JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2015, 66, 3, 449-462
www.jpp.krakow.pl

S. PETER[1], G. MICHEL[2], A. HAHN[1], M. IBRAHIM[1], A. LUBKE-BECKER[3], M. JUNG[2], R. EINSPANIER[1], C. GABLER[1]

PUERPERAL INFLUENCE OF BOVINE UTERINE HEALTH STATUS ON THE MRNA EXPRESSION OF PRO-INFLAMMATORY FACTORS

[1]Institute of Veterinary Biochemistry, Freie Universitaet Berlin, Berlin, Germany; [2]Institute for the Reproduction of Farm Animals, Bernau, Germany; [3]Institute of Microbiology and Epizootics, Freie Universitaet Berlin, Berlin, Germany

After parturition, uterine bacterial infections lead to inflammatory processes such as subclinical/clinical endometritis with high prevalence in dairy cows. Endometrial epithelial cells participate in this immune response with the production of pro-inflammatory factors. The objective of the present study was to evaluate the endometrial mRNA expression pattern of pro inflammatory factors during a selected postpartum (pp) period. Dairy cows with three different uterine health conditions on days 24-30 pp (healthy: n = 11, subclinical endometritis: n = 10, clinical endometritis: n = 10) were sampled using the cytobrush technique. Subsequently, each cow was sampled 3 more times in weekly intervals (days 31-37 pp; days 38-44 pp; days 45-51 pp). Samples were subjected to mRNA analysis performed by RT-qPCR. Additionally, an analysis of cultivable bacteria was performed at the early/late stage of the selected puerperal period. mRNA expression of 16 candidate genes was analyzed by using two different approaches. The first approach referred to the initial grouping on days 24-30 pp to reveal long-term effects of the uterine health on the subsequent puerperal period. The second approach considered the current uterine health status at each sampling to elucidate the impact of different points in time. Long-term effects seem to appear for chemokines, prostacyclin synthase and prostaglandin D2 synthase. If related to the current uterine health, the majority of candidate genes were significantly higher expressed in endometritic cows on days 45-51 pp in contrast to earlier stages of the puerperium. Microbiological analysis revealed the significantly higher prevalence of *Trueperella pyogenes* findings in cows with clinical endometritis on days 24-30 pp, but no correlations were found on days 45-51 pp. In conclusion, a strong immune response to subclinical/clinical endometritis in the late puerperium may be related to the negative impact of these conditions on reproductive performance in dairy cows.

Key words: *endometritis, dairy cow, puerperium, gene expression, chemokines, metallopeptidase-1, platelet activating factor, prostaglandin E_2*

INTRODUCTION

Endometritis in the postpartum period is one of the most prevalent disorders in high producing dairy cows (1, 2). This is associated with a negative impact on reproductive performance resulting in decreased conception rates, prolonged calving intervals, reduced milk yield and higher culling rates (3-5). A main cause for endometritis is the invasion of aerobic and anaerobic bacteria into the bovine uterus during and after parturition due to the disintegration of anatomical barriers (6-8). Endometrial luminal epithelial cells react by recognizing invading pathogens (9) and support the immune system with the production of pro-inflammatory factors such as cytokines and chemokines (10).

Chemokines are chemotactic proteins that play a central role in the initiation of the innate immune response regulating the migration of immune cells to the site of infection. This early recruitment of polymorphonuclear neutrophils (PMN) is essential for the containment of pathogens (11). The chemokine (C-X-C motif) ligands 1 (CXCL1), CXCL2, CXCL3 and CXCL5 are strong chemoattractants that all bind to the chemokine receptor 2 (CXCR2) and stimulate the recruitment and activation of neutrophils (12, 13). CXCL5 could already be emerged as a time-dependent modulator in the course of the postpartum period and is involved in immunoregulatory processes in case of subclinical or clinical endometritis (14, 15). Another potent chemoattractant of PMN that also binds to CXCR2 is interleukin 8 (IL8) (16). Its mRNA expression was observed to be dependent on the health status of the bovine endometrium along with other pro-inflammatory cytokines such as *IL1A*, *IL1B*, *IL6* and tumor necrosis factor (*TNF*) (15, 17, 18).

Besides chemokines and cytokines, prostaglandins (PGs) are another group of important inflammatory mediators in addition to their contribution to a diversity of physiological reproductive events. In previous studies, the expression of enzymes of the PG synthesis such as prostaglandin-endoperoxide synthase 2 (*PTGS2*), prostaglandin E synthase 3 (*PTGES3*) and prostaglandin D2 synthase (*PTGDS*) was linked to the health status of the puerperal bovine uterus (14, 19).

Furthermore, a potent phospholipid mediator of intercellular signaling in case of inflammation is the platelet-activating factor (PAF) (20). It is synthesized by a broad range of cell types and

activates a variety of cells including endometrial and immune cells by binding to the corresponding receptor (PTAFR) (21, 22).

In combination with the activation of potent pro-inflammatory factors, pathological events such as bacterial invasion of the uterus require repair mechanisms for the tissue. Matrix metallopeptidase 1 (MMP1) is an enzyme involved in the remodelling of tissue stimulated by pro-inflammatory cytokines (23).

The capability of the puerperal bovine endometrium for a sufficient immune response to uterine infection, cellular mechanisms and the involvement of pro-inflammatory factors are still a matter of interest and intensive research.

Therefore, the objective of the present study was to elucidate the mRNA expression pattern of candidate genes during the postpartum (pp) period in bovine endometrial samples taken at four points in time starting on days 24-30 pp. Two statistical approaches were performed with the obtained data: 1) analyzing possible long-term effects of the initial uterine health condition (healthy, subclinical endometritis and clinical endometritis) and 2) analyzing the influence of the actual bovine uterine health status at each point in time of sampling.

MATERIALS AND METHODS

Examination and classification of cows

Lactating Holstein cows included in this study were kept on a dairy farm in Brandenburg (Germany) housing around 200 cows in accordance with the guidelines of the National Animal Welfare Legislation.

At the first examination (Exam 1 [E1]) on days 24-30 pp, cows were carefully examined by inspection of the vulva, vaginoscopy, transrectal palpation and ultrasonography (Tringa Linear, Esaote, Germany) of the uterus and ovaries. Only cows (n = 31; 7 primiparous and 24 multiparous) that were not showing signs of estrus (presence of a Graafian follicle and/or discharge of clear and cohesive mucus from the vulva) were included in this study.

At E1, cows were divided into 3 groups dependent on the health status of the uterus (24).

With signs of clinical endometritis (purulent or mucopurulent uterine discharge detectable in the vagina), cows were enrolled in the first group (Clinical Endometritis [CE]; n = 10).

The second group included cows with subclinical endometritis (Subclinical Endometritis [SCE]; n = 10). These cows had no (muco)purulent discharge in the vagina, but the content of PMN in the cytological sample was ≥ 5% (19).

No (muco)purulent discharge and a PMN content of <5% classified cows as healthy (Healthy [H]; n = 11) and they were enrolled in the third group.

Each cow was examined three more times in weekly intervals in the same manner as done at E1 (days 24-30 pp). These examinations were performed on days 31-37 pp (Exam 2 [E2]), on days 38-44 pp (Exam 3 [E3]) and on days 45-51 pp (Exam 4 [E4]). After the examination of each cow, endometrial samples were collected at E1, E2, E3 and E4 as described below.

The first approach of analysis was to evaluate a long-term effect of the uterine health status at E1 on the subsequent mRNA expression pattern of the candidate genes. Therefore, cows were classified into the health groups at E1 as described above and this initial grouping remained for E2, E3 and E4.

The second approach of analysis considered the fact that the uterine health status of most cows changed after E1. Therefore, the cows were reclassified into groups considering their current uterine health at each point in time of sampling after E1. These are the same points in time of examination (E2, E3 and E4), but for the analysis the denotation was changed to Exam 2 Current Health (E2CH on days 31-37 pp), Exam 3 Current Health (E3CH on days 38-44 pp) and Exam 4 Current Health (E4CH on days 45-51 pp). This new labeling was done to distinguish this second approach of analysis from the first approach.

The stage of the estrous cycle was specified ultrasonographically. At E1, a corpus luteum was detectable in 22 cows (luteal phase). Without the presence of a corpus luteum and a Graafian follicle at E1, cows were considered to be prior to their first estrus phase after parturition (n = 9). Considering the status of the estrous cycle at the following points in time of sampling, only 9 cows were ultrasonographically classified as not being in the luteal phase due to the absence of a corpus luteum and the presence of a Graafian follicle. Seven of these cows were classified as being in pro-estrus (three cows at E2/E2CH, three cows at E3/E3CH and one cow at E4/E4CH) and two cows as being in estrus (one cow at E2/E2CH and one cow at E3/E3CH).

The cows showing signs of clinical endometritis at E1 were injected intra muscular with 25 mg $PGF_{2\alpha}$ (Dinolytic, Pfizer, Berlin, Germany) at E1 and E3 after sampling.

Collection of endometrial samples

Endometrial epithelium samples were obtained by using the cytobrush technique (19, 25). Briefly, the vulva was cleansed with dry paper towel. A sterile cytobrush (Celltip, Servoprax, Wesel, Germany), screwed on a 55 cm long rod, was inserted into the uterine body through a sterile metallic catheter, which was protected by a plastic cover (Hygienic Sheath, Minitube, Tiefenbach, Germany) to avoid contaminations from the vulva and vagina. Using a new cytobrush each time, separate samples were taken from each cow first for cytological analysis, second for microbiological analysis (at E1 and E4/E4CH) and third for isolation of RNA.

Cytological slides were prepared on the farm by rolling the cytobrush on a clean glass microscope slide. After transportation to the laboratory, the air-dried cytology slides were fixed and stained with the Hemacolor staining set (Merck, Darmstadt, Germany) following the manufacturer's protocol. All slides were examined in a meandering pattern by light microscopy (Zeiss, Axioskop, Oberkochen, Germany) at ×1000 magnification with oil. A total of 300 cells (endometrial epithelial cells, PMNs and lymphocytes) were counted to determine the percentage of PMN. Cows without (muco)purulent discharge were defined as having SCE with a PMN content of ≥ 5% or as being healthy (H) with a PMN content of <5% (19).

The cytobrush for microbiological analysis was placed in an Amies medium containing tube (Heinz Herenz, Hamburg, Germany) and transported at room temperature to the laboratory.

For mRNA analysis, one cytobrush was placed in a cryotube, which was immediately immersed into liquid nitrogen and stored at -80°C until use.

Microbiological analysis

The cytobrush samples were analyzed by aerobic and anaerobic cultivation after direct inoculation of the cytobrush with the suitable agar plates and also after the enrichment in a medium with subsequent cultivation on agar (all agar purchased from Oxoid, Wesel, Germany).

For the detection of aerobic bacteria, Columbia blood agar (5% sheep blood), Gassner agar and Brilliance UTI Clarity agar were inoculated with the cytobrush within 12 hours after sampling for 24-48 hours (aerobic, 36°C). For the evaluation of the presence of obligate anaerobic bacteria, the inoculation was

carried out on Columbia blood agar (5% sheep blood) with added L-cysteine (Merck), haemin (Sigma-Aldrich, Hamburg, Germany), vitamin K1 (Roche, Mannheim, Germany) and lysed sheep blood 0.5% (Oxoid) and on an additionally plate with the same agar and added gentamicin (Hexal, Holzkirchen, Germany), both for 48-72 hours (anaerobic, 36°C). Chocolate agar was used for cultivation of fastidious bacteria for 24-48 hours (36°C, microaerobic, 7% CO_2). In addition, an enrichment culture with brain heart infusion broth (Oxoid) was started from each cytobrush at 37°C for 24 hours, which was transferred to Columbia blood agar (5% sheep blood).

The identification of the species was carried out by Gram stain, oxidase and catalase tests according to standard protocols (26) and API strips (bioMérieux, Nürtingen, Germany).

Extraction of total RNA and reverse transcription

Total RNA was extracted from cytobrush samples by using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). The first steps of the manufacturer's protocol were slightly modified by adding the lysis buffer directly to each cytobrush and subsequent thoroughly vortexing to disrupt cells. The cytobrush was removed with clean forceps and the remaining sample was then processed as described in the manufacturer's protocol. The yield of obtained total RNA was quantified by spectrophotometry at the wavelength of 260 nm. Samples were stored at –80°C until further use.

To validate the integrity of RNA, 1 µl of total RNA was loaded onto a RNA 6000 Nano Chip using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) following the manufacturer's protocol.

100 ng total RNA per sample was used to generate single stranded cDNA by using 200 U RevertAid Reverse Transcriptase and 2.5 µM random hexamer primers (both Thermo Scientific, Schwerte, Germany) in a total volume of 60 µl (27). Before reverse transcription, a DNase treatment was performed for the removal of genomic DNA (28). Samples without addition of reverse transcriptase were also prepared as negative controls to confirm the absence of any genomic DNA or contaminations. Samples were stored in aliquots at -20°C until further analysis.

Real-time polymerase chain reaction (PCR)

To assess the mRNA expression of the candidate genes, quantitative real-time PCR was performed as previously reported (27) following the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (29). The used primer pairs were synthesized by Eurofins MWG Operon (Ebersberg, Germany) and are listed in *Table 1*. Unpublished primer pairs were first evaluated by a gradient-PCR to determine the optimal annealing temperature and obtained PCR products were submitted to commercial DNA sequencing (GATC Biotech, Konstanz, Germany) for confirmation of 100% homology to the published bovine sequences (27).

A total reaction volume of 10 µl per sample for real-time PCR contained 1 µl cDNA, 0.4 µM of each primer (forward and reverse; details given in *Table 1*) and 5 µl 2× SensiMix SYBR Low-ROX (Bioline, Luckenwalde, Germany). Amplification was carried out using the Rotor Gene 3000 (Corbett Research, Mortlake, Australia) and the following protocol. A denaturation step of 95°C for 10 min was followed by a three-step amplification in 45 cycles (except 25 cycles for *18S rRNA*): denaturation at 95°C for 15 s, annealing for 20 s (temperatures are indicated in *Table 1*) and extension at 72°C for 30 s. A subsequent melting curve program (50-99°C) with continuous fluorescence measurement was carried out to confirm specific amplification followed by a final cooling step to 40°C. A dilution series with known concentrations of the purified specific PCR products was amplified simultaneously to generate a standard curve. Rotor Gene 6.1 software (Corbett Research) was used to calculate the contents of specific mRNA in comparison with the standard curves.

Statistical analysis

Using the geNorm tool, 18S ribosomal RNA (*18S rRNA*) and suppressor of zeste 12 homolog (*SUZ12*) were chosen to normalize the expression of the genes of interest for each sample (30) and inter-run calibration was performed based on 10 inter-run calibrator samples (31). Normalized and calibrated values were used to generate box plots, which are presenting the median values with 50% of all data within the box.

Normal distribution was tested with the Shapiro-Wilk test. Neither the percentages of the PMN nor the values of any of the genes of interest were normally distributed.

Normalized and calibrated values were analyzed by the Mann-Whitney U test to compare samples from healthy cows with samples from cows with either subclinical or clinical endometritis at each point in time of examination. The level of significance was set at $P < 0.05$.

Fold changes were calculated as the approximate ratio of the mean value of normalized mRNA expression for the SCE or CE group to the mean value of the H group, respectively.

Correlations between the content of PMN with the selected factors as well as between each of the factors was calculated by the Spearman-rho test with the level of significance set at $P < 0.01$.

The Fisher's exact test was used to calculate the prevalence of *Trueperella pyogenes* and *Escherichia coli* in the group with purulent vaginal discharge (CE) in relation to the groups without purulent vaginal discharge (H and SCE) with $P < 0.05$.

All statistical evaluations and the generation of the box plots were performed using IBM SPSS Statistics 20.0 (SPSS, Chicago, USA).

RESULTS

Microbiological analysis

The evaluation of the aerobic cultivation of the cytobrush samples taken at E1 and E4CH revealed that specific pathogenic bacteria (e.g. *E. coli*, *T. pyogenes*) could not always be detected in the SCE and CE group.

At E1, *E. coli* was cultivable from only few samples of all groups. In detail, *E. coli* was detected in one sample of the H group, in three samples of the SCE group and in one sample from a cow with CE. *T. pyogenes* was not detected in the SCE group at E1, but in one sample of the H group and in four samples from cows with CE, which signifies a higher prevalence of *T. pyogenes* findings in cows with CE ($P = 0.027$).

At E4CH, no specific pathogenic bacteria were cultivable except *E. coli* in one cow of the SCE group (along with *Aerococcus* sp. and *Lactobacillus* sp.) and in one cow of the H group.

All five cows that were tested positive for *E. coli* at E1, even if they had SCE or CE, were healthy at the following examinations. In contrast, all five cows that had *T. pyogenes* cultivated from their cytobrush sample at E1, showed signs of subclinical or clinical endometritis at the following examinations with only one exception at E3CH when this cow was classified as healthy.

Additionally, aerobic cultivation revealed the sporadic presence of *Histophilus somni* in the SCE group in one cow at

Table 1. Selected gene transcripts, primer sequences and annealing temperatures used for RT-qPCR with resulting amplicon length.

Gene	Primer sequence	Reference/ GenBank accession no.	Fragment size	Annealing temperature
18S rRNA	For 5'-GAG AAA CGG CTA CCA CAT CCA A-3'	(27)	337 bp	61ºC
	Rev 5'-GAC ACT CAG CTA AGA GCA TCG A-3'			
SUZ12	For 5'-TTC GTT GGA CAG GAG AGA CC-3'	NM_001205587	286 bp	60ºC
	Rev 5'-GTG CAC CAA GGG CAA TGT AG-3'			
CXCL1/2	For 5'-GAC CTT GCA GGG GAT TCA CCT C-3'	(54)	125 bp	60ºC
	Rev 5'-CGG GGT TGA GAC ACA CTT CCT G-3'			
CXCL3	For 5'-GCC ATT GCC TGC AAA CTT-3'	(54)	189 bp	56ºC
	Rev 5'-TGC TGC CCT TGT TTA GCA- 3'			
CXCL5	For 5'-TGA GAC TGC TAT CCA GCC G- 3'	(15)	193 bp	61ºC
	Rev 5'-AGA TCA CTG ACC GTT TTG GG- 3'			
CXCR2	For 5'-AAC AGA CTC TGC CCC ATG TC-3'	(54)	151 bp	60ºC
	Rev 5'-AGT GAC AGA GCG ACC AAT CC-3'			
IL1A	For 5'-TCA TCC ACC AGG AAT GCA TC-3'	(19)	300 bp	59ºC
	Rev 5'-AGC CAT GCT TTT CCC AGA AG-3'			
IL1B	For 5'-CAA GGA GAG GAA AGA GAC A- 3'	(55)	236 bp	56ºC
	Rev 5'-TGA GAA GTG CTG ATG TAC CA- 3'			
IL6	For 5'-TCC AGA ACG AGT ATG AGG-3'	(55)	236 bp	56ºC
	Rev 5'-CAT CCG AAT AGC TCT CAG-3'			
IL8	For 5'-CGA TGC CAA TGC ATA AAA AC-3'	(15)	153 bp	56ºC
	Rev 5'-CTT TTC CTT GGG GTT TAG GC-3'			
PTGS1	For 5'-CAG ATG CGG AGT TTC TGA GTC G-3'	(27)	313 bp	60ºC
	Rev 5'-GGG TAG TGC ATC AGC ACG G-3'			
PTGS2	For 5'-CTC TTC CTC CTG TGC CTG AT-3'	(27)	359 bp	60ºC
	Rev 5'-CTG AGT ATC TTT GAC TGT GGG AG-3'			
PTGIS	For 5'-CTG TTG TCC CCA ACC AGG-3'	NM_174444	311 bp	60ºC
	Rev 5'-CTG TCC AGC ACA GGC ATG-3'			
PTGES3	For 5'-TGC AAA GTG GTA CGA TCG G-3'	(19)	253 bp	61ºC
	Rev 5'-TAA CCT TGG CCA TGA CTG G-3'			
PTGDS	For 5'-TGA GAC GCG GAC CTT ACT G-3'	(19)	193 bp	61ºC
	Rev 5'-CTG GGA GCG GCT GTA GAG-3'			
TNF	For 5'-CAA GTA ACA AGC CGG TAG CC-3'	(56)	354 bp	60ºC
	Rev 5'-GCT GGA AGA CTC CTC CCT G -3'			
MMP1	For 5'-GAT GAT GAT GAA TGG TGG ACC-3'	(57)	347 bp	60ºC
	Rev 5'-TCC ACT TCT GGG TAC AAG GG-3'			
PTAFR	For 5'-CAA CCA GGG TGA CTG GAT TC-3'	NM_001040538	338 bp	59ºC
	Rev 5'-GGA AAA TGT GGA TGG TGA GG-3'			

E1 and in one cow at E4CH, of *Streptococcus uberis* in one cow of the H group at E4CH and of *Staphylococcus* sp. in one cow of the CE group at E1.

The result of the anaerobic cultivation showed no cultivable bacteria in the H and SCE groups (except *Clostridium perfringens* in one SCE cow at E4CH). At E1, *Prevotella* sp. and *P. melaninogenica* were cultivable each in one cow with CE associated with the presence of *T. pyogenes*.

Current uterine health status at each sampling

The current uterine health status of each of the cows at each sampling is shown in *Fig. 1*.

Subsequent to E1, the number of cows in the groups dependent on their current uterine health was distributed as follows (H/SCE/CE): E2CH (n = 17/3/11), E3CH (n = 20/5/6) and E4CH (n = 16/8/7).

Most of the cows that were classified healthy at E1 were found in that same uterine health group continuously later in the puerperium. The majority of cows that had SCE at E1 were found in the healthy group at E2CH. In contrast, all of the five cows that were found in the SCE group at E3CH were also found in that group one week later. None of the cows continuously had subclinical endometritis. Most of the cows with CE at E1 were diagnosed with SCE or CE in the following puerperal period.

mRNA expression analysis

Endometrial epithelial mRNA expression of all selected candidate genes was detected in all uterine health groups and at all points in time of sampling. However, mRNA expression for *PTGS1, PTGS2, PTGIS, IL1A, IL6, TNF* and *MMP1* could not be found in each sample. In the following text, mainly significant differences in normalized mRNA expression are described.

The status of the estrous cycle from totally 124 examinations (luteal phase: n = 103, prior to first ovulation: n = 12, pro-estrus: n = 7, estrus: n = 2) did not have a statistical significant influence on the mRNA expression values.

mRNA expression of key enzymes of the prostaglandin synthesis

No differential mRNA expression for the analyzed enzymes of the prostaglandin synthesis in the endometrial epithelium was

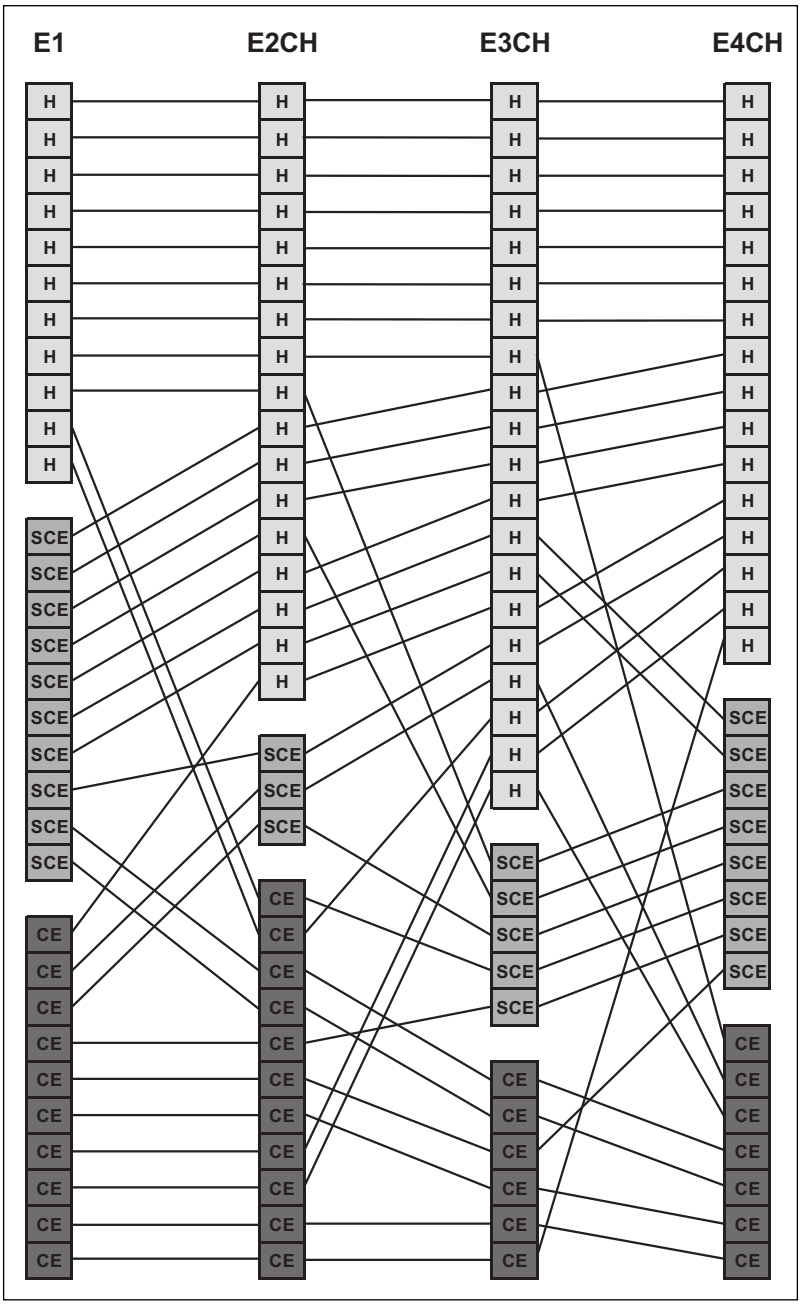

Fig. 1. Change of the uterine health status (H: healthy cow; SCE: cow with subclinical endometritis; CE: cow with clinical endometritis) of each of the cows over the period of sampling. E1 - days 24-30 pp; E2CH - days 31-37 pp; E3CH - days 38-44 pp; E4CH - days 45-51 pp.

detected on days 24-44 pp if the current uterine health status was considered. However, differences were found at the last examination (E4CH) for the mRNA expression of *PTGS1* and *PTGS2*. A fourfold higher expression (P = 0.042) was observed for *PTGS1* mRNA in the SCE group and for *PTGS2* mRNA in the CE group (P = 0.023), both compared with healthy cows (*Fig. 2A* and *2B*).

The mRNA expression of *PTGES3* did not reach any statistical differences between the groups of current uterine health at any point in time (*Fig. 2C*), also not for days 31-51 pp if evaluated referring to the initial grouping (E2, E3, E4; data not shown).

Following up the same cows dependent on the initial grouping at E1, no significant differences in *PTGIS* contents were found at E2 and E4. However at E3, *PTGIS* mRNA expression was eightfold higher (P = 0.009) in the CE group if compared with the H group (*Fig. 3A*). A similar expression pattern was found for *PTGDS* mRNA at the same time of sampling (ninefold higher; P = 0.001) with an additional threefold higher expression (P = 0.049) in the SCE group in comparison to the H group (*Fig. 3B*). At E2, the *PTGDS* transcript amount was also twofold higher (P = 0.011) in cows with CE in relation to healthy cows. Considering the actual health status at E4CH, *PTGDS* mRNA was higher expressed in both SCE (tenfold higher; P = 0.012) and CE (fivefold higher; P = 0.009) groups compared with cows of the H group.

mRNA expression of chemokines and their receptor

Sequences for *CXCL1* and *CXCL2* are to 99% homologous. Obtained sequences for the amplicons are to 100% homologous to *CXCL1* and *CXCL2*. Therefore, the primers named *CXCL1/2* were specific for *CXCL1* as well as for *CXCL2*.

At E1, *CXCL1/2* was fivefold higher (P = 0.011) expressed in SCE cows compared with H cows (*Fig. 4A*). Retaining the grouping from E1, the mRNA expression was sixfold higher (P = 0.029) in endometrial epithelium cells from cows with CE in

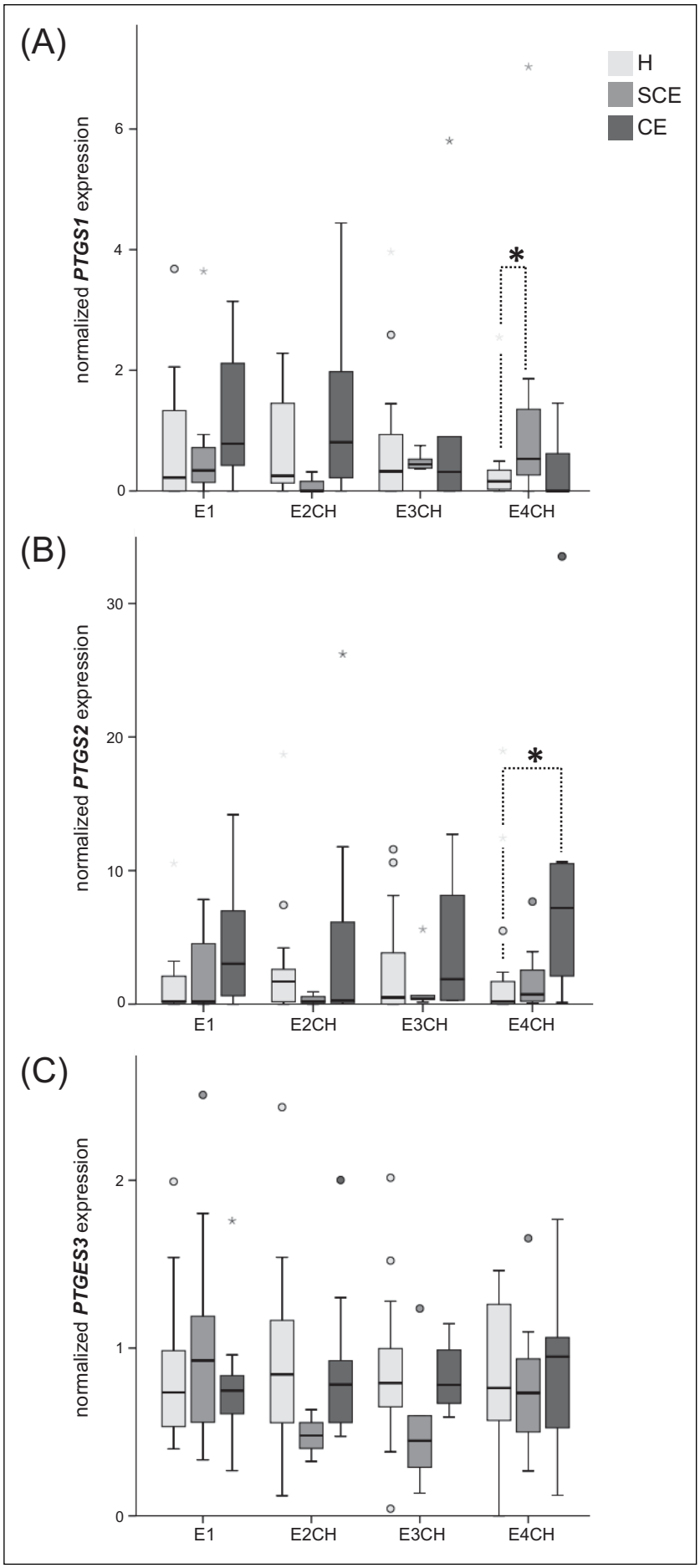

Fig. 2. Normalized mRNA expression of (A) *PTGS1*, (B) *PTGS2* and (C) *PTGES3* in bovine endometrial cytobrush samples harvested from dairy cows depending on the current status of uterine health (H: healthy, SCE: subclinical endometritis, CE - clinical endometritis) on days 24-30 pp (E1), on days 31-37 pp (E2CH), on days 38-44 pp (E3CH) and on days 45-51 pp (E4CH). Bold asterisks over dotted lines indicate significant differences between the groups ($P < 0.05$). Extreme values are diagrammed as asterisks, outliers as circles. Extreme values are not shown for *PTGS1* (9.02 and 9.76) obtained from two cows of the H group at E2CH.

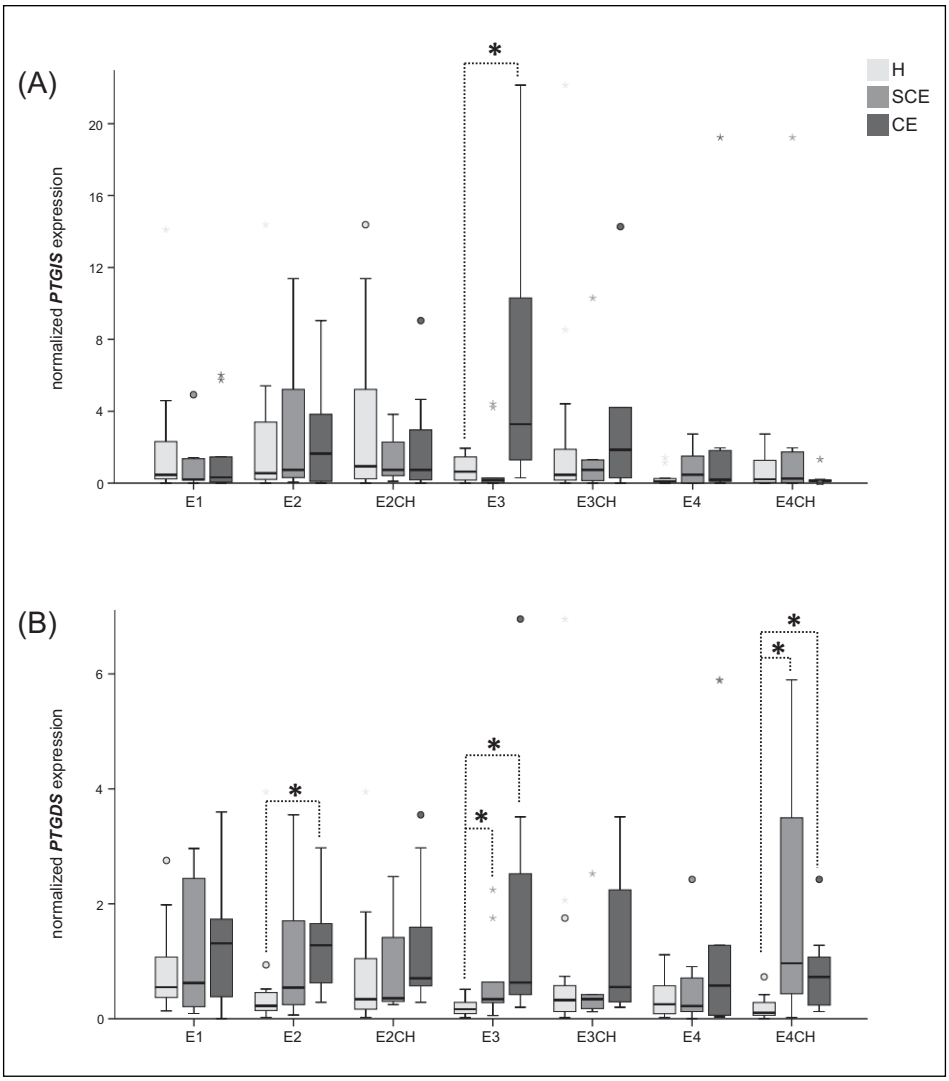

Fig. 3. Normalized mRNA expression of (A) *PTGIS* and (B) *PTGDS* in bovine endometrial cytobrush samples harvested from dairy cows depending on the status of uterine health (H: healthy, SCE: subclinical endometritis, CE: clinical endometritis) on days 24-30 pp (E1), on days 31-37 pp (E2/E2CH), on days 38-44 pp (E3/E3CH) and on days 45-51 pp (E4/E4CH). Bold asterisks over dotted lines indicate significant differences between the groups ($P < 0.05$). Extreme values are diagrammed as asterisks, outliers as circles.

comparison to the H group at E4. If the cows were classified dependent on their current health status at the time of sampling, the pattern for *CXCL1/2* mRNA additionally showed a significant correlation to the uterine health at E4CH both regarding SCE cows (threefold higher; P = 0.012) and CE cows (about 20-fold higher; P = 0.009) compared with the H group.

CXCL3 reached a twofold higher expression (P = 0.002) in CE cows compared with H cows at E1 and almost similar at E4CH (threefold higher; P = 0.013) considering the actual uterine health status (*Fig. 4B*).

Comparing transcript amounts of the CE group to the H group, *CXCL5* mRNA was significantly higher expressed at E1 (fivefold higher; P = 0.011), at E2CH (sixfold higher; P = 0.02), at E4 (sevenfold higher; P = 0.029) and at E4CH (20-fold higher; P = 0.016) (*Fig. 4C*). No differences were observed for *CXCL3* and *CXCL5* if comparing the SCE group to the H group.

The *CXCR2* mRNA expression pattern showed significant differences ($P < 0.05$) between the same groups as shown for *CXCL1/2*, with the addition that *CXCR2* was 30-fold higher expressed (P = 0.014) in SCE cows in relation to H cows at E3CH (*Fig. 4D*).

mRNA expression of interleukins

The endometrial epithelial mRNA expression of the analyzed interleukins was only correlated to the current uterine health status. A long-term effect of the health status was not observed when diagnosed at E1.

IL1A mRNA was correlated to the health status at E1, E3CH and E4CH (*Fig. 5A*). In comparison to the H group it was expressed about six- to sevenfold higher in cows with SCE, both at E1 (P = 0.02) and E3CH (P = 0.042). Later at E4CH, the mRNA expression was 100-fold higher (P = 0.011) in CE cows in comparison to the H group, but no significant differences for the SCE group were observed.

IL1B mRNA was higher expressed in cows with SCE at the early (E1; 15-fold higher; P = 0.006) and late (E4CH; 20-fold higher; P = 0.002) stage of the selected puerperial period. In addition and similar to *IL1A* expression, the highest fold change was observed also at E4CH in samples from cows with CE (200-fold higher; P = 0.003) in relation to the H group (*Fig. 5B*).

The transcript amounts of *IL6* were twofold higher (P = 0.023) in SCE cows at E3CH and sixfold higher (P = 0.022) in cows with CE at E4CH, both related to the H group (*Fig. 5C*).

The *IL8* mRNA expression pattern showed significant differences between the same groups at the same points in time of sampling as shown for *IL1A*, with the addition of a higher content of *IL8* mRNA in uterine samples from SCE cows compared with H cows at E4CH (15-fold higher; P = 0.023) (*Fig. 5D*). The expression was 25- and 10-fold higher in SCE cows at E1 (P = 0.006) and E3CH (P = 0.012), respectively. Comparable to the *IL1A* and *IL1B* expression pattern, the highest

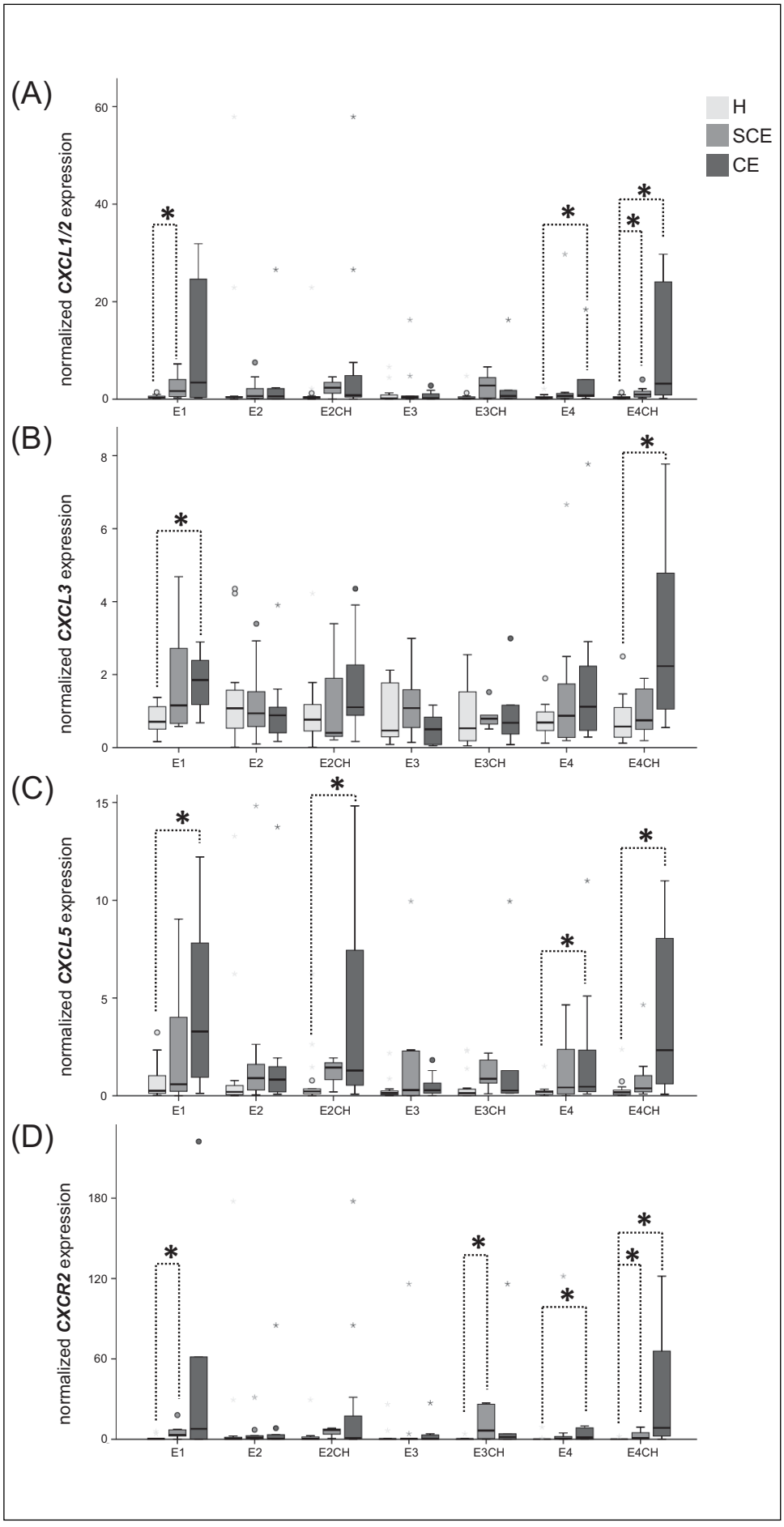

Fig. 4. Normalized mRNA expression of (A) *CXCL1/2*, (B) *CXCL3*, (C) *CXCL5* and (D) *CXCR2* in bovine endometrial cytobrush samples harvested from dairy cows depending on the status of uterine health (H: healthy, SCE: subclinical endometritis, CE: clinical endometritis) on days 24-30 pp (E1), on days 31-37 pp (E2/E2CH), on days 38-44 pp (E3/E3CH) and on days 45-51 pp (E4/E4CH). Bold asterisks over dotted lines indicate significant differences between the groups ($P < 0.05$). Extreme values are diagrammed as asterisks, outliers as circles. Extreme values are not shown for *CXCL1/2* (134.53) and *CXCR2* (321.89) obtained from a cow in the CE group at E4/E4CH and *CXCL5* (24.24) obtained from a cow in the SCE group at E4 and in the CE group at E4CH.

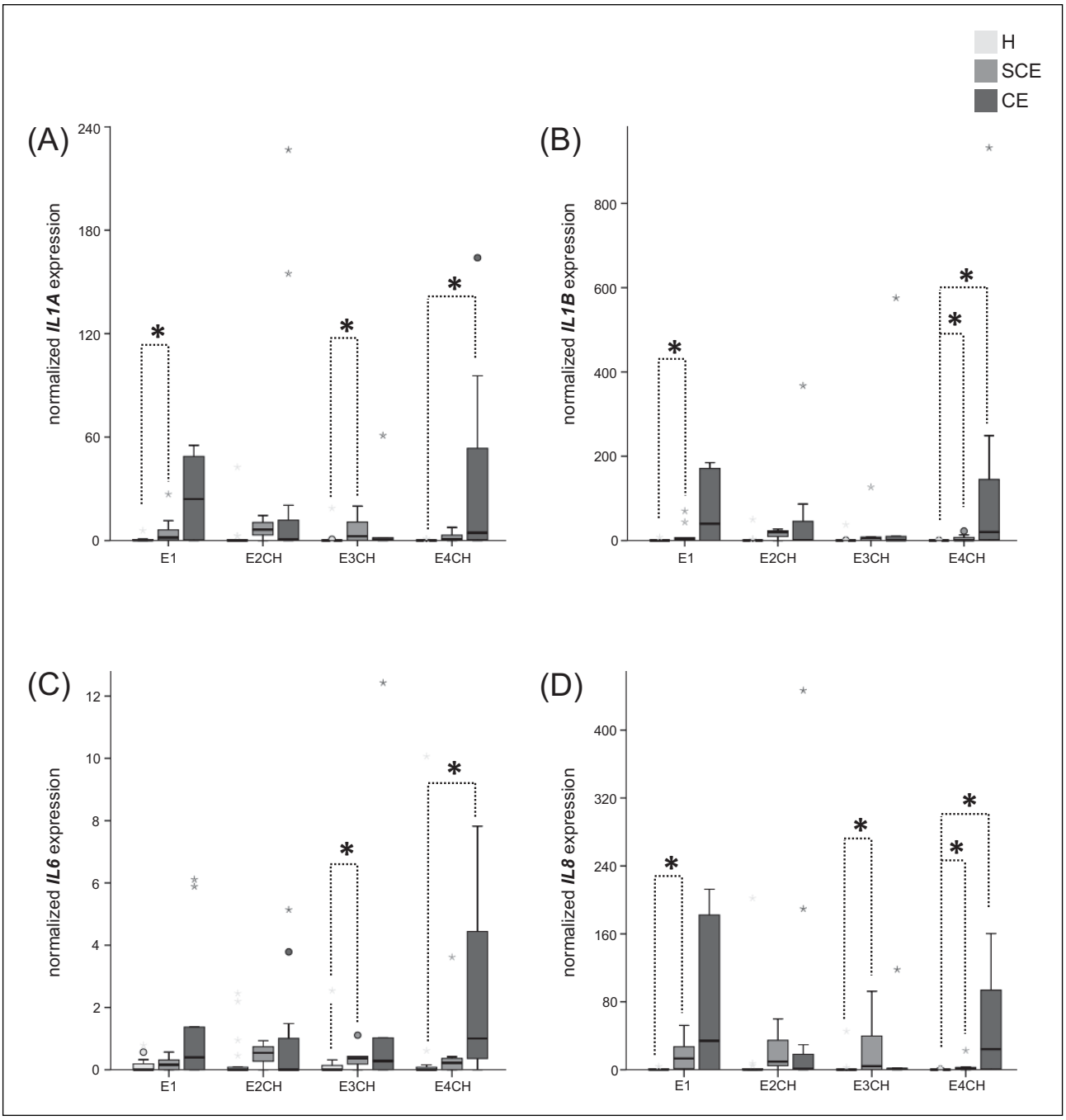

Fig. 5. Normalized mRNA expression of (A) *IL1A*, (B) *IL1B*, (C) *IL6* and (D) *IL8* in bovine endometrial cytobrush samples harvested from dairy cows depending on the current status of uterine health (H: healthy, SCE: subclinical endometritis, CE: clinical endometritis) on days 24-30 pp (E1), on days 31-37 pp (E2CH), on days 38-44 pp (E3CH) and on days 45-51 pp (E4CH). Bold asterisks over dotted lines indicate significant differences between the groups ($P < 0.05$). Extreme values are diagrammed as asterisks, outliers as circles. Extreme values are not shown for *IL1A* (323.80) and *IL1B* (1897.99) obtained from a cow with CE at E1 and for *IL1B* (1901.71) from a cow with CE at E2CH. Extreme values are also not shown for *IL6* (16.83) and *IL8* (940.72) obtained from a cow in the CE group at E4CH.

fold change (120-fold) in the CE group in relation to healthy cows was observed at E4CH ($P = 0.002$).

mRNA expression of tumor necrosis factor (TNF), matrix metallopeptidase 1 (MMP1) and platelet-activating factor receptor (PTAFR)

For the transcript amount of these selected candidate genes significant differences were also noted if the current uterine health status was considered.

The mRNA expression of *TNF* in luminal endometrial epithelium was affected at E3CH and E4CH (*Fig. 6A*). At E3CH, *TNF* was higher ($P = 0.034$) expressed in cows with SCE and at E4CH, the mRNA content of *TNF* was 15-fold higher ($P = 0.005$) only in the CE group in comparison to the H group.

MMP1 mRNA expression was dependent on the current health status starting on days 31-37 pp. Compared with the H group, a higher mRNA expression was detected in CE cows at E2CH (60-fold higher; $P = 0.01$), at E3CH (20-fold higher; $P = 0.019$) and at E4CH (150-fold higher; $P = 0.024$) and in

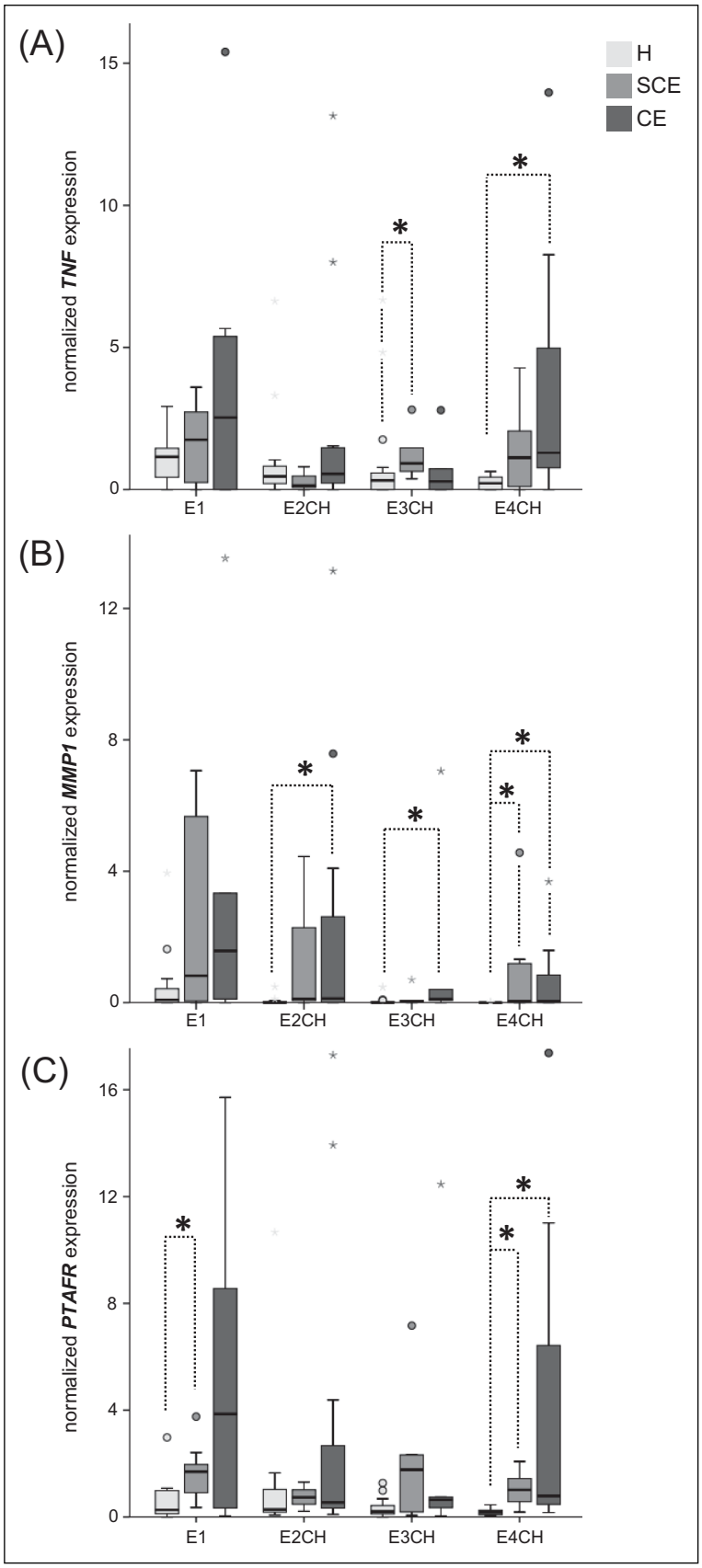

Fig. 6. Normalized mRNA expression of (A) *TNF*, (B) *MMP1* and (C) *PTAFR* in bovine endometrial cytobrush samples harvested from dairy cows depending on the current status of uterine health (H: healthy, SCE: subclinical endometritis, CE: clinical endometritis) on days 24-30 pp (E1), on days 31-37 pp (E2CH), on days 38-44 pp (E3CH) and on days 45-51 pp (E4CH). Bold asterisks over dotted lines indicate significant differences between the groups ($P < 0.05$). Extreme values are diagrammed as asterisks, outliers as circles. One extreme values is not shown for *MMP1* (107.35) obtained from a cow with CE at E1.

addition in SCE cows (180-fold higher; P = 0.047) at E4CH (*Fig. 6B*).

In comparison to the H group, *PTAFR* was twofold higher (P = 0.017) expressed in SCE cows at E1 (*Fig. 6C*). At E4CH, a direct correlation of *PTAFR* mRNA expression to the uterine health was observed with a higher expression in the endometrial epithelium in both SCE cows (fivefold higher; P = 0.001) and CE cows (25-fold higher; P = 0.003) in comparison to the H group.

Table 2. Significant correlation coefficients between the content of PMN with the mRNA expression of the selected factors as well as between each of the factors in endometrial cytobrush samples during days 24-51 pp presented as a matrix format ($P < 0.01$). When no value was indicated, no significant correlation was observed.

	PMN	*PTGS1*	*PTGS2*	*PTGIS*	*PTGES3*	*PTGDS*	*CXCL1/2*	*CXCR2*	*CXCL3*	*CXCL5*	*IL1A*	*IL1B*	*IL6*	*IL8*	*TNF*	*MMP1*	*PTAFR*
PMN			0.280			0.360	0.552	0.704	0.323	0.545	0.675	0.655	0.484	0.634	0.378	0.493	0.587
PTGS1				0.409		0.407											0.311
PTGS2	0.280					0.311	0.338	0.312	0.240	0.314	0.366	0.357	0.453	0.340	0.375	0.319	0.366
PTGIS		0.409				0.284					- 0.252	- 0.276		- 0.354			
PTGES3																	
PTGDS	0.360	0.407	0.311	0.284				0.420		0.420	0.306	0.402	0.237	0.258	0.323	0.350	0.500
CXCL1/2	0.552		0.338					0.802	0.694	0.779	0.807	0.803	0.610	0.835	0.483	0.471	0.718
CXCR2	0.704		0.312			0.420	0.802		0.462	0.738	0.791	0.877	0.598	0.843	0.473	0.506	0.740
CXCL3	0.323		0.240				0.694	0.463		0.618	0.526	0.487	0.413	0.509	0.436	0.368	0.467
CXCL5	0.545		0.314			0.420	0.779	0.738	0.618		0.702	0.718	0.523	0.699	0.553	0.500	0.696
IL1A	0.675		0.366	- 0.252		0.306	0.807	0.791	0.526	0.702		0.851	0.604	0.872	0.530	0.602	0.715
IL1B	0.655		0.357	- 0.276		0.402	0.803	0.877	0.487	0.718	0.851		0.645	0.887	0.476	0.515	0.660
IL6	0.484		0.453			0.237	0.610	0.598	0.413	0.523	0.604	0.645		0.624	0.370	0.485	0.484
IL8	0.634		0.340	- 0.354		0.258	0.835	0.843	0.509	0.699	0.872	0.887	0.624		0.459	0.552	0.608
TNF	0.378		0.375			0.323	0.483	0.473	0.436	0.553	0.530	0.476	0.370	0.459		0.389	0.591
MMP1	0.493		0.319			0.350	0.471	0.506	0.368	0.500	0.602	0.515	0.485	0.552	0.389		0.519
PTAFR	0.587	0.311	0.366			0.500	0.781	0.740	0.467	0.696	0.715	0.660	0.484	0.608	0.591	0.519	

Correlation analysis

Significant correlations are listed in *Table 2*. The percentage of PMN was highest correlated to mRNA expression of *CXCR2*, *IL1A*, *IL1B* and *IL8*. *PTGS1*, *PTGIS* and *PTGES3* were the only investigated factors whose mRNA expression did not show significant correlation to the number of PMN.

Especially the mRNA content of the factors that mainly attract PMNs showed high correlations among each other (*IL1A*, *IL1B*, *IL8*, *CXCL1/2* and *CXCR2*) with the highest correlation between *IL1B* and *IL8* mRNA expression. In contrast, the attractants for PMNs had none or only weak to moderate correlations to the enzymes of the synthesis of prostaglandins. *IL1A*, *IL1B* and *IL8* were negatively correlated to *PTGIS*.

The enzymes of the synthesis of PGs also showed either none or mostly only weak to moderate correlations among each other and to other factors. For *PTGES3*, no correlations at all could be observed. The only enzyme of PG synthesis that is correlated to all of the other groups (chemokines, interleukins, *TNF*, *MMP1* and *PTAFR*) is *PTGS2*.

DISCUSSION

The influence of the immune system on reproductive processes in the female was reported for many species (32-35). In the bovine, an upregulation of the local immune system for the clearance of invaded bacteria and lochia as well as for the repair of the epithelium is considered as a physiological process in the early postpartum period as part of involution of the uterus. This process often seems to be inefficient and the transition from a physiological to a more pathological status is associated with a shifted duration and severity of the immune response to later stages of the puerperium (10).

Several former studies already correlated the mRNA expression of pro-inflammatory factors to the uterine health, but mainly focused on only one point in time of the puerperium. Cows with clinical or subclinical endometritis had a higher mRNA expression of pro-inflammatory factors on days 21-27 pp (15, 19), on days 28-35 pp (36) and on days 28-41 pp (18). However, the present study design allowed a repeated sampling of the same cows. Therefore, it was possible to differentiate the expression patterns of pro-inflammatory factors at different points in time during a longer period of the puerperium (days 24-51 pp). This provided additional information especially about later stages of the puerperium.

The results show that in contrast to earlier points in time, almost all of the analyzed factors (except *PTGIS* and *PTGES3*) were affected by the uterine health status on days 45-51 pp. Interestingly, the fold changes were mostly higher if compared with earlier stages. This suggests that the severity of inflammation might differ during the time course of the puerperium. Clinical and subclinical endometritis on days 45-51 pp seem to cause a more severe inflammatory response than at earlier stages. This is supported by the findings of Galvao *et al.* (17) who also found an increase of *IL1B*, *IL6* and *IL8* mRNA expression in uterine biopsies from cows with endometritis around the same stage of the puerperium (week 7 after calving), even though there were no correlations to the uterine health at earlier stages. This implies that gene analysis around days 45-51 pp might be more informative when correlating pro-inflammatory factors to subclinical and/or clinical endometritis. When evaluating the effect of new applicable therapy strategies for bovine uterine diseases, this impact of the point in time of the puerperium on the mRNA expression pattern should be considered.

In this context, especially the enzymes for the PG synthesis seem to be of special interest. Although *PTGS2* mRNA expression showed a puerperal time-dependency with a significant peak on day 17 pp (14), another study did not reveal a dependency on the expression of *PTGS2* mRNA to the uterine health for the earlier puerperal stage (days 21-27 pp) (15). However, the results of the present study show that *PTGS1*, *PTGS2* and *PTGDS* were affected on days 45-51 pp. This suggests that the inflammatory response seems to be delayed on the part of PG production. Although enzymes of the synthesis of PGs did not show a significant dependency on the uterine health status on days 24-30 pp, the mRNA expression of *PTGDS* was affected on days 31-44 pp and of *PTGIS* on days 38-44 pp in cows with CE if referring to the initial grouping. Similar long-term effects failed to appear for most of the other analyzed factors. However, it has to be considered that cows with CE were injected with $PGF_{2\alpha}$ after the sampling on days 24-30 pp (E1) and on days 38-44 pp (E3). Therefore, an effect of the first $PGF_{2\alpha}$ injection on the increased mRNA expression of *PTGDS* and *PTGIS* cannot be excluded.

A special role among the PGs regarding reproductive events is awarded to PGE_2. A dysregulated too high production in combination with its luteotrophic effect (37) might lead to disrupted luteolysis and a prolonged luteal phase (38). High

concentrations of PGE_2 in the peripheral blood plasma, stimulated by the administration of a high dose of TNF, prolonged the estrous cycle in dairy cows (39). Extended postpartum luteal phases can be linked to uterine diseases (40), probably caused by the switch of the secretion from $PGF_{2\alpha}$ to PGE_2 after bacterial infection (41), whereas the absolute concentrations might be less decisive than the resulting higher $PGE_2/PGF_{2\alpha}$ ratio (42). Baranski *et al.* (43) found a significantly higher content of PGE_2 in cytobrush samples from cows with clinical endometritis on days 21-28 pp. Another study showed significantly higher concentrations of PGE_2 in the uterine fluid of cows with severe clinical endometritis compared with mild endometritis cases (44). In addition, a significantly stimulated PGE_2 output from endometrial tissue in the luteal phase or early pregnancy after the addition of pro-inflammatory IL1A as being a potent stimulator on PG production in bovine endometrial stromal cells was observed (45-47). In this context, peroxisome proliferator activated receptors were shown to be mediators for PGE_2 release from porcine endometrial explants harvested during the luteal phase of the estrous cycle or during the time of implantation (48).

However, a dependency on the mRNA expression of *PTGES3* on the uterine health status during the whole period (days 24-51 pp) was not observed in the present study. Thereby, it has to be considered that there are three different synthases for PGE_2. While PTGES1 can especially be linked to inflammation, female reproduction and tissue repair, PTGES2 and PTGES3 are more constitutive and unaffected by pro-inflammatory stimuli (49). Therefore, *PTGES1* mRNA expression analysis on days 24-51 pp should be of prospective interest.

The results of the microbiological analysis suggest that a sampling at a later stage of the puerperium is not expedient. On days 45-51 pp, almost no pathogenic bacteria at all could be detected, even if cows had purulent vaginal discharge. Therefore, no correlations to the health groups were found in strong contrast to the mRNA expression analysis. Consistent with the finding of Westermann *et al.* (50) who showed a significant increased number of samples positive for *T. pyogenes* in relation to increasing vaginal discharge score, *T. pyogenes* was found more often in cows with CE in the present study, but only on days 24-30 pp. Interestingly, cows positive for *T. pyogenes* mainly remained in either the SCE or CE group afterwards. In contrast, all cows positive for *E. coli* on days 24-30 pp switched to the H group. This is comparable to the results of Sens and Heuwieser (51) whose study revealed the higher odds of having >18% PMN on 21-27 days in milk (DIM) for cows with a *T. pyogenes* infection at 10 ± 1 DIM, but that an *E. coli* infection at 10 ± 1 DIM had no effect on the prevalence of SCE later in the puerperium. Thereby it has to be considered that the detected *E. coli* may belong to rather non-pathogenic strains than to endometrial pathogenic *E. coli* (52). In addition, there were also no bacteriological findings with significant prevalence for the SCE group. This supports the statement of McDougall *et al.* (53) that the quantification of the percentage of PMN in endometrial cytology is better suitable to predict reproductive performance than conventional intra-uterine bacteriology or the scoring of vaginal contents.

In conclusion, the attempt of revealing possible long-term effects of the initial uterine health status on days 24-30 pp on the subsequent mRNA expression pattern of the candidate genes showed effects on chemokines, *PTGDS* and *PTGIS*. If correlated to the current uterine health at each sampling, mRNA expression analysis of pro-inflammatory factors suggests a different severity of inflammatory response during days 24-51 pp with the strongest immune response at the later stage (on days 45-51 pp). In contrast, microbiological analysis of uterine samples was not meaningful around that time of the puerperium.

The present study contributes to a better understanding of inflammatory processes in the puerperal bovine endometrium, which might help with the development of new strategies of treatment for endometritis. However, the supposed differences in the severity of inflammatory response shown by the mRNA expression patterns should be substantiated by analyzing protein levels in a prospective study.

Acknowledgements: We thank the form (Groß Kreutz, Germany) for the opportunity to harvest the samples. We are also grateful to Christoph Holder for technical assistance.

This work was supported by the DFG (GA 1077/5-1), the Ministry of Infrastructure and Agriculture of the Federal State of Brandenburg and the Saxon State Office for the Environment, Agriculture and Geology.

Conflict of interests: None declared.

REFERENCES

1. Sheldon IM, Williams EJ, Miller AN, Nash DM, Herath S. Uterine diseases in cattle after parturition. *Vet J* 2008; 176: 115-121.
2. Gilbert RO, Shin ST, Guard CL, Erb HN, Frajblat M. Prevalence of endometritis and its effects on reproductive performance of dairy cows. *Theriogenology* 2005; 64: 1879-1888.
3. Baranski W, Podhalicz-Dziegielewska M, Zdunczyk S, Janowski T. The diagnosis and prevalence of subclinical endometritis in cows evaluated by different cytologic thresholds. *Theriogenology* 2012; 78: 1939-1947.
4. Barlund CS, Carruthers TD, Waldner CL, Palmer CW. A comparison of diagnostic techniques for postpartum endometritis in dairy cattle. *Theriogenology* 2008; 69: 714-723.
5. McDougall S, Macaulay R, Compton C. Association between endometritis diagnosis using a novel intravaginal device and reproductive performance in dairy cattle. *Anim Reprod Sci* 2007; 99: 9-23.
6. Singh J, Murray RD, Mshelia G, Woldehiwet Z. The immune status of the bovine uterus during the peripartum period. *Vet J* 2008; 175: 301-309.
7. Williams EJ, Fischer DP, Pfeiffer DU, *et al.* Clinical evaluation of postpartum vaginal mucus reflects uterine bacterial infection and the immune response in cattle. *Theriogenology* 2005; 63: 102-117.
8. Foldi J, Kulcsar M, Pecsi A, *et al.* Bacterial complications of postpartum uterine involution in cattle. *Anim Reprod Sci* 2006; 96: 265-281.
9. Sheldon IM, Cronin JG, Healey GD, *et al.* Innate immunity and inflammation of the bovine female reproductive tract in health and disease. *Reproduction* 2014; 148: R41-R51.
10. LeBlanc SJ. Interactions of metabolism, inflammation, and reproductive tract health in the postpartum period in dairy cattle. *Reprod Domest Anim* 2012; 47: 18-30.
11. Nathan C. Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol* 2006; 6: 173-182.
12. De Filippo K, Dudeck A, Hasenberg M, *et al.* Mast cell and macrophage chemokines CXCL1/CXCL2 control the early stage of neutrophil recruitment during tissue inflammation. *Blood* 2013; 121: 4930-4937.
13. Olson TS, Ley K. Chemokines and chemokine receptors in leukocyte trafficking. *Am J Physiol Regul Integr Comp Physiol* 2002; 283: R7-R28.
14. Gabler C, Fischer C, Drillich M, Einspanier R, Heuwieser W. Time-dependent mRNA expression of selected pro-inflammatory factors in the endometrium of primiparous cows postpartum. *Reprod Biol Endocrinol* 2010; 8: 152.

15. Fischer C, Drillich M, Odau S, Heuwieser W, Einspanier R, Gabler C. Selected pro-inflammatory factor transcripts in bovine endometrial epithelial cells are regulated during the oestrous cycle and elevated in case of subclinical or clinical endometritis. *Reprod Fertil Dev* 2010; 22: 818-829.
16. Moser B, Wolf M, Walz A, Loetscher P. Chemokines: multiple levels of leukocyte migration control. *Trends Immunol* 2004; 25: 75-84.
17. Galvao KN, Santos NR, Galvao JS, Gilbert RO. Association between endometritis and endometrial cytokine expression in postpartum Holstein cows. *Theriogenology* 2011; 76: 290-299.
18. Ghasemi F, Gonzalez-Cano P, Griebel PJ, Palmer C. Proinflammatory cytokine gene expression in endometrial cytobrush samples harvested from cows with and without subclinical endometritis. *Theriogenology* 2012; 78: 1538-1547.
19. Gabler C, Drillich M, Fischer C, *et al.* Endometrial expression of selected transcripts involved in prostaglandin synthesis in cows with endometritis. *Theriogenology* 2009; 71: 993-1004.
20. Prescott SM, Zimmerman GA, Stafforini DM, McIntyre TM. Platelet-activating factor and related lipid mediators. *Annu Rev Biochem* 2000; 69: 419-445.
21. Ahmed A, Dearn S, Shams M, *et al.* Localization, quantification, and activation of platelet-activating factor receptor in human endometrium during the menstrual cycle: PAF stimulates NO, VEGF, and FAKpp125. *FASEB J* 1998; 12: 831-843.
22. Ishii S, Nagase T, Shimizu T. Platelet-activating factor receptor. *Prostaglandins Other Lipid Mediat* 2002; 68-69: 599-609.
23. Salamonsen LA, Zhang J, Brasted M. Leukocyte networks and human endometrial remodelling. *J Reprod Immunol* 2002; 57: 95-108.
24. Sheldon IM, Lewis GS, LeBlanc S, Gilbert RO. Defining postpartum uterine disease in cattle. *Theriogenology* 2006; 65: 1516-1530.
25. Kasimanickam R, Duffield TF, Foster RA, *et al.* Endometrial cytology and ultrasonography for the detection of subclinical endometritis in postpartum dairy cows. *Theriogenology* 2004; 62: 9-23.
26. Versalovic J, Carroll KC, Funke G, Jorgensen JH, Landry ML, Warnock DW. Manual of Clinical Microbiology. Washington DC, American Society of Microbiology, 2011.
27. Odau S, Gabler C, Holder C, Einspanier R. Differential expression of cyclooxygenase 1 and cyclooxygenase 2 in the bovine oviduct. *J Endocrinol* 2006; 191: 263-274.
28. Huang ZQ, Fasco MJ, Kaminsky LS. Optimization of DNase I removal of contaminating DNA from RNA for use in quantitative RNA-PCR. *Biotechniques* 1996; 20: 1012-1014, 1016, 1018-1020.
29. Bustin SA, Benes V, Garson JA, *et al.* The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 2009; 55: 611-622.
30. Vandesompele J, De Preter K, Pattyn F, *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002; 3: research0034.
31. Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol* 2007; 8: R19.
32. Chelmonska-Soyta A, Ozgo M, Lepczynski A, *et al.* Proteome of spleen CD4 lymphocytes in mouse preimplantation pregnancy. *J Physiol Pharmacol* 2014; 65: 719-731.
33. Lee SK, Kim CJ, Kim DJ, Kang JH. Immune cells in the female reproductive tract. *Immune Netw* 2015; 15: 16-26.
34. Fair T. The contribution of the maternal immune system to the establishment of pregnancy in cattle. *Front Immunol* 2015; 6: 7.
35. Talbott H, Delaney A, Zhang P, *et al.* Effects of IL8 and immune cells on the regulation of luteal progesterone secretion. *Reproduction* 2014; 148: 21-31.
36. Kasimanickam R, Kasimanickam V, Kastelic JP. Mucin 1 and cytokines mRNA in endometrium of dairy cows with postpartum uterine disease or repeat breeding. *Theriogenology* 2014; 81: 952-958.
37. Magness RR, Huie JM, Hoyer GL, *et al.* Effect of chronic ipsilateral or contralateral intrauterine infusion of prostaglandin E_2 (PGE_2) on luteal function of unilaterally ovariectomized ewes. *Prostaglandins Med* 1981; 6: 389-401.
38. Sheldon IM, Price SB, Cronin J, Gilbert RO, Gadsby JE. Mechanisms of infertility associated with clinical and subclinical endometritis in high producing dairy cattle. *Reprod Domest Anim* 2009; 44 (Suppl. 3): 1-9.
39. Skarzynski DJ, Bah MM, Deptula KM, *et al.* Roles of tumor necrosis factor-alpha of the estrous cycle in cattle: an in vivo study. *Biol Reprod* 2003; 69: 1907-1913.
40. Opsomer G, Grohn YT, Hertl J, Coryn M, Deluyker H, de Kruif A. Risk factors for post partum ovarian dysfunction in high producing dairy cows in Belgium: a field study. *Theriogenology* 2000; 53: 841-857.
41. Herath S, Lilly ST, Fischer DP, *et al.* Bacterial lipopolysaccharide induces an endocrine switch from prostaglandin F_{2alpha} to prostaglandin E_2 in bovine endometrium. *Endocrinology* 2009; 150: 1912-1920.
42. Murakami S, Miyamoto Y, Skarzynski DJ, Okuda K. Effects of tumor necrosis factor-α on secretion of prostaglandins E_2 and $F_{2\alpha}$ in bovine endometrium throughout the estrous cycle. *Theriogenology* 2001; 55: 1667-1678.
43. Baranski W, Lukasik K, Skarzynski D, Sztachanska M, Zdunczyk S, Janowski T. Secretion of prostaglandins and leukotrienes by endometrial cells in cows with subclinical and clinical endometritis. *Theriogenology* 2013; 80: 766-772.
44. Mateus L, Lopes da Costa L, Diniz P, Ziecik AJ. Relationship between endotoxin and prostaglandin (PGE_2 and PGFM) concentrations and ovarian function in dairy cows with puerperal endometritis. *Anim Reprod Sci* 2003; 76: 143-154.
45. Tanikawa M, Lee HY, Watanabe K, *et al.* Regulation of prostaglandin biosynthesis by interleukin-1 in cultured bovine endometrial cells. *J Endocrinol* 2008; 199: 425-434.
46. Tanikawa M, Acosta TJ, Fukui T, *et al.* Regulation of prostaglandin synthesis by interleukin-1α in bovine endometrium during the estrous cycle. *Prostaglandins Other Lipid Mediat* 2005; 78: 279-290.
47. Majewska M, Woclawek-Potocka I, Bah MM, *et al.* Is interleukin-1α a luteotrophic or luteolytic agent in cattle? *Reproduction* 2010; 139: 665-672.
48. Bogacka I, Bogacki M, Gaglewska M, Kurzynska A, Wasielak M. In vitro effect of peroxisome proliferator activated receptor (PPAR) ligands on prostaglandin E_2 synthesis and secretion by porcine endometrium during the estrous cycle and early pregnancy. *J Physiol Pharmacol* 2013; 64: 47-54.
49. Kudo I, Murakami M. Prostaglandin E synthase, a terminal enzyme for prostaglandin E2 biosynthesis. *J Biochem Mol Biol* 2005; 38: 633-638.
50. Westermann S, Drillich M, Kaufmann TB, Madoz LV, Heuwieser W. A clinical approach to determine false positive findings of clinical endometritis by vaginoscopy by the use of uterine bacteriology and cytology in dairy cows. *Theriogenology* 2010; 74: 1248-1255.
51. Sens A, Heuwieser W. Presence of Escherichia coli, Trueperella pyogenes, alpha-hemolytic streptococci, and

coagulase-negative staphylococci and prevalence of subclinical endometritis. *J Dairy Sci* 2013; 96: 6347-6354.
52. Sheldon IM, Rycroft AN, Dogan B, *et al.* Specific strains of Escherichia coli are pathogenic for the endometrium of cattle and cause pelvic inflammatory disease in cattle and mice. *PLoS One* 2010; 5: e9192.
53. McDougall S, Hussein H, Aberdein D, *et al.* Relationships between cytology, bacteriology and vaginal discharge scores and reproductive performance in dairy cattle. *Theriogenology* 2011; 76: 229-240.
54. Gartner MA, Peter S, Jung M, Drillich M, Einspanier R, Gabler C. Increased mRNA expression of selected pro-inflammatory factors in inflamed bovine endometrium in vivo as well as in endometrial epithelial cells exposed to Bacillus pumilus in vitro. *Reprod Fertil Dev* 2015; Jan 7. doi: 10.1071/RD14219. (epub ahead of print)
55. Konnai S, Usui T, Ohashi K, Onuma M. The rapid quantitative analysis of bovine cytokine genes by real-time RT-PCR. *Vet Microbiol* 2003; 94: 283-294.
56. Wijayagunawardane MP, Gabler C, Killian G, Miyamoto A. Tumor necrosis factor α in the bovine oviduct during the estrous cycle: messenger RNA expression and effect on secretion of prostaglandins, endothelin-1, and angiotensin II. *Biol Reprod* 2003; 69: 1341-1346.
57. Bieser B, Stojkovic M, Wolf E, Meyer H, Einspanier R. Growth factors and components for extracellular proteolysis are differentially expressed during in vitro maturation of bovine cumulus-oocyte complexes. *Biol Reprod* 1998; 59: 801-806.

Received: September 17, 2014
Accepted: March 23, 2015

Author's address: Dr. Christoph Gabler, Institute of Veterinary Biochemistry, Freie Universitaet Berlin, 19B Oertzenweg, 14163 Berlin, Germany.
E-mail: Christoph.Gabler@fu-berlin.de