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

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9 **Abstract**

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

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

39

40 **Keywords:** Endometrium, metritis, nanostring, parity.

41

42 1. Introduction

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

51 the bacteria is key to rapidly clear the infection [2]. Recruitment of hematopoietic
52 immune cells and the inflammatory response, including secretion of chemokines and
53 cytokines, all combine to clear the bacterial infection and restore homeostasis in the
54 uterus [3]. It is known that the response of the immune system against bacteria
55 (particularly lipopolysaccharide (LPS) as a major virulence factor of endometrial
56 pathogenic *E. coli* (EnPEC) [4]) causing metritis, is stimulating TLR4-dependent
57 inflammatory responses by endometrial cells. LPS-TLR4 binding activates NF- κ B and
58 leads to the secretion of proinflammatory cytokines and chemokines such as TNF α , IL-
59 1 β , or IL-8 [5]. Thus, it is key to evaluate the transcripts related with the immune
60 system, but the environment of the endometrium during the first week postpartum is
61 still not well understood. Several studies have been performed trying to understand
62 uterine immunology at peri-partum. However, several physiological processes others
63 than immune response coexist after calving and no study to our knowledge have
64 assessed more broadly the expression profile of target genes during the first week after
65 calving. The bovine uterus must undergo extensive remodeling after parturition in order
66 to restore normal tissue architecture after expulsion of the calf and the placenta [1].
67 Similarly, the endometrium is also known for undergoing extensive tissue modification
68 at various stages of pregnancy. For instance, during the pre-attachment phase of
69 gestation, the interferon-tau produced by the conceptus induces an array of changes in
70 the uterus by promoting the expression of interferon stimulated genes (ISG) [6]. These
71 genes are related to cell remodeling, adhesion and invasion, cell orientation and
72 polarization, angiogenesis, and transporters of glucose and lipids, which are indeed
73 mostly upregulated by pregnancy and progesterone [7,8]. We hypothesized that similar
74 transcripts are key to become differentially expressed during the puerperium of cows
75 diagnosed or not with metritis. We also aimed to test the effect of parity in the current

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

88 2. Material and Methods

89 2.1 Animals, experimental design and uterine biopsies

90 A prospective cohort study was conducted in the facilities of the Dairy Research and
91 Educational Centre from the University of British Columbia (UBC) in Agassiz, Canada.
92 All experimental procedures were approved by the UBC Animal Care Committee.
93 Cows were housed in free stall barns and fed a total mixed ration to meet or exceed the
94 requirements for the fresh cows weighing 620 kg and producing 40 kg/day of 3.5% fat
95 corrected milk (NRC, 2001).
96 Twenty-four Holstein cows from a group of 90 cows initially enrolled were randomly
97 selected (16 multiparous and 8 primiparous) and endometrium biopsies were collected
98 through a non-surgical process on days 1, 3, and 6 after calving. Every sick cow later
99 diagnosed with metritis was retrospectively paired with a healthy one. An epidural
100 anesthesia was provided using 100 mg of lidocaine (Lidocaine HCl 2%, Vetoquinol,

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

107 *2.2 Clinical observations and measurements*

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

118 *2.3 RNA extraction*

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

123 *2.4 Analysis of gene expression*

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

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

144 *2.5 Bioinformatics*

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

149 *2.6 Statistical analysis*

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

157 3. Results

158 3.1 Adhesion molecules

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

172 Parity was analyzed and there were 5 genes that showed differences: in all cases,
173 multiparous cows were upregulating the gene expression compared with primiparous
174 cows (Table 3). *SERPING1* was upregulated 1.40-folds ($P = 0.01$), *CDH11* 1.23-folds (P

175 = 0.01), *CADM3* 1.17-folds ($P = 0.04$), *MYH10* 1.09-folds ($P = 0.01$), and *TIMP2* 1.03-
176 folds ($P = 0.01$). The analysis showed that the interaction between disease and parity
177 was significant in the expression of *CLDN4*, *MHY10*, and *TIMP2* (Table 4). Healthy
178 multiparous cows expressed 1.35-folds more *CLDN4* than metritic cows ($P = 0.01$,
179 Table 4). No differences between healthy and metritic primiparous cows were observed
180 (Table 1). The expression of *TIMP2* was increased 1.06-fold in multiparous healthy
181 cows compared with primiparous healthy cows ($P = 0.02$). Metritic primiparous cows
182 expressed 1.15-folds more *MHY10* than healthy primiparous cows.

183

184 3.2 Immune system

185 Seventeen genes related to the immune system were analyzed. Cows in the metritis
186 group tended to over-express *MX2* compared with healthy cows (Figure 1, $P = 0.06$).
187 Sampling time was significant in 11 of those genes. *IL6* gene expression increased
188 approximately 2-fold between day 1 and day 6 ($P = 0.03$, Table 2). The mRNA fold
189 changes in *TNF α* (1.8-fold), *IL1 β* (1.6-fold), *CXCL8* (1.5-fold), and *PTX3* (1.4-fold)
190 was lower at day 1 compared with day 3 and day 6 ($P < 0.01$). The gene expression of
191 *IDO* was 1.6-folds greater on day 3 compared with day 1 ($P < 0.05$), whereas *ISG15*
192 and *MX2* gene expression was reduced on day 6 compared with day 1 and day 3 ($P <$
193 0.01 and $P = 0.02$). The expression of *CXCL10* and *NF κ B* was increased on day 3
194 compared with day 1 and day 6 ($P < 0.01$, 1.6, and 1.4-folds respectively). In the case of
195 *SLP1* gene expression, there was a reduction on day 3 compared with day 6 of 1.6-fold
196 ($P = 0.02$). The interaction between disease and sampling time was significant for *SLP1*
197 (Figure 2F, $P = 0.03$); metritic cows had a reduction of *SLP1* gene expression at day 3
198 compared with day 1 and day 6. Parity influences the gene expression of *CXCL10*, *IDO*,
199 *TRD* and *IL6* (Table 3). The mRNA fold change of *CXCL10*, *IDO*, and *TRD* was higher

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

209

210 *3.3 Steroid and prostaglandin biosynthesis regulation*

211 Fourteen genes related to steroid and prostaglandin biosynthesis regulation were
212 analyzed. No differences were observed in the expression of any gene regarding
213 disease. Sampling time affected the expression of *CYP3A4*, *PGR*, *OXTR*, *HPGD* and
214 *ERα* (table 2). The expression of *CYP3A4* was up-regulated by time 2.3-fold whereas
215 *PGR* and *OXTR* were down-regulated 1.7 and 1.3-fold, respectively. On the other hand,
216 *HPGD* was down-regulated at day 1 compared with day 3 and day 6 (1.3-fold). Gene
217 expression of *ERα* was down-regulated on day 3 compared with day 1, but not modified
218 on day 6. The interaction between disease and time tended to be significantly expressed
219 in the genes *PGR*, and *ERα* ($P=0.04$) but no differences were observed between healthy
220 and metritic cows at the different sampling times (Figure 2 CD). Parity influenced the
221 gene expression of *PGR* and *ERα* (Table 3), multiparous cows expressed more the
222 mRNA of those genes than primiparous cows (1.3 and 1.1-fold, respectively). Finally,
223 when we analyzed the interaction between disease and parity, the gene expression of
224 *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 *IGFBP1* 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-

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

251

252 3.5 Nutrient transporters

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

261

262 4. Discussion

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

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

274 *TNF α* , *IL1 β* , *CXCL8*. When the transcription factor *NF κ B* increases on day 3 the
275 signaling cascade starts up-raising the expression of the pro-inflammatory cytokines and
276 chemokines. This seems to indicate that the immune system does not react to the
277 pathogens until day 3. Interestingly, no differences were found in the expression of
278 these cytokines between metritic and healthy cows so early after parturition contrary to
279 what has been observed later on [14].

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

291 Serpin protease inhibitor G1 (*SERPING1*) encodes a highly-glycosylated plasma
292 protein involved in the regulation of the complement cascade. It has been demonstrated
293 that *SERPING1* is over-expressed in atretic follicles compared with healthy follicles
294 [16]. We have also found this gene was up-regulated in multiparous cows compared
295 with primiparous cows (Table 3). Similarly, C-X-C motif chemokine 10 (*CXCL10*) has
296 shown to exhibit antimicrobial properties [17] in addition to be involved in cell-
297 regulating the embryo-maternal recognition [18]. Levels of *CXCL10* increases in
298 intrauterine tissues during human labor compared with those in the absence of labor

299 [19]. In this study, we found an up-regulation of this gene in multiparous cows
300 compared with primiparous cows (Table 3). Indoleamine 2,3-dioxygenase (IDO1) is
301 produced by immunosuppressive macrophages in response to $\text{IFN}\gamma$ and prevents the
302 proliferation of local T cells population [20]. In this study we observed an increment of
303 its expression in multiparous cows compared with primiparous cows. T cell receptor
304 delta (TRDC) protein contributes to the gamma delta ($\gamma\delta$) chain of T cells, that increase
305 during pregnancy and play a role in regulating maternal immune function in the uteri
306 [21]. The upregulation of *TRDC* is beneficial due to the important role of $\gamma\delta$ T cells in
307 enabling early embryonic implantation by inducing maternal immune tolerance to the
308 fetus [20]. In this study, we observed an upregulation of the *TRDC* gene in multiparous
309 cows compared with primiparous cows (Table 3). Primiparous cows tended to express
310 more *IL6* than multiparous cows (Table 3). *IL6* is a typical marker for inflammation but
311 in this study, we did not find differences between metritic and healthy cows (though
312 numerically higher in metritic cows), probably because the size sample was not enough.
313 It is quite hypothetical at this point for concrete conclusions about what the differences
314 observed between cows that calved for the first time compared with older animals mean
315 to subsequent fertility. Considering that primiparous cows are inherently different (i.e.
316 metabolic challenge, previous calving exposure, more likely to suffer from dystocia and
317 uterine disease, but more likely to conceive at first breeding) when going for their first
318 calving, it is interesting to observe that a few important genes have its expression
319 modified. Coincidentally, *SERPING1*, *IDO1* and *TRDC* are all immune modulators and
320 upregulated in multiparous cows. This finding could suggest that specific immune cell
321 activity or population number is altered in older animals.

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

324 metritic cow (Figure 1) [22]. On the other hand, it has been found overexpressed in the
325 endometrium of cows with severe negative energy balance, which may cause a delay in
326 the effective immune response to the microbial challenge experienced after calving
327 [23]. *MYH10* is a non-muscle myosin involved on the regulation of cytokinesis, cell
328 motility, and cell polarity. Regarding cell motility, it plays a role in normal adherens
329 junction integrity and structure. This gene has been found upregulated in blood from
330 pregnant cows being related with macrophages motility towards the endometrium [24].
331 Accordingly, we observed a tendency of *MYH10* to be increased in the endometrium of
332 metritic cows (Figure 2), an increment in multiparous cows compared with primiparous
333 (Table 3) and a tendency for a reduction of the gene expression of *MYH10* in
334 primiparous healthy cows compared with multiparous healthy cows (Table 4).

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

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

361 The gene expression of *IGFBP1*, *IGFBP2*, and *IGFBP3* (insulin growth factor binding
362 proteins) was not affected by lactation number or disease. IGFBP1 is related to cell
363 migration and metabolism whereas IGFBP3 may regulate local IGF1 bioavailability
364 [32] or transport IGFs through the cell layer for secretion into the uterine lumen [33].

365 *IGFBP1* and *IGFBP3* were overexpressed in the endometrium on day 1 after calving
366 compared with day 3 and day 6.

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

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

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382 5. Conclusions

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

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

Function	Genes
Adhesion Molecules	<i>MMP19, CLDN4, GLYCAM1, TIMP2, SPP1, LGALS3BP, SERPING1, EMMPRIN, CDH1, MYH9, MYH10, MYL12A, CADM3, MUC4, MUC5B, MUC1</i>
Immune System	<i>IGLL1, SELL, CXCL10, PTX3, TRD, MX2, IL10, IDO, LIFR, ISHG1, SLPI, LYZ2, UHRF1, CXCL8, IL1β, TNFα, NFκB, β Defensins, B3GAT1</i>
Steroid and prostaglandin biosynthesis and regulation	<i>WISP2, OXYTOCIN, PTGES, CYP3A4, CYP4X1, CYP4F2, OXTR, PGR, ERα, ERβ, PFKFB2, PTGES2, HPGD, MOGAT1</i>
Insulin metabolism and transcription factors	<i>IGF1, IGFBP1, IGFBP2, IGFBP3, NNMT, HOXA10, CALB2, NR112, IGF1, SGK1, DGKA</i>
Nutrient transporters	<i>FOLR1, TC1, SLC27A6, SLC5A6, SLC2A5, SLC7A10</i>

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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 $P < 0.05$.

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

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

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

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

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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 \pm SEM) for parity. Mean within a row
 550 with different subscripts differ at $P < 0.05$.

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

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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), *IGFBP1* (G), and *HOX10A* (H). Bars represent mean \pm SEM for the different groups. Bars with different letters differ ($P < 0.05$).

Figure 1

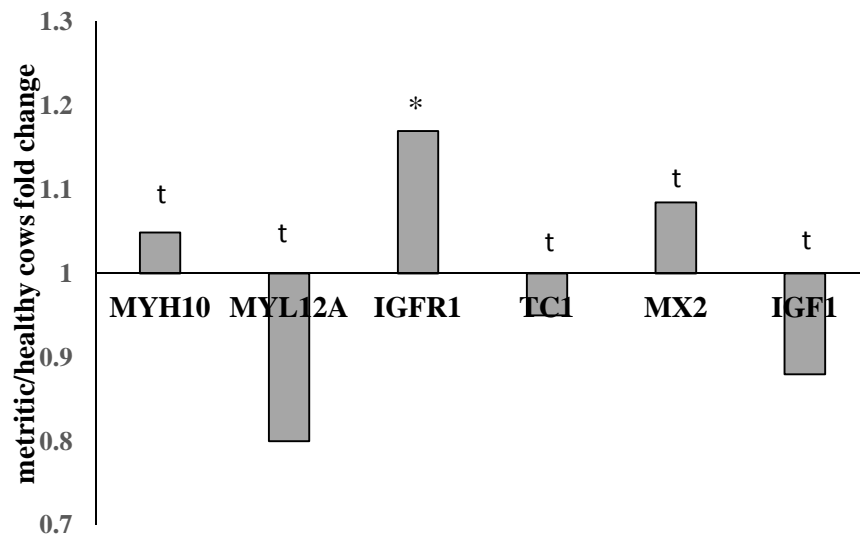
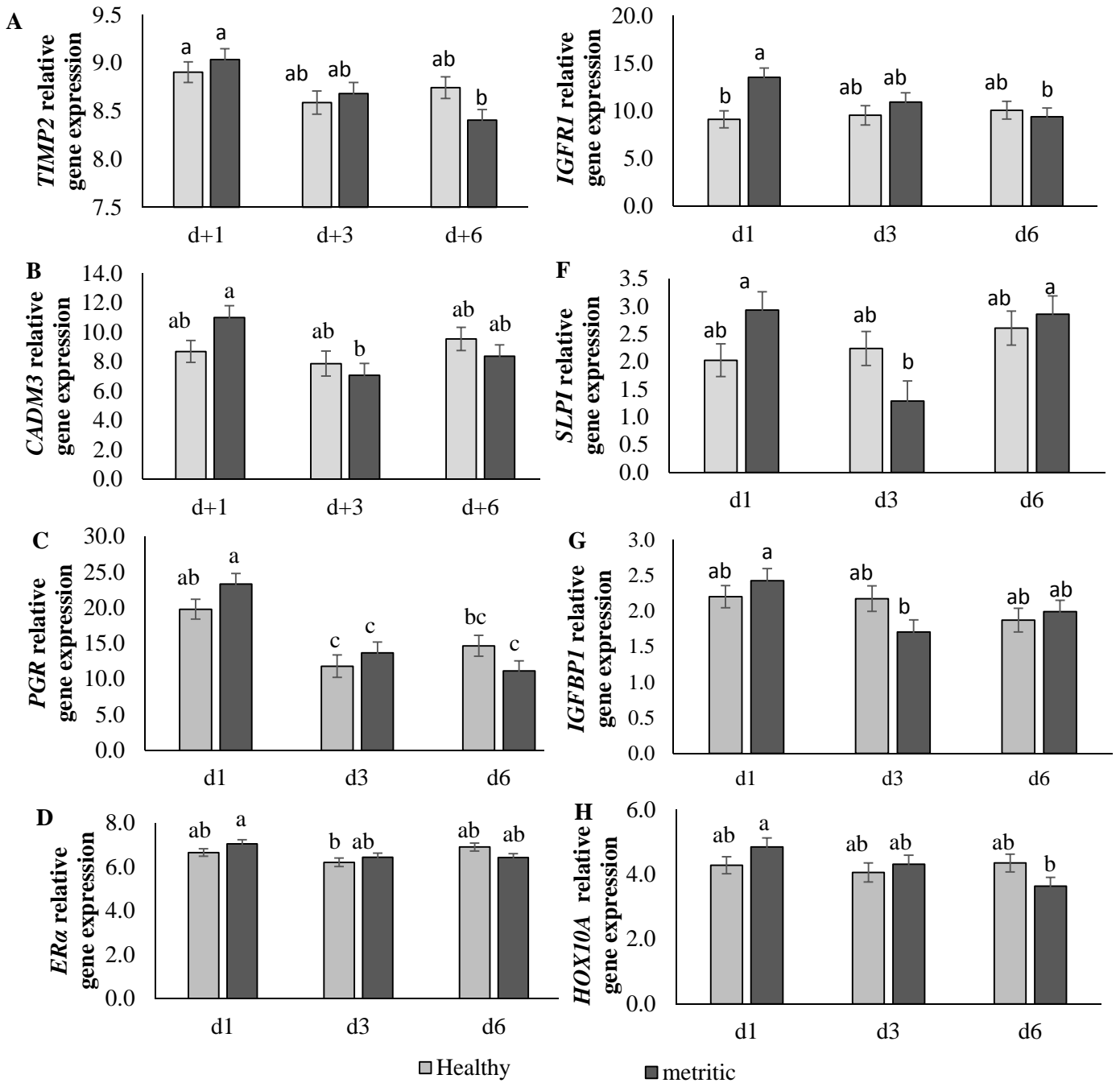


Figure 2



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.