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Transcriptomic analysis of peripheral leukocytes in dairy cows with and without evidence of metritis and associated early postpartum disease

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ABSTRACT

Objective: The aim of this study was to describe associations between peripheral leukocyte gene expression and early postpartum disease in dairy cows with clinical metritis.

Materials and Methods: The study was conducted in a conventional dairy in Washington state. Diseased cows ≤14 DIM were enrolled (n = 11) based on evidence of clinical metritis with or without comorbidity or prior treatments. Healthy cows (n = 9) were selected based on comparative DIM and lactation numbers to diseased cows. Blood was collected for a complete blood count, serum biochemistry, leukocyte isolation, and RNA extraction at enrollment and twice more at 6- to 8-d intervals.

Results and Discussion: A total of 34,625 genes were considered and 55 were associated with health status by the Boruta all-relevant feature machine learning selection algorithm. These were filtered to the 15 most representative genes within the diseased cohort as compared with the healthy cohort. The top ranked gene during every rank or error test was *PGLYRP1*. Its associated protein, peptidoglycan recognition protein 1, and other immunomodulatory molecules associated with this study selectively enhance or alter host innate immune defense mechanisms and modulate pathogen-induced inflammatory responses.

Implications and Applications: Host-defense peptides such as those associated with this study provide insight into individual and population-level markers of disease resilience that may help improve therapeutics, guide genetic selection, and clarify the burden of disease on animal well-being. This is particularly relevant to the dairy cow transition period with its metabolic and physiologic changes and associated increase in disease susceptibility.

Key words: white blood cells, metritis, postpartum, transcriptome, PGLYRP1

INTRODUCTION

Postpartum reproductive diseases negatively affect reproductive performance, productive success, and well-being of dairy cows. Bacterial contamination of the uterus is ubiquitous in the dairy cow following calving, and a substantial proportion of cows develop uterine disease including metritis (Sheldon et al., 2009; Ribeiro et al., 2013). Specifically, clinical metritis has been defined by gradients of severity. Grade 1 metritis is attributed to animals that are within 21 d postpartum and not systemically ill but have an abnormally enlarged uterus and purulent uterine discharge detectable in the vagina. Animals with grade 2 metritis have an abnormally enlarged uterus, purulent uterine discharge, and additional signs of systemic illness, such as decreased milk yield, dullness, and pyrexia. Grade 3 metritis (puerperal or toxic metritis) is an acute systemic illness defined as an abnormally enlarged uterus with a purulent or fetid watery red-brown uterine discharge, associated with signs of toxemia (e.g., inappetence, tachycardia, cold extremities, or depression) usually within 10 d after parturition (Sheldon et al., 2006; Sheldon et al., 2019). The type and severity of uterine disease depends on the immune response of the cow, as well as the species and number (load or challenge) of bacteria (Sheldon et al., 2006; Galvão et al., 2019; Pascottini and LeBlanc, 2020). Specifically, *Escherichia coli*, *Trueperella pyogenes*, *Prevotella* spp., *Fusobacterium necrophorum*, *Bacteroidetes* spp., and *Firmicutes* spp. have been identified as predominant taxa associated with postpartum uterine disease. However, these bacteria also have been identified in the uterus of healthy cows (Santos et al., 2011; Jeon et al., 2015), suggesting that uterine disease is likely dependent on the overgrowth of certain bacteria and the responsiveness of the immune system (Moore et al., 2017).

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Several risk factors have been associated with metritis including dystocia, twinning, stillbirth, retained fetal membranes, displaced abomasum, and ketosis (Bruun et al., 2002; Sheldon et al., 2006; Potter et al., 2010). Many such risk factors are associated with compromising the protective mucosa of the endometrium, and metabolic challenges such as postpartum negative energy balance may further weaken the metabolically expensive immune function (Ingvartsen and Moyes, 2015; Gilbert and Santos, 2016; Kvidera et al., 2017). The postpartum innate immune system has been studied extensively and data suggest that the postpartum functionality of the innate immune response in general, and neutrophil function in particular, is associated with the development of uterine disease (Martinez et al., 2012; Pinedo et al., 2013; Bromfield et al., 2018). Neutrophilic activity may be impaired by reduced activation, chemotaxis, adherence, pathogen ingestion, respiratory burst, and release of lytic enzymes (Aleri et al., 2016; Sordillo, 2016). Although the role of peripheral blood mononuclear cells in dairy cow uterine disease has been less well investigated, cows with active postpartum uterine infection may have alterations in the proportions and functionalities of mononuclear leukocytes in general, and various peripheral lymphocyte subsets specifically (Aleri et al., 2016; Bromfield et al., 2018).

Whether observed changes in peripheral blood leukocytes of cows with postpartum uterine disease are a cause of the pathogenesis or a consequence of the uterine inflammatory process is in need of further investigation (Düvel et al., 2014; Bromfield et al., 2018). Regardless, interactions between metabolic stress, inflammation, and immune function exist during the transition period and can affect the immune system including changes to the expression of key genes (Madsen et al., 2002; Sordillo and Aitken, 2009; Sordillo et al., 2009). Transcriptional changes in metabolically stressed periparturient dairy cows have been shown to be consistent with altered purinergic signaling and reduced neutrophil function, likely increasing the susceptibility to infectious disease (Seo et al., 2013; Crookenden et al., 2019). Altered expression profiles of neutrophil genes related to trafficking, migration, adhesion, energy metabolism, inflammatory mediators, cell survival, and apoptosis have been associated with the demands of parturition alone (Madsen et al., 2004; Burton et al., 2005; Crookenden et al., 2019) and in concert with uterine disease (Düvel et al., 2014; Pathak et al., 2015). Given that neutrophils are mediators of the inflammatory response and the main leukocyte type involved in bacterial clearance (Galvão et al., 2012), their function is closely associated with the outcome of peripartal conditions such as uterine infection (Hammon et al., 2006). However, neutrophils account for only a proportion of circulating leukocytes at calving (Paape et al., 2003; Trevisi et al., 2010), and changes in monocyte and macrophage gene expression also affect immunomodulation through the production and release of cytokines and chemokines (Galvão et al., 2012; Heiser et al., 2015). In fact, major differences have been shown in monocyte cytokine gene expression between cows with uterine disease and healthy controls (Galvão et al., 2012; Düvel et al., 2014; Bromfield et al., 2018).

There remains much to learn regarding the role of leukocytes in modulating the periparturient immune response. Integrative approaches at the molecular, cellular and animal levels may help explain the complex interactions between physiologic disturbances and immune function associated with periparturient diseases. Furthermore, enhancing our understanding of the inflammatory pathways at the molecular level and their effects on immune function could improve our ability to predict, prevent, and treat transition cow disorders (Esposito et al., 2014). Although significant gene expression changes naturally occur as an adaptation to the demands of lactation, maintenance, and uterine involution, specifically designed gene expression studies in target tissues from healthy and diseased animals can be used to prioritize candidate genes and identify novel biomarkers affecting disease (Fang et al., 2017). The aim of this study was to describe associations between peripheral leukocyte gene expression and early postpartum disease in dairy cows as evidenced by clinical metritis. In an effort to cast a wide net reflecting the fluidity of change related to metabolic stress, inflammation, and immune function, healthy cows were compared against those with uterine disease and assorted physiologic differences, comorbidities, and treatments. Our overall goal was to assess specific disease-associated changes in gene expression bridging the continuum of disease severity and resolution affecting homeorhetic regulation during the early postpartum period.

MATERIALS AND METHODS

Experimental Design and Clinical Assessments

The Washington State University (**WSU**) Institutional Animal Care and Use Committee (Pullman, WA) approved all animal manipulations associated with this study (ASAF#05061). This fixed cohort field study was conducted during June 2018, on a high-producing (average 12,780 kg of 305-d mature equivalent milk production), conventional dairy in the state of Washington with an inventory of approximately 7,500 lactating cows. All cows were housed in open dry lots with shaded loafing areas, fed a TMR, and milked twice daily. Cows within the early postpartum period up to approximately 21 DIM were evaluated by herdsmen daily for evidence of clinical disease [i.e., retained fetal membranes (**RFM**), metritis, displaced abomasum, and so on] and treated based on farm protocols.

Requirements for sample size were calculated using a negative binomial distribution suited to modeling count data such as RNA-Seq, and capable of accounting for both biological and technical variability (Hart et al., 2013). This equated to an estimate of a minimum of 5 samples within each group (diseased, healthy) based on a significance

level of 0.05, power of 80%, conservative 2-fold estimate of effect size, 30 million read depth of sequencing for a given transcript, and a conservative 0.35 CV for RNA-Seq counts within comparative groups of a relatively inbred dairy population.

Initial enrollment of eligible Holstein-Friesian cows occurred on June 4, 6, and 7, 2018. Eligible cows were ≤14 DIM and evaluated by a WSU veterinarian and veterinary student assistant for clinical or puerperal metritis as defined previously (Sheldon et al., 2006). Briefly, metritis was initially suspected based on fetid vaginal discharge and confirmed using rectal palpation to expel bloody, red-brown, milky, thin or watery discharge from an enlarged uterus. Cows (n = 11) with metritis were enrolled into the diseased cohort on the day of the initial clinical evaluation by WSU personnel during the first 14 DIM, regardless of the date of diagnoses by on-farm personnel, comorbidity, or previous treatment history. Cows (n = 9) without metritis or any other clinical disease were enrolled into the healthy cohort to provide comparisons to those cows with metritis based on equivalent DIM and lactation number. Healthy cows had no history of disease during the current lactation and no evidence of exclusionary systemic infectious or metabolic disease based on ensuing bloodwork. All enrolled diseased and healthy cows were clinically evaluated on the day of enrollment and twice more by the WSU veterinarian and student at 6- to 8-d intervals to evaluate vulvar discharge, rectal temperature, and BHB levels (CentriVet GK, ACON Laboratories Inc., San Diego, CA).

Blood Sampling

At the time of the 3 clinical evaluations, blood was collected from the coccygeal blood vessels for leukocyte isolation (~10 mL/cow), complete blood counts (**CBC**; ~10 mL/cow), and haptoglobin measurement (~10 mL/cow). Blood for a serum biochemistry panel (~10 mL/cow) was collected only at the time of the initial assessment. Blood for leukocyte isolation and CBC evaluation was collected in Covidien Monoject coated EDTA evacuated tubes with lavender stoppers (Fisher Scientific, Waltham, MA). Blood for haptoglobin and serum biochemistry analysis was collected in Covidien Monoject silicone-coated evacuated tubes with red stoppers (Fisher Scientific). All tubes were inverted multiple times and placed immediately on top of ice until further processing. Samples for CBC and biochemistry were kept chilled until submission to the WSU College of Veterinary Medicine clinical pathology laboratory within 6 h of collection.

Haptoglobin

Blood to be analyzed for haptoglobin concentrations was processed within 2 h of collection. Tubes were allowed to settle at room temperature for 30 min before centrifugation at 2,750 × *g*. Serum then was aspirated off the clot, placed in 2-mL microcentrifuge tubes, and held at −80°C until further processing via a bovine haptoglobin ELISA kit (Innovative Research Inc., Novi, MI). Serum samples were initially diluted 1/500. Concentrations above the standard curve required additional dilutions of up to 1/50,000, to ascertain values relative to a reference interval (≤140 μg/mL) established at the Kansas State University veterinary diagnostic laboratory.

Latent Class Analysis

Latent class analysis (**LCA**) was performed using R (R Project for Statistical Computing, https://www.r-project.org/) and package poLCA to identify unique classes of animals with shared clinical and physiologic parameters based on categories for normal or abnormal using reference intervals established at the WSU clinical pathology laboratory (CBC) and Kansas State University (haptoglobin). All observations from enrolled cows were used (n = 60 observations), and variables used to inform the models included rectal temperature, BHB levels, CBC data, and serum haptoglobin levels. Latent class analysis is a statistical method that uses observed categorical responses to identify underlying latent or "unobserved" groups of individuals or objects that share certain characteristics (Collins and Lanza, 2010).

Leukocyte RNA Extraction

Isolation of leukocytes from whole blood was accomplished using a modified Ficoll-Paque separation with a short red blood cell lysis stage. Using a sterile serological pipette, 7 mL of whole blood was placed into a sterile 50-mL Nunc conical sterile polypropylene centrifuge tube (Fisher Scientific) containing 7 mL dPBS(−) (Maqbool et al., 2011). The diluted blood mixture was carefully layered over 10 mL of Ficoll-Paque Premium 1.084 media (GE Healthcare Life Sciences, Marlborough, MA). The conical tubes were centrifuged for 30 min at 400 × *g* at 20°C. After removal of the plasma layer, a sterile pipette was used to transfer the cellular interface to a new 50-mL conical tube (GE, 2010). The cellular interface included the mononuclear cells, Ficoll-Paque layer, and the upper most portion of the red blood cell layer containing polymorphonuclear neutrophils (granulocytes). It was diluted 1:1 with dPBS(−) before centrifuging 10 min at 400 × *g*. Using a sterile pipette the supernatant was discarded and red blood cell contamination was removed by performing and repeating a lysis step using 5 mL each of hypotonic lysis buffer and re-equilibration buffer (Kuhns et al., 2015). The clarified cell pellet was resuspended in 1.2 mL of QIAzol from a miRNeasy kit (Qiagen, Hilden, Germany) (GMB, 2016). The pellet mixture was transferred into a 2-mL microcentrifuge tube and homogenized by vortexing for 1 min before being stored on dry ice for transport.

The QIAzol/cell mixture was held at −80°C until further processing for RNA extraction using the miRNeasy mini kit protocol under the directions for purification of total

RNA from animal cells. Samples were thawed to room temperature, and 0.025 mL of proteinase K (4 mg/mL) was added to the QIAzol/cell mixture and allowed to incubate at ambient temperature for 10 min. Appropriate adjustments were made to reagents to meet requirements for volume ratios, and remaining steps were completed following the miRNeasy kit protocol. Genomic DNA was removed using the Ambion Turbo DNA-free kit (Life Technologies, Rockville, MD). The total quantity of RNA obtained was validated using a Nanodrop 1000 Spectrophotometer (Thermo Fischer Scientific, Waltham, MA).

RNA Gene Expression and Analysis

The RNA samples were submitted to Novogene Corporation Inc. (Chula Vista, CA) for mRNA sequencing and bioinformatics. Before library construction, quality control reports were generated for each sample using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA). Samples required an RNA integrity number value of $\geq$6.8 to be selected for library preparation (Schroeder et al., 2006); anything lower was re-extracted and submitted again. Expression was performed using Illumina Novaseq and Hiseq platforms with a paired-end 150 bp sequencing strategy (Novogene, Sacramento, CA).

To identify genes relevant for health status, Illumina mRNA sequence reads were first filtered for adapter and low-quality regions using Trimmomatic (Bolger et al., 2014). Next, the resulting filtered reads were then aligned to the *Bos taurus* ARS-UCD1.2 genome (National Center for Biotechnology Information accession GCF_002263795.1) using Hisat2 (Pertea et al., 2016). Paired-end reads that lost their pairs after trimming were aligned separately, and results were combined with paired alignments using Samtools (Li et al., 2009). Gene expression values were normalized into fragments per kilobase of transcript per million mapped reads values using Stringtie (Pertea et al., 2016). The fragments per kilobase of transcript per million mapped reads values for all genes from all 60 samples were combined into a single tab-delimited file known as a gene expression matrix (**GEM**) where each column represents a sample and rows are genes. Elements in the matrix represent quantified gene expression levels. Creation of the GEM was orchestrated using GEMmaker, an open-source Nextflow workflow that automates execution of each step just described on high performance computing infrastructure (Hadish et al., 2020). GEMmaker was executed on WSU's Kamiak computing cluster. Next, the Boruta all-relevant feature selection algorithm (with a random forest model) was used to select genes relevant to LCA-designated health status (healthy vs. diseased; Python Software Foundation, Wilmington, DE; Kursa and Rudnicki, 2010). Random forest models provided to Boruta were performed with the Python scikit-learn package (scikit-learn version: 0.21, Python version: 3.6; Pedregosa et al., 2011) and configured as follows: number of estimators: 100; maximum tree depth: 4. All other parameters were left at their default assignments. The Boruta model (Kursa and Rudnicki, 2010; Python, 2019) was configured as follows: *P*-value (α cutoff): 0.05; percent threshold: 100; number of iterations: 10,000. All other parameters were left at their default assignments. Boruta identified 55 candidate genes as being predictive of LCA health status. These were filtered to include only the 15 most relevant genes, with mProbe scores indicating that no random permutations of any of the selected genes performed better as a predictor than the original gene values. Then, to identify functional themes associated with those 15 candidate genes, the GO database was used as a source for functional terms related to those genes (Gene Ontology Consortium, 2015). The GO term assignments to the ARS-UCD1.2 genes were obtained, pre-prepared, from the Gene Ontology website as Gene Annotation Format files, and functional enrichment analysis was performed using FUNC-E, a DAVID-like tool that performs Fisher's tests to identify functional enrichment followed by Kappa statistics of clustered enriched terms (Huang et al., 2009; Ficklin and Feltus, 2018). From these results, the top 5 enriched terms were chosen based on Fishers *P*-values and a Bonferroni adjustment for multiple comparisons with *P*-values below a 0.05 threshold.

RESULTS AND DISCUSSION

Enrollments and Clinical Assessments

Twenty Holstein-Friesian lactating cows were enrolled in this project and sampled 3 times each. At enrollment, the cattle were from 4 to 14 DIM and in their first, second, or third lactation (1.8–4.2 yr). Although healthy and diseased cattle were not formally matched, each healthy cow aligned with 1 or 2 of the diseased cattle in terms of lactation number and DIM ($\pm$2 d). For multiparous cows, previous DIM averaged 307 d, days dry averaged 45 d, and previous 305-d mature equivalent milk production averaged 12,688 kg (27,913 lb). Eleven cows were diagnosed with clinical metritis, 7 of which had delivered twins (n = 5) or a stillborn calf (n = 2; Table 1). Of those cows with metritis, 4 had documentation of RFM and 3 were treated for a left displaced abomasum either before (n = 2) or after (n = 1) initial samples were taken. None of the 9 healthy cows delivered twins or stillborn calves, or had RFM or left displaced abomasum diagnoses. Per farm protocols, clinical assessments, and herdsmen discretion, cases of RFM were not treated, metritis was either left untreated or treated with a course of ampicillin (i.m.) or ceftiofur (s.c.) alone or in combination, and left displaced abomasums were replaced using a toggling procedure with or without flunixin meglumine (i.v.; Table 2). Of note, this study purposely investigated cattle across a range of early DIM, parities, previous milk production levels, treatments, and postpartum disease severity and complexity in an effort to explore peripheral leukocyte gene expression signals in a diverse community of animals in field conditions. None of the 9 healthy cows had treatments admin-

Table 1. Animal demographics including freshening date, metritis status, calf details, age, parity, and for multiparous animals their previous DIM, days dry, and average 305-d mature equivalent milk production (305ME)[1]

Cow ID	Calving date (mo/d/yr)	Metritis (Y/N)[2]	Calf sex(es)[3]	Age (yr)	Parity	Previous DIM	Previous days dry	Previous 305ME [lb (kg)]
28233	5/28/2018	N	B	3.8	3	324	40	28,730 (13,059)
28460	5/26/2018	N	B	3.7	3	319	38	32,440 (14,745)
31323	5/31/2018	N	B	2.9	2	323	57	33,990 (15,450)
31409	5/30/2018	N	B	2.9	2	277	56	25,900 (11,773)
31432	5/28/2018	N	H	2.9	2	323	47	33,220 (15,100)
31624	5/25/2018	N	B	2.8	2	311	51	33,250 (15,114)
32069	5/31/2018	N	B	2.7	2	268	50	30,000 (13,636)
34848	5/24/2018	N	B	1.9	1	—	—	—
35095	5/25/2018	N	H	1.9	1	—	—	—
26914	5/23/2018	Y	HH	4.2	3	412	28	32,160 (14,618)
27931	5/23/2018	Y	HH	3.9	3	314	35	28,600 (13,000)
28308	5/28/2018	Y	HB	3.8	3	282	33	25,850 (11,750)
31398	5/31/2018	Y	BB	2.9	2	324	50	24,030 (10,923)
31623	5/24/2018	Y	BB	2.8	2	294	43	20,320 (9,236)
31727	5/31/2018	Y	B	2.8	2	269	50	26,960 (12,255)
31812	5/28/2018	Y	B	2.8	2	298	47	26,450 (12,023)
31881	5/30/2018	Y	B (DOA)	2.7	2	268	49	16,790 (7,632)
34020	5/28/2018	Y	B (DOA)	2.1	1	—	—	—
35070	5/24/2018	Y	H	1.9	1	—	—	—
35332	5/24/2018	Y	H	1.8	1	—	—	—

[1]Previous DIM, days dry, and 305ME (mature equivalent) refer to the previous lactation.

[2]Y = yes; N = no.

[3]Calf sex(es) are as follows: H = heifer; B = bull; HH = twin heifers; HB = twin heifer and bull; BB = twin bulls; DOA = dead on arrival.

istered during the sampling period. For those cows diagnosed with metritis, any discharge from the vagina and uterus was documented according to its appearance and smell for each of the 3 sampling periods (Table 2). Only 1 cow had demonstrable discharge at the time of the second or third sample. Although, antibiotics initiated on the day of enrollment were administered after the blood sample was obtained, 5 of the cows were administered antibiotics before the day of enrollment. Only 1 cow was not treated with antibiotics at all (Table 2).

Clinical assessments, serum biochemistry (Table 3), BHB, haptoglobin, and CBC data (Table 4) were used to discriminate potentially healthy cows and gradients of disease severity at enrollment. Twice repeated clinical assessments, BHB, haptoglobin, and CBC data (Table 4) were used to further discriminate disease progression or resolution and the stability of healthy cows (range 12–27 DIM). Although individual healthy cows did demonstrate some mild shifts outside serum biochemical and CBC reference ranges at the initial sampling point, the overall clinical and diagnostic assessments indicated no evidence of metritis or systemic physiological derangements that would exclude them from the healthy group. Similar to the healthy cows, serum biochemistry at the time of enrollment was not particularly discriminating in diseased cows although creatinine and albumin levels were typically low (Table 3). On the other hand, CBC results for diseased cows provided a nuanced perspective on the severity and progression of inflammatory and immunologic responses at the time of enrollment and across sampling points (Table 4).

LCA

Latent class analysis was used to validate the separation between healthy and diseased cows, and to provide insight into the effect of physiological changes from one sampling period to the next. Eight Nclasses were explored, and the lowest Akaike's information criterion (473.78) with Nclass = 3 was used to distinguish unique groups from the 60 observations of the 20 enrolled cows (Table 4). Latent class 1 (**LC** = 1) membership (n = 38) described substantially normal or mild changes to physiologic, biochemical, or CBC parameters; LC = 2 (n = 9) indicated moderate changes to health parameters; and LC = 3 (n = 13) described overt systemic inflammatory and immunologic changes. All of the healthy cows were designated to the same class (LC = 1) for all 3 sampling points, aside from one individual without any discernable clinical illness that was classified as LC = 2 at the third sample due to marginal shifts in hemoglobin, packed cell volume, and platelet numbers. At enrollment, 10 of the diseased

Table 2. Disease diagnoses, treatment information, and descriptions of vaginal and uterine discharge at the time of sampling for those cows with metritis and associated early postpartum disease[1]

Cow ID	Clinical diagnoses DIM	Clinical diagnoses[2]	Treatment (route of delivery)	Sampling DIM	Vaginal and uterine discharge on sample day	Antibiotics initiated before sample (d)
26914	2	RFM	No treatment	12	Fetid, red-brown	4
	8	Metritis	Ampicillin (i.m.)	20	None	12
	—	—	—	27	None	19
27931	3	RFM	No treatment	14	Mild smell, bloody	4
	10	Metritis	Ampicillin (i.m.)	20	None	10
	—	—	—	27	None	17
28308	7	Metritis	Ampicillin (i.m.)	7	Fetid, red-brown, copious	—
	—	—	—	15	None	8
	—	—	—	22	None	15
31398	4	Metritis	Ampicillin (i.m.) and	4	Mild smell, thin, watery	—
	—	—	Ceftiofur (s.c.)	12	None	8
	12	LDA	Toggle only	19	None	15
31623	2	Metritis	Ampicillin (i.m.) and	13	Fetid, red-brown	11
	—	—	Ceftiofur (s.c.)	19	None	17
	—	—	—	26	None	24
31727	6	Metritis	Ampicillin (i.m.)	6	Mild smell, copious	—
	—	—	—	12	None	6
	—	—	—	19	None	13
31812	2	RFM	No treatment	10	Fetid, bloody	—
	5	LDA	Toggle; flunixin (i.v.)	17	Foul, bloody	7
	10	Metritis	Ampicillin (i.m.)	24	Mild smell	14
31881	2	RFM	No treatment	8	Fetid, milky	—
	6	LDA	Toggle only	15	None	7
	8	Metritis	Ampicillin (i.m.)	22	None	14
34020	8	Metritis	Ampicillin (i.m.)	9	Fetid, red-brown	1
	—	—	—	15	None	7
	—	—	—	22	None	14
35070	14	Metritis	No treatment	14	Fetid, milky	—
	—	—	—	21	None	—
	—	—	—	28	None	—
35332	9	Metritis	Ceftiofur (s.c.)	11	Fetid, red-brown	2
	—	—	—	19	None	10
	—	—	—	26	None	17

[1]Antibiotics initiated on the day of enrollment were administered after the blood sample was obtained.

[2]RFM = retained fetal membranes; LDA = left displaced abomasum.

cows were designated to LC = 3, and 1 diseased cow was allocated to LC = 2. As can be seen in Table 4, as the sampling periods progressed, there were numerous changes in LC membership for the 11 diseased cows due to variable inflammatory and immunologic parameters. By the third sampling point, 5 diseased cows were assigned to LC = 2 and none remained in LC = 3. These results aligned with clinical perceptions in that the variable appearance and smell of vaginal and uterine discharge at the time of enrollment and thereafter was suggestive of variations in the nature and severity of infection that would naturally affect physiologic responses. Overall, diseased cows demonstrated expected pathophysiologic gradients of disease severity and resolution indicative of variations in uterine infection, comorbidity, and treatments.

Gene Expression Analysis

All 60 RNA samples submitted to Novogene had RNA integrity number values of at least 8 (range 8.0–9.9; mean and median 9.5) with an average pure RNA quantity of 221 ± 120 ng/μL (range 82–691). A total of 34,625 genes were considered in the gene expression matrix. Illumina 16S mRNA sequence data has been deposited in the National Center for Biotechnology Information database under study accession number PRJNA639363. All 55 genes that were associated with the LCA-designated health status were identified using Boruta. Ultimately, 15 genes were most represented within the diseased cohort as compared with the healthy cohort using machine learning feature selection (Table 5). These genes are primarily involved

Table 3. Serum biochemical data at the time of enrollment for cows with and without metritis and associated early postpartum disease[1]

Cow ID	Metritis (Y/N)	Sampling dates (mo/d/yr)	DIM	SDH (0–24 U/L)	GGT (12–80 U/L)	AST (50–320 U/L)	ALP (13–183 U/L)	CK (30–360 U/L)	BUN (7–19 mg/dL)	Creatinine (0.8–1.4 mg/dL)	Glucose (40–80 mg/dL)	TP (5.8–8.3 g/dL)
28233	N	6/4/2018	7	10	38	129	36	247	16	0.8	42	7.3
28460	N	6/7/2018	12	18	38	126	62	218	16	0.8	41	8.2
31323	N	6/4/2018	4	22	24	121	98	131	17	0.9	45	6.9
31409	N	6/7/2018	8	16	51	124	68	128	20[2]	0.9	37[2]	7.2
31432	N	6/7/2018	10	29[2]	40	127	67	193	14	0.8	33[2]	7.7
31624	N	6/7/2018	13	20	36	129	44	232	15	0.9	33[2]	7.9
32069	N	6/6/2018	6	15	42	90	58	157	13	0.8	51	7.2
34848	N	6/7/2018	14	9	28	90	79	194	16	0.8	59	8
35095	N	6/4/2018	10	10	25	87	51	167	12	0.8	63	7.5
26914	Y	6/4/2018	12	12	32	145	38	249	7	0.6[2]	51	6.7
27931	Y	6/6/2018	14	7	23	123	35	761[2]	10	0.6[2]	54	6.3
28308	Y	6/4/2018	7	32[2]	33	136	35	148	15	0.8	56	6.2
31398	Y	6/4/2018	4	<1[2]	29	141	47	570[2]	17	1.1	66	6.8
31623	Y	6/6/2018	13	19	29	115	34	155	9	0.5[2]	55	7
31727	Y	6/6/2018	6	8	24	199	35	368[2]	14	0.8	48	6.4
31812	Y	6/7/2018	10	3	25	180	52	106	10	0.6[2]	43	6.1
31881	Y	6/7/2018	8	8	31	131	60	153	11	0.7[2]	54	6.4
34020	Y	6/6/2018	9	11	30	123	52	206	14	0.8	61	5.7[2]
35070	Y	6/7/2018	14	5	33	97	63	332	10	0.7[2]	40	7.3
35332	Y	6/4/2018	11	6	27	109	45	241	10	0.7[2]	54	6.3

Cow ID	Metritis (Y/N)	Sampling dates (mo/d/yr)	Albumin (2.9–4.1 g/dL)	Globulin (2.5–4.9 g/dL)	Ca (8.4–10.8 mg/dL)	P (3.6–8.1 mg/dL)	Mg (1.6–3.0 mg/dL)	Na (133–148 mEq/L)	K (3.7–5.8 mEq/L)	Chloride (94–109 mEq/L)	CO_2 (18–34 mEq/L)	Anion gap (12–28 mEq/L)
28233	N	6/4/2018	3.4	3.9	9.7	5.9	1.7	142	4.5	100	28.3	18.2
28460	N	6/7/2018	3.3	4.9	9.4	6.4	2.1	139	5.1	99	27.3	17.8
31323	N	6/4/2018	3.1	3.8	10.1	6.2	1.7	145	4.8	102	32.2	15.5
31409	N	6/7/2018	2.9	4.3	9.2	5.5	2	139	5.2	98	31.3	14.9
31432	N	6/7/2018	3.5	4.2	9.8	5.2	1.8	139	5.3	97	29.7	17.6
31624	N	6/7/2018	3.4	4.5	8.8	7.1	2.6	142	5.2	101	26.6	19.6
32069	N	6/6/2018	3.5	3.7	10.1	5.3	2	147	5.4	106	30	16.4
34848	N	6/7/2018	3.4	4.6	9.9	7.2	2.4	139	4.6	99	26.4	18.2
35095	N	6/4/2018	2.7[2]	4.8	9.6	8	2.1	142	5.7	103	29.2	15.5
26914	Y	6/4/2018	2.5[2]	4.2	8.7	4.9	2	140	5.3	100	28.9	16.4
27931	Y	6/6/2018	2.5[2]	3.8	8.7	4.9	2.1	142	5.9[2]	103	30.6	14.3
28308	Y	6/4/2018	2.6[2]	3.6	9.7	7.5	0.9[2]	144	4.3	99	32.1	17.2
31398	Y	6/4/2018	3.1	3.7	8.6	4.9	1.9	144	5.8	104	29.5	16.3
31623	Y	6/6/2018	2.3[2]	4.7	8.8	6	1.9	141	4.7	102	30.5	13.2
31727	Y	6/6/2018	2.8[2]	3.6	8.6	6.4	1.6	145	4.6	104	28.4	17.2
31812	Y	6/7/2018	2[2]	4.1	8.7	5	1.6	141	5.4	102	28	16.4
31881	Y	6/7/2018	2.6[2]	3.8	8.4	6.7	1.9	140	4.5	99	27.7	17.8
34020	Y	6/6/2018	2.3[2]	3.4	9.1	7.4	1.6	146	4.5	105	29.6	15.9
35070	Y	6/7/2018	2.6[2]	4.7	9	5.4	1.8	140	5.1	99	28.4	17.7
35332	Y	6/4/2018	2.4[2]	3.9	9.2	6.5	1.9	146	4.6	105	29.8	15.8

[1]Reference ranges are provided within parentheses. Y = yes; N = no; SDH = sorbitol dehydrogenase; GGT = gamma-glutamyl transferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase; CK = creatine kinase; TP = total protein.

[2]Value is outside the established reference range.

Table 4. Diagnostic results including latent classes, rectal temperature, serum BHB, serum haptoglobin levels, and complete blood count data at all sampling points for cows with and without metritis and associated early postpartum disease[1]

Cow ID	Metritis (Y/N)	Sampling dates (mo/d/yr)	DIM	Latent class (1–3)	Rectal temp (<39.5°C)	BHB (<1.2 mmol/L)	Hapt (≤140 µg/mL)	WBC (5.5–13.5 ×10³/µL)	Band# (<0.1 ×10³/µL)	Seg# (1.4–8.0 ×10³/µL)
28233	N	6/4/2018	7	1	38.6	1.3[2]	485.3[2]	8.2	0.00	3.94
		6/12/2018	15	1	38.8	0.6	33.2	7.3	0.00	3.72
		6/19/2018	22	1	38.9	0.5	1,011.7[2]	7.1	0.00	4.40
28460	N	6/7/2018	12	1	38.7	0.5	15.8	8.3	0.08	3.49
		6/14/2018	19	1	38.7	0.2	1,330.9[2]	10.1	0.00	5.76
		6/21/2018	26	1	38.4	0.2	11.5	7.3	0.00	2.34
31323	N	6/4/2018	4	1	38.4	0.7	127.6	7.7	0.00	1.31[2]
		6/12/2018	12	1	38.7	0.4	99.2	11.1	0.00	4.44
		6/19/2018	19	1	38.6	0.3	7.2	11.1	0.00	9.21[2]
31409	N	6/7/2018	8	1	38.8	0.6	109.9	9.6	0.00	3.65
		6/14/2018	15	1	38.7	0.4	28.5	8.9	0.00	4.81
		6/21/2018	22	2	39.1	0.4	19.2	8.4	0.00	3.70
31432	N	6/7/2018	10	1	38.6	0.5	18.1	8.6	0.00	4.99
		6/14/2018	17	1	38.3	0.2	10.2	7.0	0.00	3.64
		6/21/2018	24	1	38.4	0.8	260.2[2]	8.4	0.00	6.80
31624	N	6/7/2018	13	1	38.8	0.5	12.4	8.3	0.00	2.91
		6/14/2018	20	1	38.4	0	17.4	7.1	0.00	4.54
		6/21/2018	27	1	38.9	0.5	26.9	8.1	0.08	3.89
32069	N	6/6/2018	6	1	38.6	0.8	47.6	6.0	0.00	3.00
		6/12/2018	12	1	38.5	0.4	37.1	8.0	0.00	4.64
		6/19/2018	19	1	38.9	0.3	10.2	8.0	0.24[2]	5.20
34848	N	6/7/2018	14	1	38.7	0.3	10.5	8.0	0.00	3.12
		6/14/2018	21	1	38.9	0	15.9	8.0	0.00	4.32
		6/21/2018	28	1	38.8	0	14.0	7.9	0.08	4.35
35095	N	6/4/2018	10	1	38.6	0.1	218.4[2]	9.3	0.00	3.91
		6/12/2018	18	1	38.8	0	15.6	7.8	0.00	2.18
		6/19/2018	25	1	38.6	0.1	61.1	7.7	0.00	3.54
26914	Y	6/4/2018	12	3	39.2	1.6[2]	477.9[2]	3.4[2]	0.14[2]	1.09[2]
		6/12/2018	20	1	38.4	0.3	18.8	5.6	0.00	2.35
		6/19/2018	27	1	38.9	0.2	20.3	9.9	0.00	6.14
27931	Y	6/6/2018	14	2	39.1	0.5	155.4[2]	8.9	0.45[2]	6.05
		6/12/2018	20	2	38.4	0.5	15.4	7.6	0.00	4.33
		6/19/2018	27	2	38.4	0.8	118.3	5.7	0.00	4.05
28308	Y	6/4/2018	7	3	38.5	1.5[2]	5,148.4[2]	7.1	0.57[2]	2.06
		6/12/2018	15	1	38.7	0.2	10.9	5.8	0.00	1.68
		6/19/2018	22	1	38.9	0.2	9.1	6.0	0.00	1.62
31398	Y	6/4/2018	4	3	39.4	0.6	4,343.8[2]	2.2[2]	0.22[2]	0.73[2]
		6/12/2018	12	3	38.2	2.4[2]	499.3[2]	2.5[2]	0.00	0.33[2]
		6/19/2018	19	2	38.7	0.8	461.7[2]	3.8[2]	0.00	2.09
31623	Y	6/6/2018	13	3	38.6	0	199.1[2]	5.8	0.12[2]	1.28[2]
		6/12/2018	19	1	38.6	0.2	11.4	6.8	0.00	2.38
		6/19/2018	26	2	38.8	0.1	9.3	14.6[2]	0.00	12.70[2]
31727	Y	6/6/2018	6	3	39.7[2]	0.8	2,244.2[2]	7.5	0.75[2]	0.60[2]
		6/12/2018	12	3	38.6	0.2	1,868.3[2]	9.9	0.50[2]	4.65
		6/19/2018	19	1	38.8	0.5	107.5	11.8	0.00	8.73[2]
31812	Y	6/7/2018	10	3	39.6[2]	0.1	3,023.4[2]	9.9	1.29[2]	4.36
		6/14/2018	17	2	38.9	0	19,941.2[2]	13.9[2]	0.00	10.43[2]
		6/21/2018	24	2	38.5	0	5,507.8[2]	5.5	0.00	2.97
31881	Y	6/7/2018	8	3	39.4	1.5[2]	1,610.0[2]	9.7	0.39[2]	1.94
		6/14/2018	15	1	39.2	0.1	2,339.2[2]	12.6	0.00	4.91
		6/21/2018	22	2	38.8	0.2	2,015.6[2]	10.3	0.31[2]	4.84
34020	Y	6/6/2018	9	3	38.7	0.7	1,315.0[2]	5.3[2]	0.58[2]	1.17[2]
		6/12/2018	15	3	38.7	0.4	29.0	4.3[2]	0.00	0.77[2]
		6/19/2018	22	1	38.7	0.1	119.8	6.6	0.07	2.71
35070	Y	6/7/2018	14	3	39.2	0.9	2,6071.4[2]	11.9	0.36[2]	4.88
		6/14/2018	21	1	39.4	0.5	2539.1[2]	15.7[2]	0.00	9.11[2]
		6/21/2018	28	1	39.2	0.2	786.7[2]	10.1	0.00	7.07
35332	Y	6/4/2018	11	3	38.4	0.5	891.2[2]	8.2	0.25[2]	1.56
		6/12/2018	19	1	38.7	0.1	16.8	11.6	0.00	3.71
		6/19/2018	26	1	38.9	0.1	9.6	14.9[2]	0.00	4.62

Continued

Table 4 (Continued). Diagnostic results including latent classes, rectal temperature, serum BHB, serum haptoglobin levels, and complete blood count data at all sampling points for cows with and without metritis and associated early postpartum disease[1]

Cow ID	Metritis (Y/N)	Sampling dates (mo/d/yr)	Lymph# (2.3–6.6 ×10³/µL)	RBC (5.6–8.1 ×10⁶/µL)	Hgb (9.5–12.6 g/dL)	PCV (25–33 %)	Pprot (7.0–8.5 g/dL)	Fibrinogen (200–600 mg/dL)	Platelets (135–650 ×10³/µL)	MPV (4.7–8.0 fL)
28233	N	6/4/2018	3.69	6.0	10.3	29	7.5	500	508	7.1
		6/12/2018	2.77	5.7	9.7	27	7.6	300	617	8
		6/19/2018	2.13[2]	5.9	9.8	28	8.1	400	824[2]	6.8
28460	N	6/7/2018	3.74	5.8	9.9	27	8	500	432	7.6
		6/14/2018	3.23	5.5[2]	9.2[2]	25	8.7[2]	700[2]	466	7.1
		6/21/2018	3.58	6.2	10.3	28	8.8[2]	400	493	6.4
31323	N	6/4/2018	6.16	5.6[2]	10.4	28	7	400	369	7.4
		6/12/2018	3.66	5.8	10.5	29	8	600	543	7.4
		6/19/2018	1.44[2]	5.4[2]	9.8	27	7.9	300	600	6.9
31409	N	6/7/2018	4.22	6.6	9.9	28	7.4	600	486	7
		6/14/2018	2.94	6.1	9.1[2]	25	7.5	500	693[2]	6.9
		6/21/2018	3.95	5.8	8.7[2]	24[2]	7.4	400	888[2]	6.2
31432	N	6/7/2018	2.92	6.4	10.7	29	7.5	300	434	6.8
		6/14/2018	2.38	6.4	10.9	28	8	300	497	6.9
		6/21/2018	1.34[2]	6.2	10.4	27	8.4	500	539	6.4
31624	N	6/7/2018	4.90	6.7	11.0	31	7.6	200	101[2]	7.5
		6/14/2018	2.06[2]	6.6	10.9	30	8	400	403	7.3
		6/21/2018	3.65	6.4	10.3	29	8.6[2]	500	456	7.1
32069	N	6/6/2018	2.52	5.8	10.3	29	7.4	500	496	6.9
		6/12/2018	2.32	5.5[2]	9.9	27	7.7	500	445	7.6
		6/19/2018	1.68[2]	5.5[2]	9.9	27	7.9	500	583	6.8
34848	N	6/7/2018	4.16	6.2	11.2	30	7.7	400	290	8.5[2]
		6/14/2018	2.88	5.9	10.3	28	7.7	600	427	8
		6/21/2018	3.24	5.9	10.5	28	7.9	300	358	7.8
35095	N	6/4/2018	5.21	6.1	9.5	27	7.4	500	597	6.6
		6/12/2018	5.07	6.2	9.8	27	7.8	500	661[2]	6.9
		6/19/2018	3.70	6.1	9.7	27	7.8	300	665[2]	6.4
26914	Y	6/4/2018	1.97[2]	5.6[2]	10.0	28	6.9[2]	600	459	7.3
		6/12/2018	2.86	5.3[2]	9.6	26	7.3	400	152	12.5[2]
		6/19/2018	2.67	5.4[2]	9.6	27	7.9	400	525	6.7
27931	Y	6/6/2018	2.14[2]	4.7[2]	8.6[2]	24[2]	6.3[2]	300	170	6.9
		6/12/2018	2.13[2]	4.4[2]	7.8[2]	22[2]	7.1	500	450	7.4
		6/19/2018	1.31[2]	4.6[2]	8.6[2]	24[2]	7.8	500	445	7.3
28308	Y	6/4/2018	4.40	6.4	10.8	29	6.5[2]	600	274	7.9
		6/12/2018	3.83	5.5[2]	9.0[2]	25	6.3[2]	200	469	9[2]
		6/19/2018	4.08	5.4[2]	8.9[2]	25	7.4	600	216	7.1
31398	Y	6/4/2018	1.06[2]	5.6[2]	9.9	27	7.3	900[2]	255	7.3
		6/12/2018	1.75[2]	5.0[2]	—[2]	24[2]	6.2[2]	500	381	9[2]
		6/19/2018	1.41[2]	4.7[2]	8.1[2]	22[2]	7.1	500	584	7
31623	Y	6/6/2018	4.00	6.1	9.5	27	7.1	500	488	6.5
		6/12/2018	4.08	5.7	9.0[2]	25	7.3	300	605	7.6
		6/19/2018	1.75[2]	5.4[2]	8.5[2]	24[2]	8	500	627	7.1
31727	Y	6/6/2018	5.10	6.3	10.3	29	6.6[2]	500	467	7.3
		6/12/2018	5.05	5.9	9.6	26	7.3	800[2]	599	8.6[2]
		6/19/2018	2.60	5.9	9.6	26	7.7	600	882[2]	7
31812	Y	6/7/2018	3.56	5.9	8.8[2]	25	6.5[2]	900[2]	354	8.8[2]
		6/14/2018	2.78	5.1[2]	7.6[2]	21[2]	7.4	800[2]	867[2]	7.4
		6/21/2018	2.09[2]	5.0[2]	7.4[2]	21[2]	8.5	800[2]	1140[2]	6.3
31881	Y	6/7/2018	6.60	6.8	10.8	30	6.5[2]	600	513	7
		6/14/2018	7.06[2]	6.0	9.5	26	7.6	700[2]	524	7.4
		6/21/2018	4.84	5.3[2]	8.4[2]	24[2]	8.3	800[2]	686[2]	6.7
34020	Y	6/6/2018	3.50	5.8	9.7	27	6[2]	600	320	8.7[2]
		6/12/2018	3.01	5.8	9.7	27	6.8[2]	400	583	7.6
		6/19/2018	3.43	5.5[2]	9.1[2]	25	7.3	400	718[2]	6.9
35070	Y	6/7/2018	6.19	5.7	9.8	27	7.7	900[2]	488	7.3
		6/14/2018	5.65	5.8	9.9	28	8.9[2]	800[2]	967[2]	7.3
		6/21/2018	2.83	5.9	9.9	28	9[2]	800[2]	904[2]	6.8
35332	Y	6/4/2018	5.90	6.2	9.6	28	6.6[2]	600	229	7.2
		6/12/2018	7.31[2]	6.2	10.0	28	7.6	500	367	8
		6/19/2018	9.09[2]	6.4	10.4	29	8.1	700[2]	235	7.3

[1]Reference ranges are provided within parentheses. Y = yes; N = no; temp = temperature; Hapt = haptoglobin; WBC = white blood cells; Band# = band cells; Seg# = segmented cells; Lymph# = lymphocytes; RBC = red blood cells; Hgb = hemoglobin; PCV = packed cell volume; Pprot = plasma protein; MPV = mean platelet volume.

[2]Value is outside the established reference range.

Table 5. The 15 most represented genes selected using Boruta (Kursa and Rudnicki, 2010) with a random forest model within a diseased cohort of cows (n = 11) with and without metritis and associated early postpartum disease, as compared with a healthy cohort of cows (n = 9)[1]

Gene symbol	Gene name	Random forest importance mean	Random forest importance SD	mProbe mean	mProbe SD
BTLA	B and T lymphocyte associated	0.0422	0.0060	0.0000	0.0000
CATHL6	Cathelicidin 6	0.0547	0.0081	0.0000	0.0000
EAF2	ELL associated factor 2	0.0569	0.0089	0.0025	0.0050
IGF2BP3	Insulin like growth factor 2 mRNA binding protein 3	0.1344	0.0104	0.0000	0.0000
IL17D	Interleukin 17D	0.0718	0.0095	0.0000	0.0000
KCNJ16	Inward rectifier potassium channel 16	0.0521	0.0066	0.0000	0.0000
KLHDC8A	Kelch domain-containing 8A	0.0477	0.0066	0.0025	0.0050
KLRF2	Killer cell lectin-like receptor F2	0.0850	0.0096	0.0000	0.0000
LCN2	Lipocalin 2	0.0637	0.0080	0.0000	0.0000
LOC100847835	Uncharacterized	0.0674	0.0078	0.0000	0.0000
LRBA	LPS responsive beige-like anchor protein	0.0407	0.0060	0.0050	0.0061
NRCAM	Neuronal cell adhesion molecule	0.0697	0.0079	0.0000	0.0000
PGLYRP1	Peptidoglycan recognition protein 1	0.0923	0.0098	0.0000	0.0000
PLPPR5	Phospholipid phosphatase related 5	0.0651	0.0096	0.0000	0.0000
ZNF432	Zinc finger protein 432	0.0565	0.0082	0.0025	0.0050

[1]mProbe scores provide an estimation of the family-wise error rate.

in immune cell and receptor function, tissue repair, and cell signaling based on functional enrichment analysis and gene ontology annotations (enrichment Fisher's P-value $<$ 0.01). The top ranked gene during every rank or error test was *PGLYRP1*, which encodes for peptidoglycan recognition protein 1 (PGLYRP-1). Additional analysis using the GO database identified the top 5 enrichment terms most frequently associated with the 15 genes as a whole (Bonferroni P-value < 0.05): killing of cells of other organism, defense response to fungus, antimicrobial humoral immune response mediated by antimicrobial peptide, innate immune response, and defense response to gram-positive bacterium (Table 6).

Gene Functions

Peptidoglycan recognition proteins are important pattern recognition molecules of the innate immune system. They have been shown to act similar to glycopeptide antibiotics that kill bacteria by directly interacting with cell wall peptidoglycans, or by binding the cell wall or outer membrane to exploit bacterial stress defenses (Cho et al., 2007; Kashyap et al., 2011). Specifically, bovine PGLYRP-1 has been shown to have both gram-positive (*Staphylococcus aureus* and *Listeria monocytogenes*) and gram-negative (*Escherichia coli* and *Salmonella typhimurium*) bactericidal effects (Tydell et al., 2002; Tydell et al.,

Table 6. GO[1] terms enriched in the 15 most significant Boruta (Kursa and Rudnicki, 2010) selected genes

Term	Definition	Mod count[2]	Background count[3]	Fishers *P*-value	Bonferroni *P*-value
GO:0031640	Killing of cells of other organism	2	8	<0.0001	0.0009
GO:0050832	Defense response to fungus	2	12	<0.0001	0.0018
GO:0061844	Antimicrobial humoral immune response mediated by antimicrobial peptide	2	25	0.0001	0.0069
GO:0045087	Innate immune response	3	168	0.0001	0.0083
GO:0050830	Defense response to gram-positive bacterium	2	56	0.0005	0.0323

[1]Gene Ontology Consortium (2015).

[2]The number of times that a term was annotated to any of the 15 genes.

[3]The number of times that a term was found annotated to any gene in the genome.

2006; Wang et al., 2007). Furthermore, *PGLYRP1* has been associated previously with varying disease susceptibilities indicating different roles for PGLYRP-1 in diverse diseases (Pant et al., 2011; Wang et al., 2013).

Similar to PGLYRP-1, the cathelicidin-6 protein (CATHL6) is a member of a major group of host-defense antimicrobial peptides and has been shown to exert a potent antimicrobial activity against gram-negative and gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* and *E. coli* (Tomasinsig et al., 2010; Whelehan et al., 2014). Lipocalin-2 (LCN2) is also a member of a highly diverse group of proteins that participate in modulating the immune response (Flower et al., 2000). In fact, LCN2 rapidly increases during bacterial infections and inflammatory conditions (Schmidt-Ott et al., 2007), acts as a natural bacteriostatic agent through the elimination of iron ions that are necessary for the growth of pathogenic bacteria (Goetz et al., 2002), and has been recommended as a potential marker of infection for bovine mastitis (Pokorska et al., 2019).

Although several of the genes in this study have not been explored to date with regard to bovine disease states, their underlying immunologic functions have been demonstrated in other species. For example, *BTLA* encodes B and T lymphocyte associated protein (BTLA), which is a lymphoid-specific cell surface receptor expressed by B and T cells, dendritic cells, macrophages, and natural killer cells (Watanabe et al., 2003; Krieg et al., 2005). BTLA is an Ig superfamily member that has been shown to exert both inhibitory and stimulatory effects on B and T lymphocytes in mice and humans depending on circumstances such as pathogen infections and autoimmunity (Gavrieli et al., 2003; Hurchla et al., 2007). Interleukin-17D (IL-17D) belongs to the IL-17 family of cytokines that have been implicated in inflammation and host defense (Pappu et al., 2011). However, Lee et al. (2019) recently determined that IL-17D is a critical cytokine in mice during intracellular bacterial and viral infections that may compromise host-defense function through the suppression of CD8 T cell activity in part by suppressing dendritic cells. Killer cell lectin-like receptor F2 (*KLRF2*) is a C-type lectin-like receptor encoded by the natural killer gene complex and purported to be involved in natural killer cell mediated cytotoxicity and cytokine secretion (Yokoyama and Plougastel, 2003; Spreu et al., 2010). Although less is known regarding lipopolysaccharide-responsive and beige-like anchor protein (*LRBA*), it is induced after LPS stimulation of B cells and macrophages and may be involved in coupling signal transduction and vesicle trafficking to enable polarized secretion and membrane deposition of immune effector molecules (Wang et al., 2001).

Other genes associated with bovine metritis and associated early postpartum disease in our study have demonstrated a variety of molecular functions in other species. For example, the ELL associated factor 2 protein (*EAF2*) influences the regulation of RNA polymerase II transcription (Kong et al., 2005). Insulin like growth factor 2 mRNA binding protein 3 (*IGF2BP3*) contributes to RNA-binding, subcellular sorting and protein-complex formation (Wachter et al., 2013). Inward rectifier potassium channel 16 (*KCNJ16*) potentially influences the regulation of fluid and pH balance (Pessia et al., 1996). Neuronal immunoglobulin cell adhesion molecules (*NRCAM*) play a role in facilitating cell-cell contacts in the brain and peripheral nervous system (Eshed et al., 2005). Though poorly described, phospholipid phosphatase related 5 protein (*PLPPR5*) may promote neurite growth in a CDC42-independent manner (The UniProt Consortium, 2019), zinc finger protein 432 (*ZNF432*) may be involved in transcriptional regulation (The UniProt Consortium, 2019), and kelch domain-containing protein 8A (*KLHDC8A*) may play a role in tumor formation and maintenance, and the maintenance of the neural stem cell population (Mukasa et al., 2010).

One gene of interest (*LOC100847835*) remains uncharacterized and is responsible for a long noncoding RNA (**lncRNA**). Noncoding RNA are abundant and functional RNA molecules that are transcribed but not translated into proteins (Koufariotis et al., 2015). Few functional lncRNA have been well characterized, but they are implicated in a variety of biological functions (Wang and Chang, 2011; Wapinski and Chang, 2011), such as protein synthesis, RNA maturation and transport, and transcriptional gene silencing (Bernstein and Allis, 2005; Whitehead et al., 2009). Although limited information is available regarding bovine lncRNA and their biological functions, the regulatory roles of lncRNA imply that they may be involved in disease manifestation (Huang et al., 2012).

The specific transcriptional changes documented in this study speak to the complex interactions between physiologic disturbances and immune function associated with metritis and diverse early postpartum disease. More broadly, the GO terms assigned to the genes encapsulate the response to postpartum uterine disease in terms of mounting humoral and innate defenses against other organisms such as gram-positive bacteria. A specific component of that defense is attributed to cationic host-defense (antimicrobial) peptides that are not only directly microbicidal, but also possess the potential to bind highly conserved pathogen-associated molecular patterns. For example, the antimicrobial peptide PGLYRP-1 associated with this study has been shown to bind to a variety of pathogen-associated molecular patterns including lipopolysaccharide, lipoteichoic acid, and peptidoglycan (Pant et al., 2011). Cathelicidin-6 also has demonstrated anti-endotoxin responses that may protect against endotoxemia in vivo (Mookherjee and Hancock, 2007).

Cathelicidins and PGLYRP-1 have been identified previously as indicator proteins for endometritis in the presence of *T. pyogenes* and *Streptococcus agalactiae* (Ledgard et al., 2015). Given that uterine contamination with pathogens such as *T. pyogenes* increases the risk of develop-

ing more severe endometritis or other sequelae that might prolong or increase the severity of disease, the presence of such peptides offers a range of useful antimicrobial and immunomodulatory activities (Hancock, 2001). However, thorough elucidation of their biological importance in innate immunity and realization of their full clinical potential will require additional efforts. This includes efforts to improve host genetics through selective breeding targeting genes involved in innate immunity as putative candidate loci. In fact, *PGLYRP1* is considered a particularly important candidate gene that may ultimately aid in the suppression of economically important diseases in food-animal populations, due to its diverse role in bovine innate immunity and differential susceptibility to gram-positive bacteria, gram-negative bacteria, and even fungi (Seabury and Womack, 2008).

APPLICATIONS

Overall, the suite of genes identified in this study encompass a range of immunologic and cellular functions responsive to the effects of metritis and associated early postpartum disease on homeorhetic regulation. Gene *PGLYRP1* was the top ranked gene during every rank or error test within this study. Its associated protein, PGLYRP-1, and other immunomodulatory molecules (e.g., CATHL6, LCN2, BTLA, IL-17D, and so on) represent principal molecules that selectively enhance or alter host innate immune defense mechanisms and modulate pathogen-induced inflammatory responses. Furthermore, these proteins provide insight into individual and population-level markers of disease resilience (resistance and tolerance) that may help guide improvements in host genetic selection and clarify the burden of disease on animal well-being. Although additional research is required to explain their functional mechanisms and bioactivity across dairy populations, many of these molecules also represent potential alternatives to antimicrobials for infection management. All told, antimicrobial peptides such as those associated with this study are emerging as attractive candidates for treatment of a variety of pathogenic conditions given that they do not appear to target pathogens directly, nor do they modify single inflammatory mediators. As per the GO terms aligned with our findings and highlighted elsewhere, the central theme is that these host-defense peptides boost specific innate immune responses and exert selective immunomodulatory effects on immune cells upon exposure to pathogenic challenges (Mookherjee and Hancock, 2007). Although the diagnostic and therapeutic utility of these findings remains to be seen, defining relationships between molecular regulatory features and phenotypic traits ultimately may help reduce disease incidence and antibiotic use in animal production. This is particularly relevant to the dairy cow transition period with its metabolic and physiologic changes and associated increase in disease susceptibility.

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