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Effect of metritis on endometrium tissue transcriptome during puerperium in Holstein lactating cows

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Abstract

The objective of this prospective cohort study was to evaluate the effect of parity and uterine health status postpartum on the gene expression profile of the endometrium early post-partum. Twenty-four Holstein cows were randomly selected (16 multiparous (MP) and 8 primiparous (PP)) and endometrium biopsies were collected on days 1, 3, and 6 after calving and clinically monitored for metritis. Rectal temperature was measured twice and fever was defined as a temperature ≥ 39.5°C. A case of metritis was diagnosed with the presence of red-brown watery, foul-smelling uterine discharge or a purulent discharge with more than 50% pus and fever between days 1 and 6 postpartum. Cows were then retrospectively selected (cows diagnosed with metritis were paired with healthy ones) to analyze the expression of 66 genes measured on the NanoString nCounter Analysis System. The genes selected were related with adhesion, immune system, steroid and prostaglandin biosynthesis regulation, insulin metabolism and transcription factors, and nutrient transporters. The results indicated a different pattern on genes related to immune function by parity. *PTX3,* involved in antigen presentation, was increased in healthy MP compared with healthy PP whereas inflammatory cytokine *TNF*α and complement-related protein *SERPING1* was upregulated in MP compared

with PP (P < 0.05). As expected, presence of a metritis condition affected the expression of genes related to immune function. There was an increased expression of the antiviral factor *MX2* and *MYH10* gene, which is involved in macrophages recruitment, in metritic compared with healthy cows ($P < 0.05$). Differences in uterine involution from cows diagnosed with metritis were reflected by the downregulation of *IGF1* ($P < 0.10$), involved in endometrium remodeling, and a possible compensatory upregulation of its receptor *IGFR1* ($P < 0.05$). A greater expression of prostaglandins and oxytocin receptors (*PGR* and *OXTR*), involved in the involution process, were observed in metritic PP compared with healthy PP ($P < 0.05$). Overall, it seems that metritis significantly modulate processes closely tied with the physical involution of the uterus early post-partum (*IGF1, IGFR1, PGR, OXTR*), whereas both metritis and multiparous cows tended to upregulate genes related to immune response (*PTX3, TNFα, SERPING1, MX2, MYH10*).

Keywords: Endometrium, metritis, nanostring, parity.

1. Introduction

Metritis is the inflammation of the uterus due to bacterial infection, occurring within 21 days (most commonly within 7 days) of parturition. It is characterized by systemic signs of sickness that can include all or a combination of fever, red-brown watery foul-smelling uterine discharge, dullness, inappetence, elevated heart rate, and low milk production [5]. The endometrium is the first line of defense of the uterus against microbial infections and the resolution of post-partum uterine infection and inflammation has been identified as one of the most important events needed to establishment a successful pregnancy in dairy cattle [1]. The innate immune response to

the bacteria is key to rapidly clear the infection [2]. Recruitment of hematopoietic immune cells and the inflammatory response, including secretion of chemokines and cytokines, all combine to clear the bacterial infection and restore homeostasis in the uterus [3]. It is known that the response of the immune system against bacteria (particularly lipopolysaccharide (LPS) as a major virulence factor of endometrial pathogenic *E. coli* (EnPEC) [4]) causing metritis, is stimulating TLR4-dependent inflammatory responses by endometrial cells. LPS-TLR4 binding activates NF-κB and leads to the secretion of proinflammatory cytokines and chemokines such as TNFα, IL-1β, or IL-8 [5]. Thus, it is key to evaluate the transcripts related with the immune system, but the environment of the endometrium during the first week postpartum is still not well understood. Several studies have been performed trying to understand uterine immunology at peri-partum. However, several physiological processes others than immune response coexist after calving and no study to our knowledge have assessed more broadly the expression profile of target genes during the first week after calving. The bovine uterus must undergo extensive remodeling after parturition in order to restore normal tissue architecture after expulsion of the calf and the placenta [1]. Similarly, the endometrium is also known for undergoing extensive tissue modification at various stages of pregnancy. For instance, during the pre-attachment phase of gestation, the interferon-tau produced by the conceptus induces an array of changes in the uterus by promoting the expression of interferon stimulated genes (ISG) [6]. These genes are related to cell remodeling, adhesion and invasion, cell orientation and polarization, angiogenesis, and transporters of glucose and lipids, which are indeed mostly upregulated by pregnancy and progesterone [7,8]. We hypothesized that similar transcripts are key to become differentially expressed during the puerperium of cows diagnosed or not with metritis. We also aimed to test the effect of parity in the current

study. Dairy heifers usually calve for the first time at 24 months of age as this maximizes the economic benefit [9], however, some of these animals might not be physically mature at this stage [10]. Cows approaching their first parturition have a different metabolic status [11] and a possibly different endometrial gene expression. Moreover, the uterus of primiparous cows has not been challenged by cellular stresses such as uterine involution, regeneration of the endometrium, elimination of bacterial contamination [12]. Thus, the objective of this study was to describe the modulation of the endometrium transcriptome in the first wk post-partum associated with metritis and parity. The gene expression analyses focused on endometrial transcripts from functional groups associated with extensive tissue remodeling, such as adhesion molecules, immune function, nutrient transporters, as well as steroids and prostaglandin biosynthesis.

2. Material and Methods

2.1 Animals, experimental design and uterine biopsies

A prospective cohort study was conducted in the facilities of the Dairy Research and Educational Centre from the University of British Columbia (UBC) in Agassiz, Canada. All experimental procedures were approved by the UBC Animal Care Committee. Cows were housed in free stall barns and fed a total mixed ration to meet or exceed the requirements for the fresh cows weighing 620 kg and producing 40 kg/day of 3.5% fat corrected milk (NRC, 2001).

Twenty-four Holstein cows from a group of 90 cows initially enrolled were randomly selected (16 multiparous and 8 primiparous) and endometrium biopsies were collected through a non-surgical process on days 1, 3, and 6 after calving. Every sick cow later diagnosed with metritis was retrospectively paired with a healthy one. An epidural anesthesia was provided using 100 mg of lidocaine (Lidocaine HCl 2%, Vetoquinol,

Lavaltrie, QC). The vulva was cleaned, and a disinfected guarded biopsy instrument (crocodile-type biopsy forceps, Aries Surgical, Davies, CA) was introduced via the cervix in the body of the uterus via vaginal (day 1) or *per rectum* (days 3 and 6) manipulations. Tissue collected was submerged in 500 μL of RNAlater (ThermoFisher Scientific, Cramlington, UK) and kept overnight at 4°C. Then RNAlater was removed and the tissue was stored at -80°C until further analysis.

2.2 Clinical observations and measurements

All cows were clinically monitored at days 1, 3, and 6 postpartum for metritis. Rectal temperature was measured twice at days 1, 3, and 6, and fever was defined as a temperature equal or greater than 39.5°C. A case of metritis was defined as a cow with a red-brown watery foul-smelling uterine discharge or a purulent discharge with more than 50% pus and fever on days 1 or 6 postpartum. The Metricheck device, a soft rubber hemisphere connected to a stainless steel rod, was inserted into the vaginal canal to assess the discharge. Vaginal discharge was evaluated after retracting the device caudally [13] and score from 1 to 4 was assigned. Score 1 was clear mucus, score 2 mucus containing flecks of white or of-white pus, score 3 exudate containing < 50% pus, and score 4 exudate containing > 50% pus).

2.3 RNA extraction

Total RNA was extracted from endometrial biopsies using total RNA isolation solution, Tri Reagent (Invitrogen, Carlsbad, CA, USA), and the commercial kit PureLink (Invitrogen, Carlsbad, CA, USA). The RNA was quantified using a Nanodrop 2000 instrument (Thermo scientific, Wilmington, DE, USA).

2.4 Analysis of gene expression

Twenty-four cows were selected to analyze the gene expression. Twelve of them had metritis, with 5 primiparous cows and 7 multiparous, while the gene expression of 12

healthy cows was analyzed (3 primiparous and 9 multiparous). The mRNA expression of 66 target transcripts (Table 1) from endometrial biopsy samples were measured on the NanoString nCounter Analysis System (NanoString Technologies, Seattle, WA, USA). The target mRNA (Supplementary material S1) was mixed in solution with a large excess of the reporter and capture probe pairs, so each targeted transcript found its corresponding probe pair. After hybridization, excess unbound probes were washed away and the tripartite complexes, comprising target mRNA bound to specific reporter-capture probe pairs, were isolated. The biotin level at the 3' end of the capture probes was used to attach the complexes to streptavidin-coated slides. An electric field was applied to orient and extend the tripartite complexes on the surface of the slide to facilitate imaging and detection of the color-coded molecules. A microscope objective and a CCD camera were then used to image the immobilized complexes using four different excitation wavelengths (480, 545, 580, and 622 nm) corresponding to the four fluorescent dyes. The different combinations of the four distinct colors allows for a large diversity of color-based barcodes, each designating a different gene transcript. The expression level of a gene is measured by counting the number of the specific barcode detected. The protocol was performed from start to finish, including hybridization processing and digital data acquisition, on the nCounter System.

2.5 Bioinformatics

To analyze the gene expression data, filtering of samples using quality control criteria was performed according to manufacturer's recommendations. Row counts of quality control-passed samples were normalized using four reference genes as internal controls (*GAPDH, ACTB, RPL19*, and *PGK1*).

2.6 Statistical analysis

Previous to statistical analysis, data were either log- or square root-transformed when necessary to achieve a normal distribution of the residuals. Results herein are expressed as the means of non-transformed ± SEM obtained with normalized data (except otherwise indicated). An ANOVA for repeated measures using proc MIXED of SAS (SAS 9.4, SAS Institute Inc., Cary, NC, USA) was used to analyze gene expression considering disease, day, and parity as fixed effects and animal as random. Tukey-Kramer's test was used for post hoc analysis to correct for family-wise error rate.

3. Results

3.1 Adhesion molecules

Fifteen genes related to adhesion were analyzed. *MYH10* tended to be more expressed in metritic than healthy cows (P = 0.07; Figure 1), while the gene expression of *MYL12A* tended to be higher in healthy than metritic cows (P = 0.07; Figure 1). The interaction between disease and time tended to be significant when *TIMP2* and *CADM3* gene expression was analyzed (Figure 2AB, P = 0.05, P = 0.07). Specifically, metritic cows reduced *TIMP2* expression on day 6 compared with day 1 and *CADM3* on day 3 compared with day 1. Five of the analyzed genes showed differences regarding sampling time (Table 2). *CLDN4* expression was reduced at day 6 compared with day 3 and day 1 (P < 0.01, 1.42, and 1.33-folds respectively), and *CADM3* expression was reduced at day 3 compared with day 1 (P = 0.02, 1.31-folds). The expression of *MYH10* increased by time, being different at day 6 (P = 0.01). *TIMP2* expression was decreased at day 3 and day 6 compared with day 1 (P < 0.01), and a tendency to decrease at day 3 is observed with *MYL12A* (P = 0.09).

Parity was analyzed and there were 5 genes that showed differences: in all cases, multiparous cows were upregulating the gene expression compared with primiparous cows (Table 3). *SERPING1* was upregulated 1.40-folds (P = 0.01), *CDH1*1.23-folds (P

= 0.01), *CADM3* 1.17-folds (P = 0.04), *MYH10* 1.09-folds (P = 0.01), and *TIMP2* 1.03-folds (P = 0.01). The analysis showed that the interaction between disease and parity was significant in the expression of *CLDN4*, *MHY10*, and *TIMP2* (Table 4). Healthy multiparous cows expressed 1.35-folds more *CLDN4* than metritic cows (P = 0.01, Table 4). No differences between healthy and metritic primiparous cows were observed (Table 1). The expression of *TIMP2* was increased 1.06-fold in multiparous healthy cows compared with primiparous healthy cows (P = 0.02). Metritic primiparous cows expressed 1.15-folds more *MHY10* than healthy primiparous cows.

3.2 Immune system

Seventeen genes related to the immune system were analyzed. Cows in the metritis group tended to over-express *MX2* compared with healthy cows (Figure 1, P = 0.06). Sampling time was significant in 11 of those genes. *IL6* gene expression increased approximately 2-fold between day 1 and day 6 (P = 0.03, Table 2). The mRNA fold changes in *TNF*α (1.8-fold), *IL1*β (1.6-fold), *CXCL8* (1.5-fold), and *PTX3* (1.4-fold) was lower at day 1 compared with day 3 and day 6 (P < 0.01). The gene expression of *IDO* was 1.6-folds greater on day 3 compared with day 1 (P < 0.05), whereas *ISG15* and *MX2* gene expression was reduced on day 6 compared with day 1 and day 3 (P < 0.01 and P = 0.02). The expression of *CXCL10* and *NFkB* was increased on day 3 compared with day 1 and day 6 (P < 0.01, 1.6, and 1.4-folds respectively). In the case of *SLP1* gene expression, there was a reduction on day 3 compared with day 6 of 1.6-fold (P = 0.02). The interaction between disease and sampling time was significant for *SLP1* (Figure 2F, P = 0.03); metritic cows had a reduction of *SLP1* gene expression at day 3 compared with day 1 and day 6. Parity influences the gene expression of *CXCL10*, *IDO*, *TRD* and *IL6* (Table 3). The mRNA fold change of *CXCL10*, *IDO*, and *TRD* was higher

in the endometrium of multiparous cows than primiparous cows (1.4, 1.4, 1.2-fold respectively, $P < 0.02$). On the other hand, *IL6* that tended to be more abundant in primiparous than multiparous cows (1.4-fold, $P = 0.09$). The interaction between disease and parity was significant for *PTX3, NFκB,* and *TNFα* (Table 4). *PTX3* gene expression was decreased in multiparous healthy cows compared with primiparous healthy cows ($P < 0.05$) while a tendency to increase *NFκB* expression in metritic multiparous cows compared with metritic primiparous cows was observed ($P = 0.09$). Healthy multiparous cows tended ($P = 0.08$) to express more *TNFα* than healthy primiparous cows.

3.3 Steroid and prostaglandin biosynthesis regulation

Fourteen genes related to steroid and prostaglandin biosynthesis regulation were analyzed. No differences were observed in the expression of any gene regarding disease. Sampling time affected the expression of *CYP3A4, PGR, OXTR, HPGD* and *ERα* (table 2). The expression of *CYP3A4* was up-regulated by time 2.3-fold whereas *PGR* and *OXTR* were down-regulated 1.7 and 1.3-fold, respectively. On the other hand, *HPGD* was down-regulated at day 1 compared with day 3 and day 6 (1.3-fold). Gene expression of *ERα* was down-regulated on day 3 compared with day 1, but not modified on day 6. The interaction between disease and time tended to be significantly expressed in the genes *PGR,* and *ERα* (P=0.04) but no differences were observed between healthy and metritic cows at the different sampling times (Figure 2 CD). Parity influenced the gene expression of *PGR* and *ERα* (Table 3), multiparous cows expressed more the mRNA of those genes than primiparous cows (1.3 and 1.1-fold, respectively). Finally, when we analyzed the interaction between disease and parity, the gene expression of *PGR, OXTR,* and *ERα* was modified (Table 4). In all cases, healthy multiparous cows

225 overexpressed the genes compared with healthy primiparous cows (1.8, 1.2, 1.1-fold,
226 respectively) while no differences were observed between primiparous and multiparous
227 cows with metritis. *PGR* and *OXTR* were up-regulated in primiparous metritic cows
228 compared with primiparous healthy cows (1.5 and 1.3-fold respectively).
229
230 *3.4 Insulin metabolism and transcription factors*
231 Eleven genes related to transcription factors were analyzed. *IGFR1* was significantly
232 increased in metritic cows compared with healthy cows while *IGF1* tended to be
233 downregulated in metritic cows (Figure 1, $P = 0.03$ and 0.08, respectively). When the
234 interaction between disease and time was analyzed, there were differences in the
235 expression of *IGFR1* and a tendency in *IGFBP1* and *HOX10A*. The expression of
236 *IGFPB1* was down-regulated on day 3 compared with day 1 (1.4-fold, $P = 0.05$) in
237 metritic cows, and 2.15-fold the expression of *HOX10A* from day 1 to day 6 ($P = 0.09$).
238 The gene expression of *IGFR1* on day 1 in metritic cows was up-regulated compared
239 with healthy cows on day 1 (Figure 2E, $P = 0.03$), and no differences between healthy
240 and metritic cows were observed on day 3 or day 6. The sampling time modified the
241 expression of *IGF1*, *IGFBP1*, *IGFBP3* (1.6, 1.2, and 1.1-fold respectively, Table 2), all
242 genes were down-regulated by time. Furthermore, the expression of *SGK1* on day 3
243 tended to be down-regulated compared with that on day 1 ($P = 0.05$). Primiparous cows
244 down-regulated the gene expression of *IGFR1*, *DGKA*, and *SGK1* compared with
245 multiparous cows (Table 3). The interaction between disease and parity modified the
246 expression of *IGFR1*, *IGF1*, *HOX10A*, and *SGKA* (Table 4). Metritic primiparous cows
247 expressed more *IGFR1* and *HOX10A* than healthy primiparous cows (1.6 and 1.2-fold,
248 respectively). On the other hand, multiparous healthy cows expressed more *IGF1* (1.3-

fold) than multiparous metritic cows and also expressed more *SGK1* than primiparous healthy animals (1.1-fold).

3.5 Nutrient transporters

Six genes encoding nutrient transporters were analyzed. *TC1* tended to be less expressed in metritic cows compared with healthy cows (Figure 1, $P = 0.10$). Sampling time affected the expression of *TC1* and *SLC2A5* (Table 2). *TC1* expression was down-regulated with time, and *SLC2A5* tended to decrease with time. No differences in any gene were observed for the interaction between disease and sampling time. Parity did not alter the expression of the analyzed genes but an interaction between disease and parity was observed with *TC1* gene expression (Table 4). Healthy multiparous cows expressed more *TC1* in the endometrium than metritic multiparous cows.

4. Discussion

The uterine environment at different stages of gestation is still a topic of extensive research because of its key importance to improve embryonic survival, calving conditions, and uterine health status. Understanding the differences in endometrial gene expression might allow us to better understand how the endometrium works under different conditions. Modification in the expression of transcripts related to the immune system, steroid and prostaglandin biosynthesis and to other major functional groups associated with uterine involution (e.g. nutrient transporters and insulin metabolism) caused by metritis, sampling time and parity was tested in the present study.

It is interesting to observe how small intervals between sampling times change the expression of the genes. In the case of genes related with the immune system we observed similar curves in most of the genes. Same pattern was observed for *IL6*,

TNFα, *IL1β*, *CXCL8*. When the transcription factor *NFκB* increases on day 3 the signaling cascade starts up-raising the expression of the pro-inflammatory cytokines and chemokines. This seems to indicate that the immune system does not react to the pathogens until day 3. Interestingly, no differences were found in the expression of these cytokines between metritic and healthy cows so early after parturition contrary to what has been observed later on [14].

The results indicated a different pattern on genes related to the immune function by parity. Pentraxin-related protein (*PTX3)* binds with high affinity to TNF-stimulated gene 6 (*TSG-6*) and facilitates pathogen recognition by macrophages and dendritic cells. Based on that, we expected to observe the same increase or decrease expression pattern on both genes. On the contrary, we observed a decrease of *PTX3* in multiparous healthy cows compared with primiparous healthy cows, whereas *TNFα* (tumor necrosis factor α) was expressed 1.8-fold more in healthy multiparous cows than in healthy primiparous cows. It has been reported that an increased endometrial expression of *PTX3* may lead to a recruitment and/or activation of macrophages and dendritic cells enhancing a feedback effect on *PTX3* expression [15]. However, no differences have been observed between healthy and metritic cows.

Serpin protease inhibitor G1 (SERPING1) encodes a highly-glycosylated plasma protein involved in the regulation of the complement cascade. It has been demonstrated that *SERPING1* is over-expressed in atretic follicles compared with healthy follicles [16]. We have also found this gene was up-regulated in multiparous cows compared with primiparous cows (Table 3). Similarly, C-X-C motif chemokine 10 (CXCL10) has shown to exhibit antimicrobial properties [17] in addition to be involved in cell-regulating the embryo-maternal recognition [18]. Levels of *CXCL10* increases in intrauterine tissues during human labor compared with those in the absence of labor

[19]. In this study, we found an up-regulation of this gene in multiparous cows compared with primiparous cows (Table 3). Indoleamine 2,3-dioxygenase (IDO1) is produced by immunosuppressive macrophages in response to IFNγ and prevents the proliferation of local T cells population [20]. In this study we observed an increment of its expression in multiparous cows compared with primiparous cows. T cell receptor delta (TRDC) protein contributes to the gamma delta (γδ) chain of T cells, that increase during pregnancy and play a role in regulating maternal immune function in the uteri [21]. The upregulation of *TRDC* is beneficial due to the important role of γδ T cells in enabling early embryonic implantation by inducing maternal immune tolerance to the fetus [20]. In this study, we observed an upregulation of the *TRDC* gene in multiparous cows compared with primiparous cows (Table 3). Primiparous cows tended to express more *IL6* than multiparous cows (Table 3). *IL6* is a typical marker for inflammation but in this study, we did no find differences between metritic and healthy cows (though numerically higher in metritic cows), probably because the size sample was not enough. It is quite hypothetical at this point for concrete conclusions about what the differences observed between cows that calved for the first time compared with older animals mean to subsequent fertility. Considering that primiparous cows are inherently different (i.e. metabolic challenge, previous calving exposure, more likely to suffer from dystocia and uterine disease, but more likely to conceive at first breeding) when going for their first calving, it is interesting to observe that a few important genes have its expression modified. Coincidentally, *SERPING1*, *IDO1* and *TRDC* are all immune modulators and upregulated in multiparous cows. This finding could suggest that specific immune cell activity or population number is altered in older animals.

As expected, metritis incidence affected gene expression pattern related to immune function with an increased expression of the anti-viral myxovirus resistance 2 (*MX2*) in

metritic cow (Figure 1) [22]. On the other hand, it has been found overexpressed in the endometrium of cows with severe negative energy balance, which may cause a delay in the effective immune response to the microbial challenge experienced after calving [23]. MYH10 is a non-muscle myosin involved on the regulation of cytokinesis, cell motility, and cell polarity. Regarding cell motility, it plays a role in normal adherens junction integrity and structure. This gene has been found upregulated in blood from pregnant cows being related with macrophages motility towards the endometrium [24]. Accordingly, we observed a tendency of *MYH10* to be increased in the endometrium of metritic cows (Figure 2), an increment in multiparous cows compared with primiparous (Table 3) and a tendency for a reduction of the gene expression of *MYH10* in primiparous healthy cows compared with multiparous healthy cows (Table 4).

It is known that there is an increment in negative energy balance (NEB) in postpartum cows as they cannot consume sufficient energy-yielding nutrients from voluntary dry matter intake (DMI) to meet energetic requirements for milk production. Consequently, NEB occurs for a period of days to weeks during early lactation [25]. Fat reserves are moved allowing glucose to be redirected for fetal metabolism and lactose synthesis [26]. Those metabolic adaptations lead to insulin resistance, a physiological condition where the body tissues have lower response to insulin [27]. In normal conditions, growth hormone (GH) binds to growth hormone receptor (GHR) in the liver, increasing IGF1. This results in the synthesis of pancreatic insulin that acts in the tissues to promote the glucose uptake except in the mammary gland where the glucose flows independently of insulin [28]. Near parturition, feed intake is reduced and GHR expression, and consequently IGF1, decrease avoiding the feedback against GH secretion. Circulating IGF1 is mostly bound to high affinity IGF binding proteins, which protect the hormone from proteolysis and modulate its interaction with the IGFR1 [29].

We observed differences in the expression of genes related with uterus involution. IGFR1 is a transmembrane receptor that is activated by IGF1 and by a related hormone IGF2. The receptor mediates the effects of IGF1 and it is thought to support the regression and growth of the uterine tissue during estrous cycle and throughout the regenerative processes in women following menstruation [30]. It may also play a role during uterus involution after calving. Differences in uterus involution with metritis were reflected by downregulation of *IGF1*, involved in endometrium remodeling, and a compensatory upregulation of its receptor *IGFR1* in metritic cows compared with healthy cows specially on day 1 postpartum (Figure 2, 3). It has been seen that IGF1 production increases during the wound healing process [31], stimulating the proliferation of the epithelia and the stroma during uterine involution [30]. In this study, we observed that *IGF1* is downregulated through time (Table 2).

The gene expression of *IGFBP1, IGFBP2,* and *IGFBP3* (insulin growth factor binding proteins) was not affected by lactation number or disease. IGFBP1 is related to cell migration and metabolism whereas IGFBP3 may regulate local IGF1 bioavailability [32] or transport IGFs through the cell layer for secretion into the uterine lumen [33]. *IGFBP1* and *IGFBP3* were overexpressed in the endometrium on day 1 after calving compared with day 3 and day 6.

It is known, that the uterine OXTR increases at calving in all mammalian species tested to date, including cows [34]. In the cow endometrium during pregnancy, oxytocin stimulates $PGF_{2\alpha}$ formation, which increases with gestation and correlates with oxytocin receptor binding [34]. The oxytocin receptor interacts directly with the myometrium stimulating uterine contractions. Prostaglandins (PG) regulate leukocyte function and have a role in the mechanisms of parturition, the expulsion of the placenta, and postpartum uterine involution [35]. A greater expression of receptors of prostaglandins

and oxytocin (*PGR* and *OXTR*), involved in involution processes, were observed in metritic primiparous compared with healthy primiparous ($P < 0.05$). It has been hypothesized that increasing the expression of *OXTR* in postpartum uterine cells may help in managing incomplete uterine involution [36]. It is known that in mammals, signaling oxytocin via *OXTR* in the uterus results in the initiation of parturition [37]. Consequently, we observed an increase in the expression of *PGR* and *OXTR* on d1 after parturition compared with d3 and d6.

5. Conclusions

In conclusion, there are important differences of the endometrium transcriptomes between the metritic and healthy cows. An over-expression of *IGFR1* in metritic cows may suggest a compensatory effect caused by the downregulation of *IGF1*. *MYH10* and *MX2* tend to be up-regulated in metritic cows while *MYL12A* and *TC1* tend to be increased in healthy cows. The gene expression in the endometrium during the first week postpartum also differs between primiparous and multiparous cows with main differences related to the immune system and tissue involution and remodeling. *SERPING1*, *IGFR1*, *CXCL10*, *IDO*, *PTX3*, *TNFα*, *PGR*, and *OXTR* are the transcripts with the greatest fold-change modifications caused by parity. Some key gene expression changes were found between the biopsy collection days. The substantial remodeling of the uterus does require specific timing for sample collection and correct interpretation of gene expression results. Overall these results reflect the effect of metritis in involution and immune response along with the parity influence in post calving status of the animal.

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530 **Table 1:** List of tested genes in different functional groups.

Function	Genes
Adhesion Molecules	*MMP19, CLDN4, GLYCAM1, TIMP2, SPP1, LGALS3BP, SERPING1, EMMPRIN, CDH1, MYH9, MYH10, MYL12A, CADM3, MUC4, MUC5B, MUC1*
Immune System	*IGLL1, SELL, CXCL10, PTX3, TRD, MX2, IL10, IDO, LIFR, ISHG1, SLPI, LYZ2, UHRF1, CXCL8, IL1β, TNFα, NFκB,* β Defensins, *B3GAT1*
Steroid and prostaglandin biosynthesis and regulation	*WISP2, OXYTOCIN, PTGES, CYP3A4, CYP4X1, CYP4F2, OXTR, PGR, ERα, ERβ, PFKFB2, PTGES2, HPGD, MOGAT1*
Insulin metabolism and transcription factors	*IGFR1, IGFBP1, IGFBP2, IGFBP3, NNMT, HOXA10, CALB2, NR1I2, IGF1, SGK1, DGKA*
Nutrient transporters	*FOLR1, TC1, SLC27A6, SLC5A6, SLC2A5, SLC7A10*

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534 **Table 2**: Relative expression of genes related to adhesion, immune system, steroid and
535 prostaglandin biosynthesis regulation, and insulin metabolism at days 1, 3, and 6 after
536 calving from endometrial biopsies. Means within a row with different subscripts differ
537 at $P < 0.05$.

		mean ± SEM			
Group	**Gene**	**d1**	**d3**	**d6**	***P* value**
Adhesion	*CLDN4*	1.417 ± 0.08a	1.328 ± 0.09a	0.994 ± 0.08b	0.001
	CADM3	9.835 ± 0.55a	7.501 ± 0.59b	8.901 ± 0.56ab	0.017
	MYH10	2.369 ± 0.05b	2.484 ± 0.06ab	2.606 ± 0.05a	0.011
	TIMP2	8.957 ± 0.08a	8.654 ± 0.09b	8.597 ± 0.08b	0.001
	MYHL12	2595.132 ± 196.31	1998.590 ± 211.39	2521.111 ± 199.81	0.088
Immune System	*IL6*	1.992 ± 0.48b	3.574 ± 0.51ab	3.803 ± 0.49a	0.029
	TNFa	4.560 ± 0.88b	8.252 ± 0.93a	8.253 ± 0.89a	0.009
	IL1B	1.748 ± 0.19b	2.868 ± 0.21a	3.011 ± 0.19a	<.0001
	CXCL10	1.843 ± 0.18b	2.932 ± 0.19a	2.067 ± 0.18b	0.001
	IDO	1.478 ± 0.19b	2.327 ± 0.20a	1.791 ± 0.19ab	0.016
	SLPI	2.484 ± 0.22ab	1.768 ± 0.24b	2.738 ± 0.23a	0.020
	CXCL8	2.353 ± 0.21b	3.46 ± 0.22a	3.325 ± 0.21a	0.001
	PTX3	3.918 ± 0.41b	5.539 ± 0.44a	5.522 ± 0.41a	0.006
	NFKB	16.150 ± 1.00b	22.142 ± 1.07a	18.072 ± 0.99b	<.0001
	ISG15	6.229 ± 0.25a	6.571 ± 0.27a	5.155 ± 0.25b	0.005
	MX2	2.681 ± 0.09a	2.734 ± 0.10a	2.36 ± 0.09b	0.016
Steroid and prostaglandin biosynthesis regulation	*CYP3A4*	7.216 ± 0.98a	16.764 ± 1.07b	14.764 ± 0.99b	<.0001
	PGR	21.526 ± 1.02a	12.718 ± 1.11b	12.880 ± 1.02b	<.0001
	OXTR	6.820 ± 0.17a	5.656 ± 0.19b	5.102 ± 0.17b	<.0001
	HPGD	3.020 ± 0.19b	3.820 ± 0.21a	3.781 ± 0.19a	0.007
	ERα	6.851 ± 0.13b	6.322 ± 0.14b	6.668 ± 0.13ab	0.024
Insulin metabolism and	*IGF1*	28.470 ± 1.09a	18.540 ± 1.18b	16.449 ± 1.09b	<.0001
	IGFBP1	2.314 ± 0.12a	1.940 ± 0.13b	1.932 ± 0.12b	0.037
	IGFBP3	3.781 ± 0.08a	3.303 ± 0.09b	3.290 ± 0.08b	<.0001

transcription factor	*SGK1*	5.922 ± 0.07	5.675 ± 0.07	5.795 ± 0.07	0.054
Nutrient transporters	*TC1*	3.901 ± 0.09a	3.784 ± 0.10ab	3.499 ± 0.09b	0.009
	SLC2A5	4.552 ± 0.24	3.563 ± 0.26	3.652 ± 0.24	0.088

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Table 3: Relative expression of genes related to adhesion, immune system, steroid and prostaglandin biosynthesis regulation, and insulin metabolism by parity from endometrial biopsies. Relative units of gene expression (mean ± SEM) for parity. Mean within a row with different subscripts differ at $P < 0.05$.

		Mean ± SEM parity			
Group	**Gene**	**primiparous**	**multiparous**	***P* value**	**fold change**
adhesion	*SERPING1*	1201.398 ± 126.18b	1683.336 ± 99.91a	0.004	1.40
	CDH1	5.856 ± 0.59	7.196 ± 0.45	0.082	1.23
	CADM3	8.065 ± 0.51b	9.429 ± 0.41a	0.042	1.17
	MYH10	2.384 ± 0.08b	2.588± 0.06a	0.002	1.09
	TIMP2	8.611 ± 0.07b	8.861 ± 0.07a	0.015	1.03
Immune system	*CXCL10*	1.895 ± 0.17b	2.669 ± 0.14a	0.002	1.41
	IDO	1.583 ± 0.19b	2.178 ± 0.155a	0.023	1.38
	IL6	3.631 ± 0.45	2.616 ± 0.35	0.087	1.39
	TRD	2.029 ± 0.13b	2.437 ± 0.11a	0.025	1.20
Steroid and prostaglandin biosynthesis regulation	*PGR*	13.724 ± 0.96b	17.692 ± 0.76a	0.002	1.29
	ERα	6.393 ± 0.12b	6.835 ± 0.10a	0.006	1.07
Insulin metabolism and transcription factors	*IGFR1*	9.096 ± 0.62b	11.754 ± 0.49a	0.013	1.29
	DGKA	7.686 ± 0.45b	9.100 ± 0.35a	0.016	1.18
	SGK1	5.631 ± 0.06b	5.964 ± 0.05a	0.001	1.06

546 **Table 4**: Relative expression of genes related with adhesion, immune system, steroid
547 and prostaglandin biosynthesis regulation, insulin metabolism, and nutrient transport by
548 parity (primiparous / multiparous) and disease (healthy/metritic) from endometrial
549 biopsies. Relative units of gene expression (mean ± SEM) for parity. Mean within a row
550 with different subscripts differ at $P < 0.05$.

		mean ± SEM				
		primiparous		**multiparous**		
Group	**Gene**	**healthy**	**metritic**	**healthy**	**metritic**	***P* value**
adhesion	*CLDN4*	1.148 ± 0.11ab	1.336 ± 0.10 ab	1.398 ± 0.08a	1.034 ± 0.09b	0.005
	MYH10	2.258 ± 0.08b	2.584 ± 0.05a	2.494 ± 0.07a, b(t)	2.584 ± 0.06a	0.070
	TIMP2	8.500 ± 0.11b	8.684 ± 0.10a	8.986 ± 0.08a(t), b	8.726 ± 0.09ab	0.021
Immune system	*TNFa*	4.605 ± 1.16a(t)	8.493 ± 1.16a	8.123 ± 0.79a (t)	6.865 ± 1.02a	0.021
	PTX3	5.845 ± 0.52a	4.738 ± 0.45ab	4.014 ± 0.38b	5.201 ± 0.43ab	0.017
	NFkB	19.313 ± 1.28	17.126 ± 1.13t	18.924 ± 0.94	20.930 ± 1.05t	0.072
Steroid and prostaglandine biosynthesis regulation	*PGR*	11.204 ± 1.43b	16.266 ± 1.26a	19.592 ± 0.99a	15.792 ± 1.15ab	0.006
	OXTR	5.286 ± 0.24b	6.617 ± 0.21a	6.245 ± 0.17a	5.740 ± 0.19ab	0.001
	ERα	6.194 ± 0.18b	6.591 ± 0.16ab	6.988 ± 0.12a	6.682 ± 0.14ab	0.025
Insulin metabolism and transcription factors	*IGFR1*	7.122 ± 0.92b	11.070 ± 0.82a	12.019 ± 0.64a	11.490 ± 0.74a	0.006
	IGF1	20.898 ± 1.53ab	22.070 ± 1.35ab	23.717 ± 1.06a	17.928 ± 1.22b	0.01
	HOX10A	3.764 ± 0.27b	4.531 ± 0.24ab	4.709 ± 0.19a	4.103 ± 0.22ab	0.002
	SGK1	5.521 ± 0.09b	5.742 ± 0.08b	6.032 ± 0.07a	5.895 ± 0.08ab	0.031
Nutrient transporters	*TC1*	3.689 ± 0.13	3.704 ± 0.11	3.960 ± 0.09t	3.570 ± 0.10t	0.073

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Figure 1: Gene expression fold change in metritic cows in relation to healthy ones. Bars with asterisk differ ($P < 0.05$), and with t ($P < 0.10$) between metritic and healthy cows. Genes represented are *MYH10, MYL12A, IGFR1, TC1, MX2,* and *IGF1*.

Figure 2: Gene expression of healthy cows (light grey) versus metritic ones (dark grey) at different sampling times. *TIMP2* relative gene expression (A), *CADM3* relative gene expression (B), *PGR* relative gene expression (C), *ER*α relative gene expression (D), *IGFR1* relative gene expression (E), *SLPI* relative gene expression (F), *IGFPB1* (G), and *HOX10A* (H). Bars represent mean ± SEM for the different groups. Bars with different letters differ ($P < 0.05$).

Figure 1

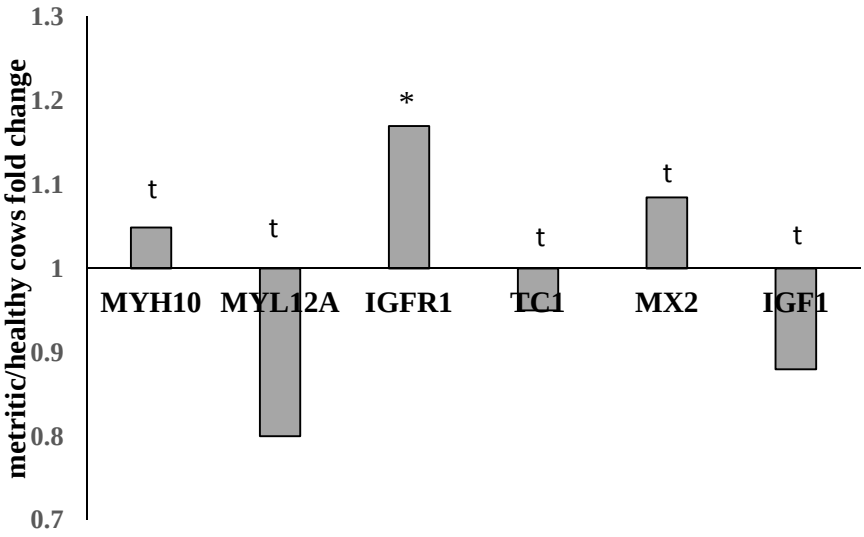
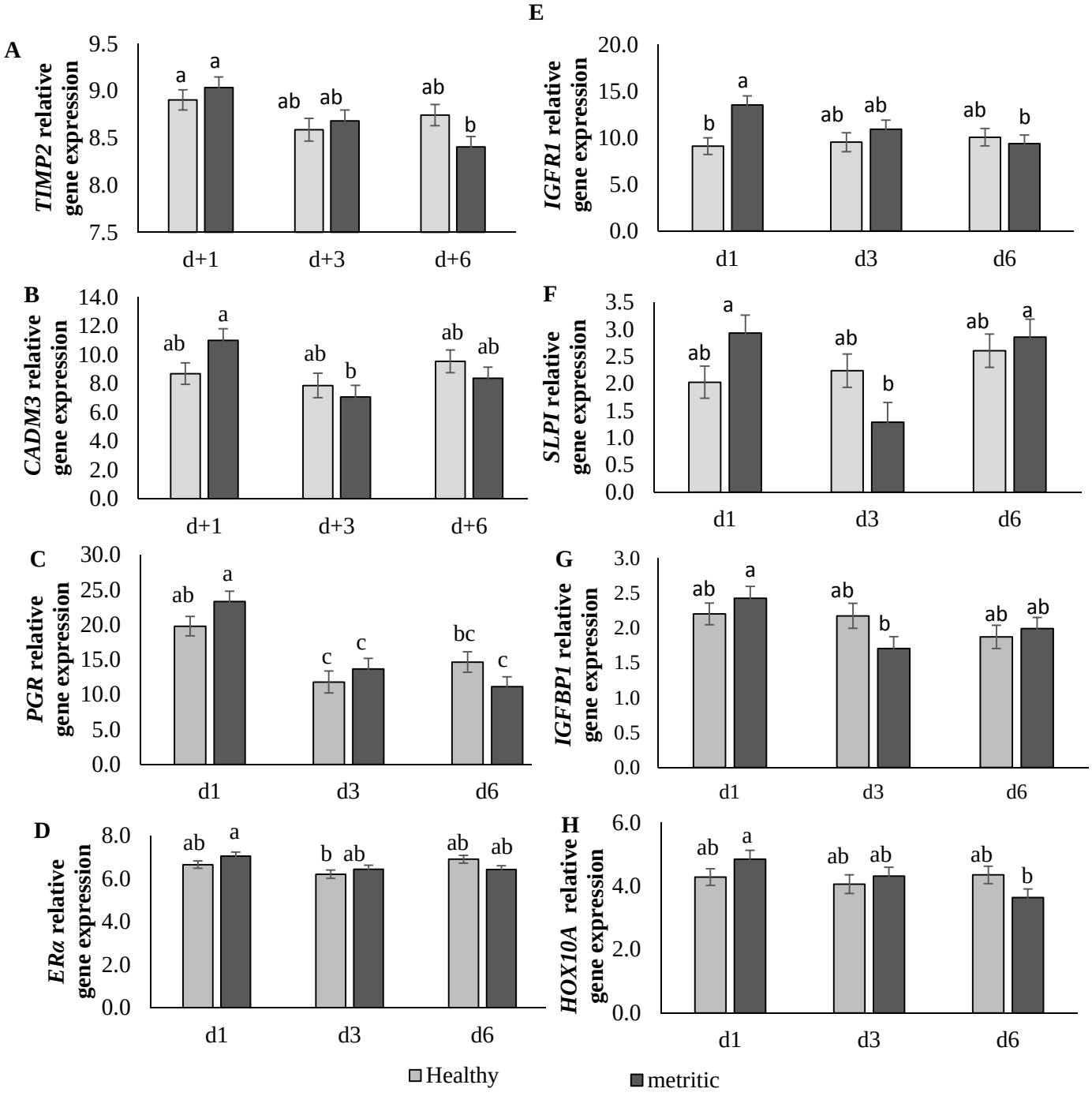


Figure 2


A
TIMP2 relative gene expression
9.5
9.0
8.5
8.0
7.5
a a ab ab ab b
d+1 d+3 d+6
B
CADM3 relative gene expression
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
ab a ab b ab ab
d+1 d+3 d+6
C
PGR relative gene expression
30.0
25.0
20.0
15.0
10.0
5.0
0.0
ab a c c bc c
d1 d3 d6
D
ERα relative gene expression
8.0
6.0
4.0
2.0
0.0
ab a b ab ab ab
d1 d3 d6
E
IGFR1 relative gene expression
20.0
15.0
10.0
5.0
0.0
b a ab ab ab b
d1 d3 d6
F
SLPI relative gene expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ab a ab b ab a
d1 d3 d6
G
IGFBP1 relative gene expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ab a ab b ab ab
d1 d3 d6
H
HOX10A relative gene expression
6.0
4.0
2.0
0.0
ab a ab ab ab b
d1 d3 d6
Healthy
metritic

Highlights:

1. Metritic cows downregulate *IGF1* compared with healthy cows, and there is a compensatory upregulation of its receptor *IGFR1*.

2. There is a greater expression of *PGR* and *OXTR*, involved in involution processes, in metritic primiparous cows than in healthy primiparous cows.

3. Metritis incidence affected gene expression pattern related to the immune function.