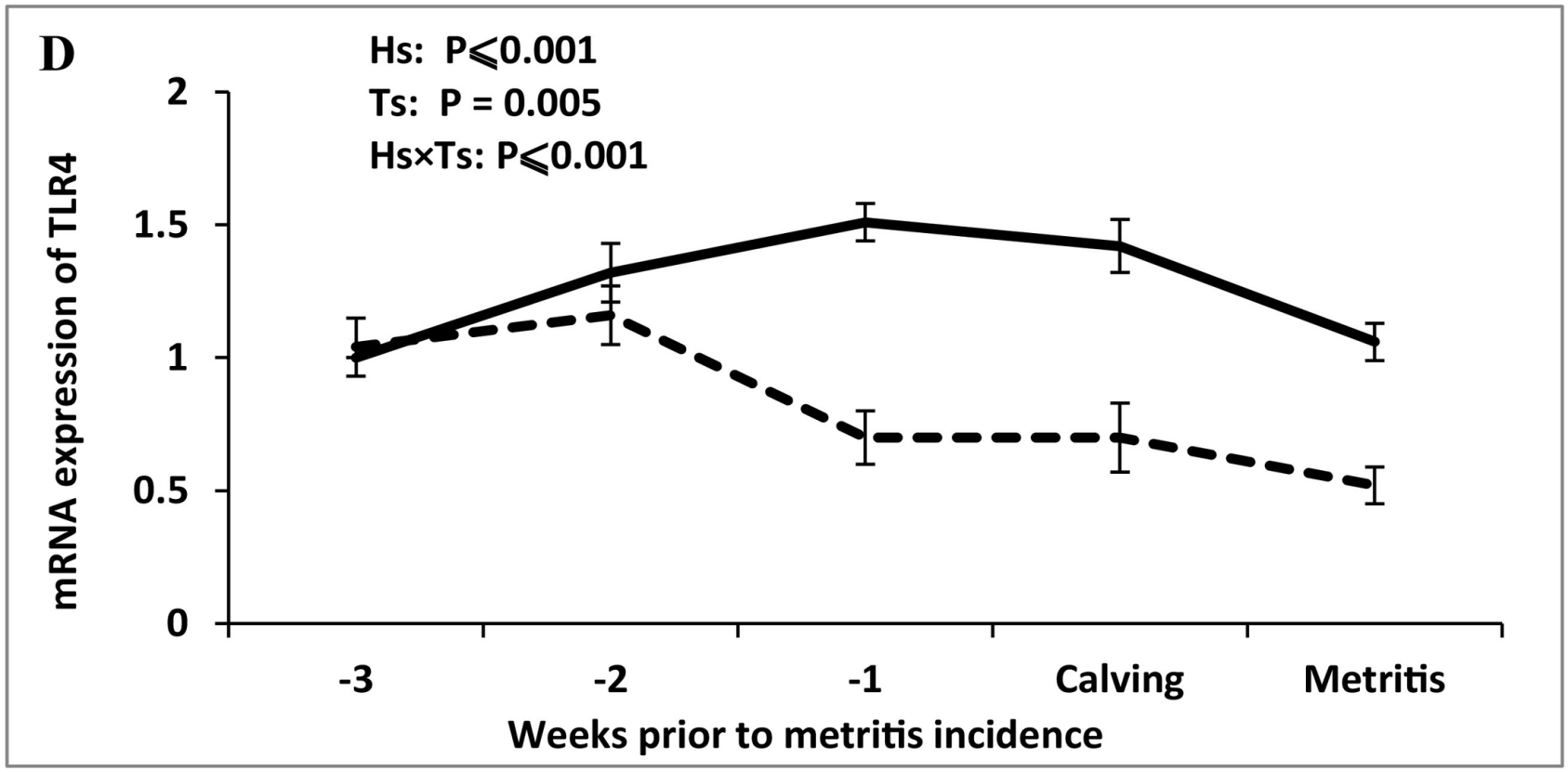

C
Hs: P⩽0.001
Ts: P = 0.005
Hs×Ts: P⩽0.001
mRNA expression of TLR2
2.5
2
1.5
1
0.5
0
-3
-2
-1
Calving
Metritis
Weeks prior to metritis incidence

Figure 3A


D
Hs: P⩽0.001
Ts: P = 0.005
Hs×Ts: P⩽0.001
mRNA expression of TLR4
2
1.5
1
0.5
0
-3
-2
-1
Calving
Metritis
Weeks prior to metritis incidence

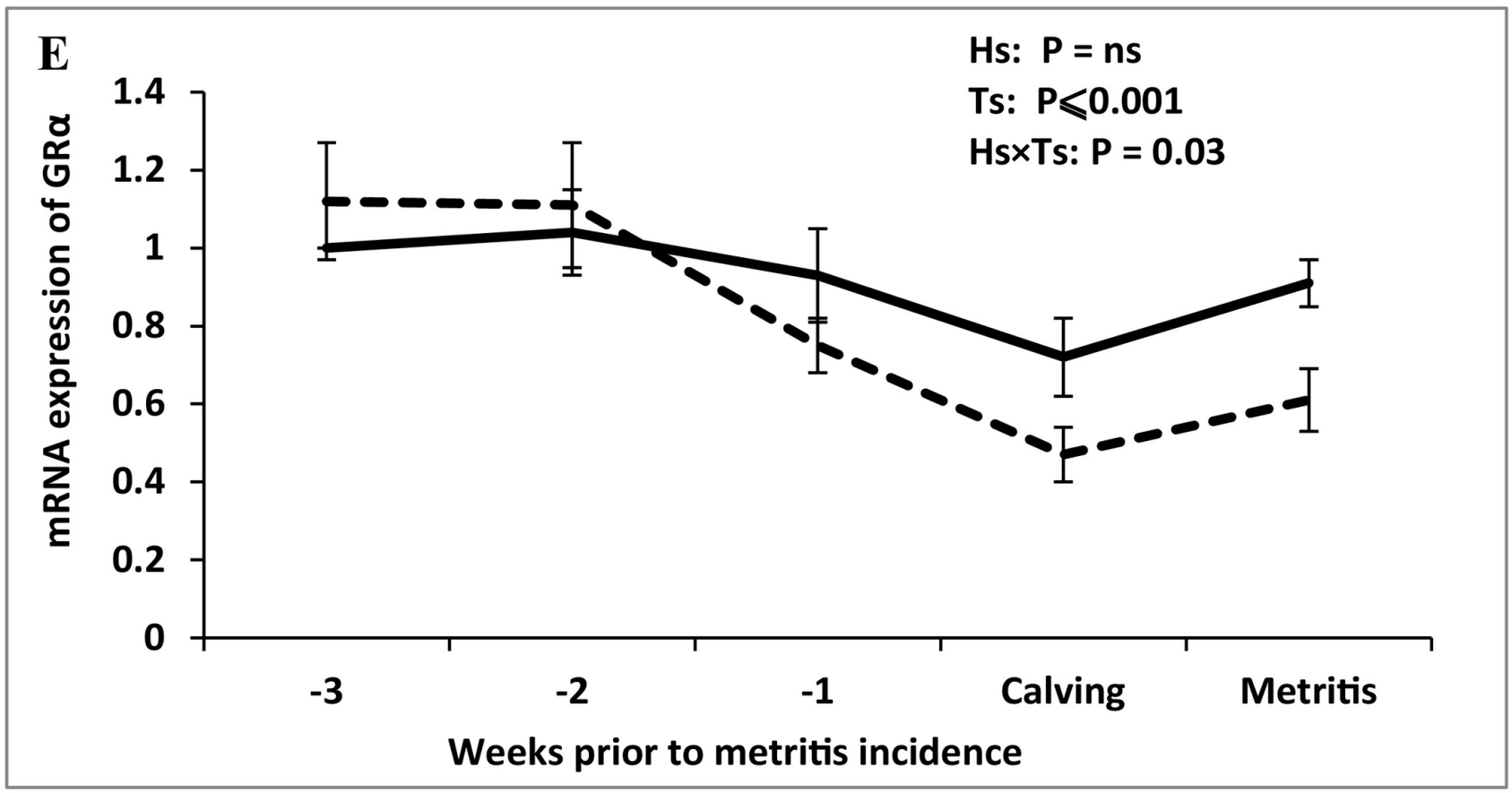
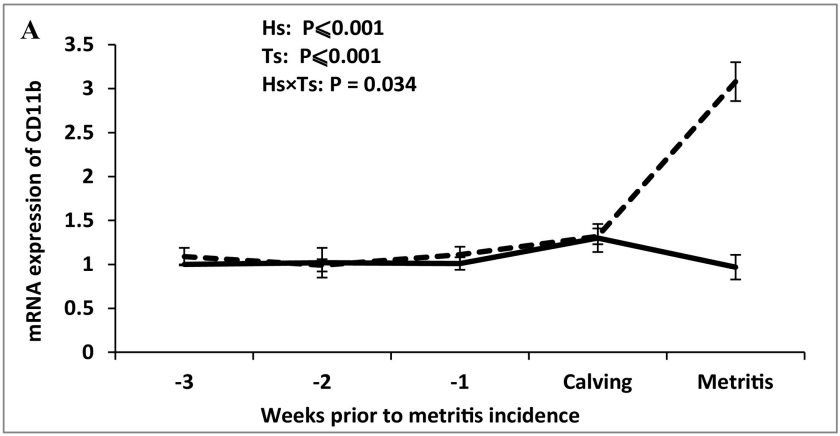
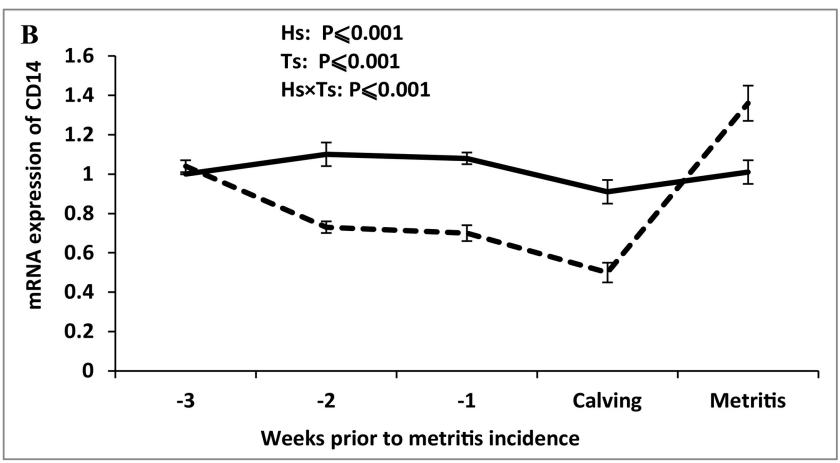

E
Hs: P = ns
Ts: P⩽0.001
Hs×Ts: P = 0.03
mRNA expression of GRα
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-3
-2
-1
Calving
Metritis
Weeks prior to metritis incidence

Figure 3B


A
Hs: P⩽0.001
Ts: P⩽0.001
Hs×Ts: P = 0.034
mRNA expression of CD11b
3.5
3
2.5
2
1.5
1
0.5
0
-3
-2
-1
Calving
Metritis
Weeks prior to metritis incidence


B
Hs: P⩽0.001
Ts: P⩽0.001
Hs×Ts: P⩽0.001
mRNA expression of CD14
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-3
-2
-1
Calving
Metritis
Weeks prior to metritis incidence

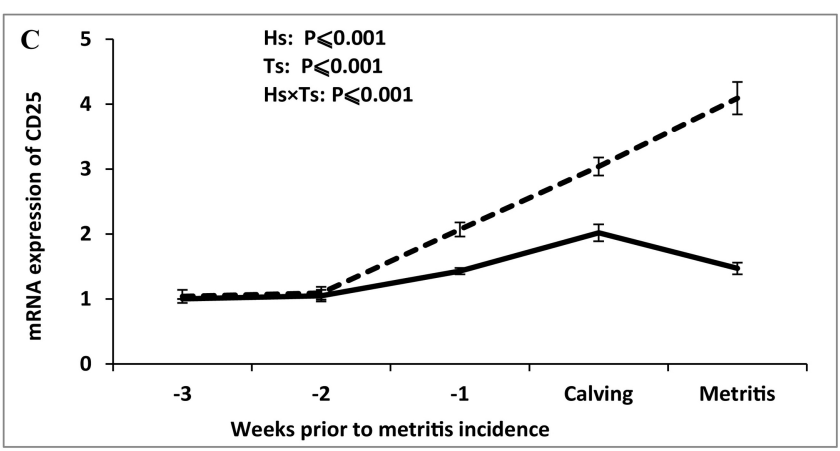
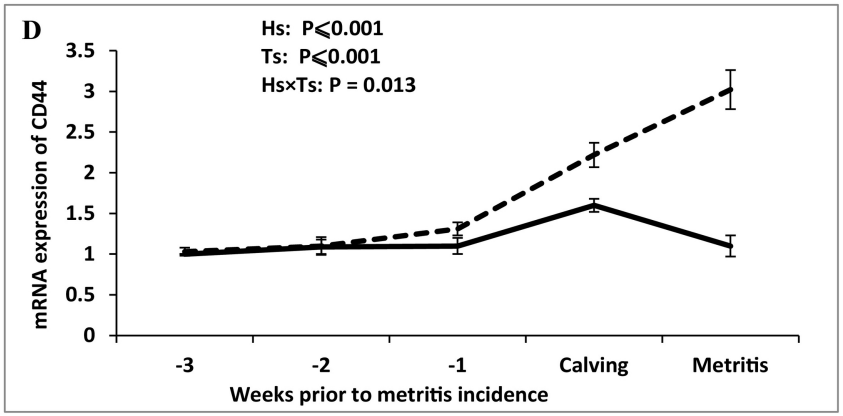
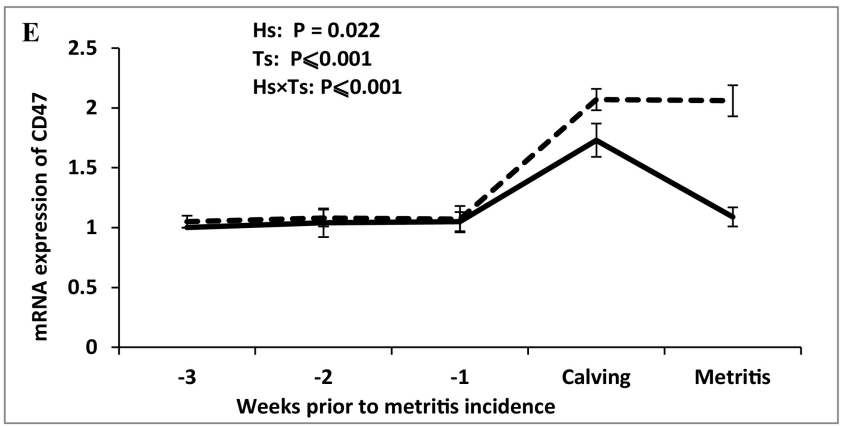

C
Hs: P⩽0.001
Ts: P⩽0.001
Hs×Ts: P⩽0.001
mRNA expression of CD25
5
4
3
2
1
0
-3
-2
-1
Calving
Metritis
Weeks prior to metritis incidence

Figure 4A


D
Hs: P⩽0.001
Ts: P⩽0.001
Hs×Ts: P = 0.013
mRNA expression of CD44
3.5
3
2.5
2
1.5
1
0.5
0
-3
-2
-1
Calving
Metritis
Weeks prior to metritis incidence


E
Hs: P = 0.022
Ts: P⩽0.001
Hs×Ts: P⩽0.001
mRNA expression of CD47
2.5
2
1.5
1
0.5
0
-3
-2
-1
Calving
Metritis
Weeks prior to metritis incidence

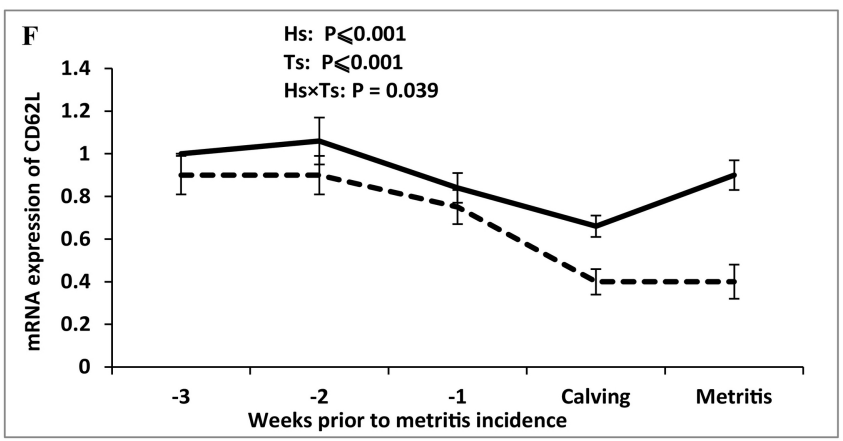

F
Hs: P⩽0.001
Ts: P⩽0.001
Hs×Ts: P = 0.039
mRNA expression of CD62L
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-3
-2
-1
Calving
Metritis
Weeks prior to metritis incidence

Figure 4B

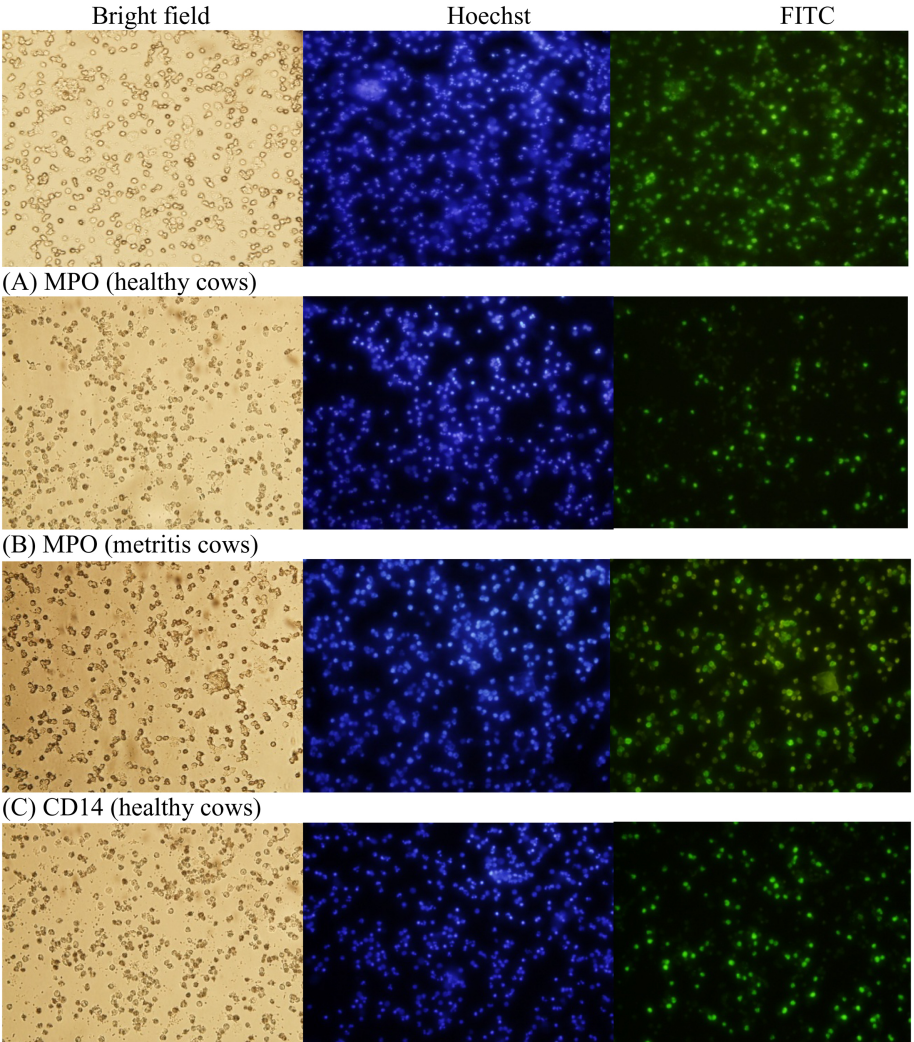

Bright field
Hoechst
FITC
(A) MPO (healthy cows)
(B) MPO (metritis cows)
(C) CD14 (healthy cows)
(D) CD14 (metritis cows)

Figure 5

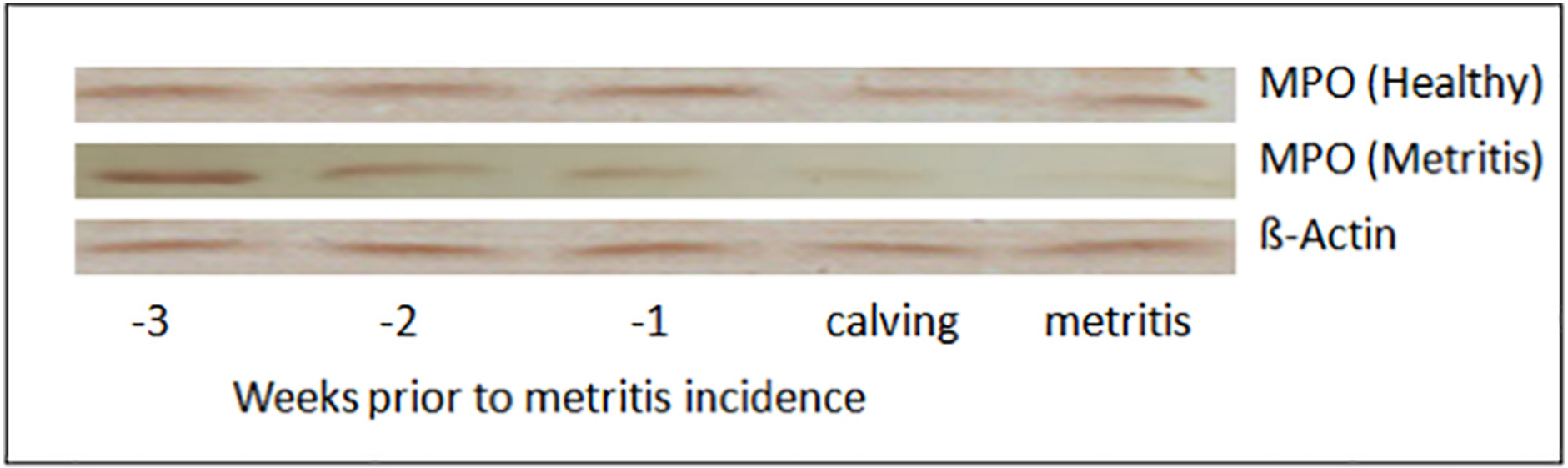

MPO (Healthy)
MPO (Metritis)
ß-Actin
-3
-2
-1
calving
metritis
Weeks prior to metritis incidence

Figure 6

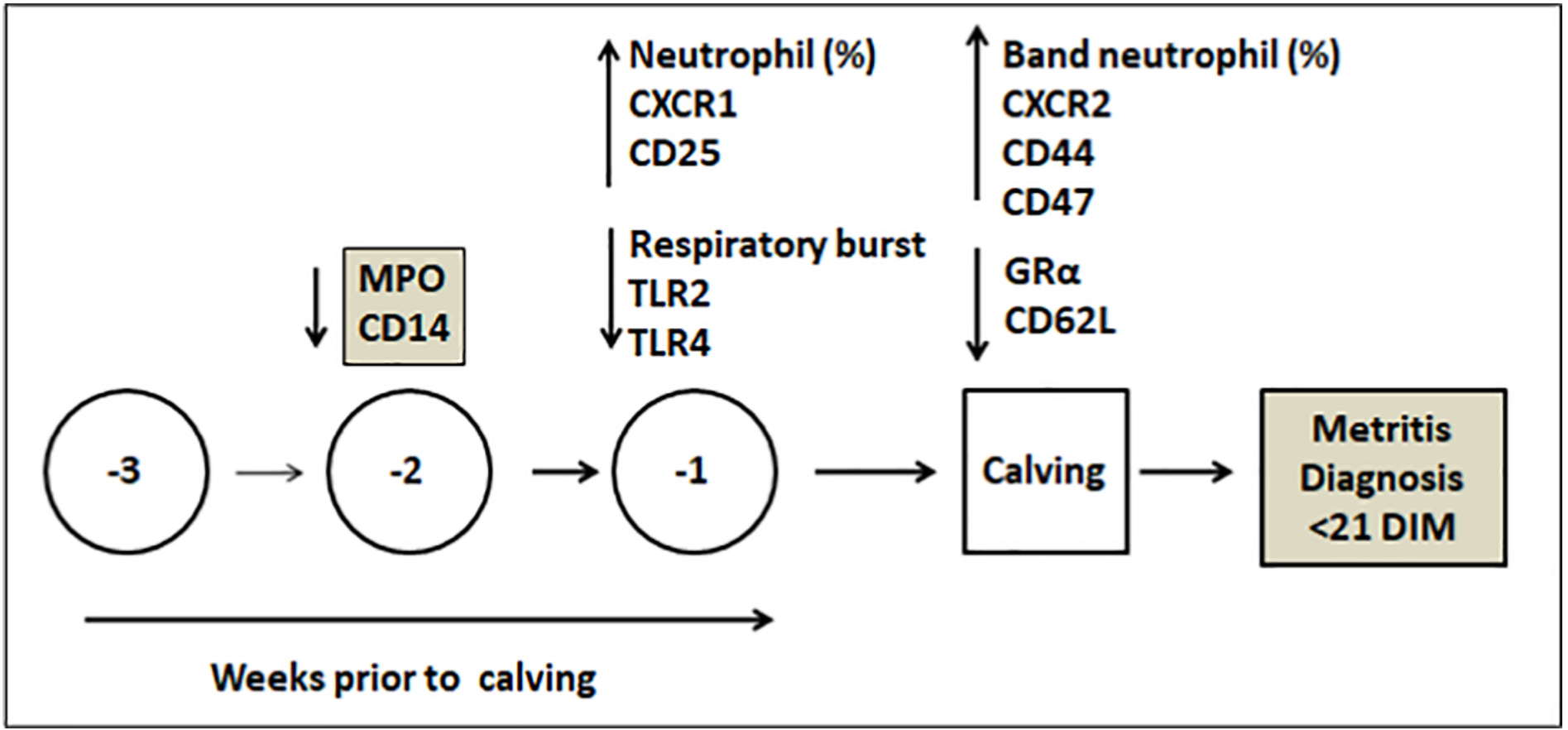

Neutrophil (%)
CXCR1
CD25
Band neutrophil (%)
CXCR2
CD44
CD47
Respiratory burst
TLR2
TLR4
MPO
CD14
GRα
CD62L
-3
-2
-1
Calving
Metritis
Diagnosis
<21 DIM
Weeks prior to calving

Figure 7