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Characterization of circulating microRNA profiles of postpartum dairy cows with persistent subclinical endometritis

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ABSTRACT

Subclinical endometritis (SCE) is an unresolved inflammation of the endometrium of postpartum dairy cows, seriously affecting fertility. Current diagnosis, which relies on uterine cytology or even more invasive biopsy sampling, would benefit from the identification of blood-based diagnostic biomarkers. Due to the known role of microRNAs (miRNAs) in other diseases, this case-control study evaluated the cell-free circulating miRNA profiles of SCE cows, and the network of transcripts predicted to interact with those miRNAs, previously identified as differentially expressed genes (DEG) in the endometrium of the same cows. Healthy (H, n = 6) and persistent SCE (n = 11) cows characterized by endometrial cytology and biopsy were blood sampled at 21 and 44 d postpartum (DPP). Following extraction of cell-free plasma miRNAs and RNA-seq analysis, differential abundance analysis of miRNAs was performed with the DESeq2 R package (adjusted p-value of 0.05), and in silico prediction of miRNA-interacting genes on a sequence complementary basis was conducted using the miRWalk database. The principal component analysis showed a clear clustering between groups of uterine health phenotypes (H vs. SCE), although the clustering between groups was less pronounced at 44 DPP than at 21 DPP. No effect of the stage (21 vs. 44 DPP) was observed. A total of 799 known circulating miRNAs were identified, from which 34 demonstrated differential abundance between H and SCE cows (12 less abundant and 22 more abundant in SCE than in H cows). These 34 miRNAs are predicted to interact with 10,104 transcripts, among which 43, 81, and 147 were previously identified as differentially expressed in, respectively, endometrial luminal epithelial, glandular epithelial, and stromal cells of the same cows. This accounts for approximately half of the DEG identified between those H and SCE cows, including genes involved in endometrial cell proliferation, angiogenesis and immune response, whose dysregulation in SCE cows may impair pregnancy establishment. From 219 miRNAs with mean normalized read counts above 100, the presence and abundance of miR-425–3p and miR-2285z had the highest discriminatory level to differentiate SCE from H cows. In conclusion, despite apparent confinement to the endometrium, SCE is associated with a distinct circulating miRNA profile, which may represent a link between the systemic changes associated with disease and the endometrial immune response. The validation of a miRNA panel consisting of circulating cell-free miR-425–3p and miR-2285z may prove a relevant advancement for the noninvasive diagnosis of persistent SCE.

Key words: blood microRNAs, subclinical endometritis, dairy cow

INTRODUCTION

During the postpartum period of dairy cows, one of the most prevalent diseases, often underdiagnosed due to lack of clinical signs, is subclinical endometritis (**SCE**; Sheldon et al., 2006). Subclinical endometritis is an inflammatory process in the endometrial lining of the uterus, characterized by increased infiltration of PMN, for which current diagnosis relies on uterine cytology or even more invasive biopsy sampling (Sheldon et al., 2006; Pascottini et al., 2016). Subclinical endometritis affects the expression of genes involved in inflammatory response, tissue remodeling and immune tolerance in the uterus (Kasimanickam et al., 2014; Salilew-Wondim et al., 2016; Raliou et al., 2019; Pereira et al., 2022). The

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comparison from full transcriptomes of laser microdissected endometrial compartments from cows suffering from persistent SCE or recovering from SCE with those from healthy ones revealed cell type-specific changes of the above genes and pathways that may subsequently affect mechanisms of pregnancy establishment (Pereira et al., 2022).

Epigenetic processes can alter gene expression patterns. Namely, numerous RNA-based mechanisms are responsible for the post-transcriptional control of gene expression involved in innate immunity (Carpenter et al., 2014). Among these, microRNAs (**miRNAs**) have emerged as key modulators of mRNA decay and translation (Filipowicz et al., 2008; Carpenter et al., 2014; Oladejo et al., 2020). These modulators are noncoding RNAs with 21 to 24 nucleotides that, coupled with argonaute proteins, form RNA-induced silencing complexes (Hutvagner and Simard, 2008) and hybridize with their interacting mRNAs (Filipowicz et al., 2008). Depending on the interacting mRNA-specific binding sequence's location at the 3′ UTR, 5′ UTR, or promoter regions, miRNAs can induce translational repression, silencing effects on gene expression, or alternatively induce transcription (O'Brien et al., 2018).

There is increasing evidence that miRNAs have a role in host defense response to bacterial infections by modulating inflammation, cell penetration, tissue remodeling, and innate and adaptive immunity (Zhou et al., 2018). The endometrial miRNA profile changes associated with postpartum SCE were investigated in uterine full tissue (Salilew-Wondim et al., 2016) and cytobrush samples (Hailemariam et al., 2014), unveiling a putative regulatory role of uterine miRNAs in the development and progression of SCE. The miRNAs can be secreted into extracellular fluids, thus functioning as chemical messengers to mediate cell-cell communication (O'Brien et al., 2018), and circulating miRNAs were described as highly stable, convenient (collection of samples from blood), and useful biomarkers of tissue function for a variety of diseases in cattle (Kasimanickam and Kastelic, 2016; Gupta et al., 2018; Miretti et al., 2020; Luoreng et al., 2021). However, to the best of our knowledge, the circulating miRNA profiles of postpartum dairy cows with persistent SCE have not been characterized to date. Following a former study describing the effect of SCE on gene expression in the main 3 endometrial cell types (Pereira et al., 2022), the objective of this study was to evaluate the effect of SCE on circulating miRNAs of postpartum dairy cows. In addition, the lists of differentially abundant miRNAs and their potentially interacting mRNAs were compared with the lists of differentially expressed genes (**DEG**) previously identified in the 3 endometrial compartments of the same cows and at the same moments (Pereira et al., 2022). The findings of the present study enlighten the changes in post-transcriptional epigenetic regulation associated with SCE and may provide novel biomarkers for the diagnosis and prognosis of SCE in postpartum dairy cows.

MATERIALS AND METHODS

Ethics Statement

The project was approved by the Institutional Animal Care and Use Committee (reference CEIE nº37/2019). All clinical procedures were conducted in compliance with the European Union legislation on the protection of animals used for scientific purposes (Directive 2010/63/EU).

Animals

Animals included in this study correspond to a subset of those enrolled in Pereira et al. (2022), where the experimental design, animal handling, and sampling procedures are fully described. Briefly, the study took place on a dairy herd in Benavente (Portugal), where postpartum Holstein dairy cows (n = 17), without signs of puerperal disease (either uterine or extrauterine) and antibiotic or anti-inflammatory therapy, were enrolled in the study. Due to the strict inclusion criteria, the enrolled animals had no signs of clinical metritis (up to 21 d postpartum [**DPP**]) or clinical endometritis (from 21 to 44 DPP), nor any clinical extrauterine disease (e.g., mastitis, metabolic, lamness, pneumonia) throughout the sampling period.

Genital Tract Evaluation and Endometrial Cytology

Cows were submitted to a gynecological examination, endometrial cytology, and blood sampling at 21 ± 0.4 and 44 ± 0.8 DPP. Following collection with a Metricheck device (EndoControl Sampler, Minitube, La Selva del Camp, Spain), vaginal discharge was graded according to Williams et al. (2005), and the genital tract was evaluated by transrectal palpation and ultrasonography. Animals with signs of clinical uterine disease (e.g., vaginal discharge score ≥1) were excluded from further exploration. Next, endometrial cytology was performed with the cytobrush technique, and the percentage of PMN was assessed by counting 400 cells. Cows were considered healthy when PMN percentage was <18% and <5% at 21 and 44 DPP, respectively (Pereira et al., 2020, 2022). Cows from the healthy group (**H**, n = 6) had a healthy uterus at both exami-

nations and subsequently became pregnant at the first insemination, whereas persistent SCE (n = 11) presented PMN percentages higher than the above cut-offs at 21 and 44 DPP, and did not become pregnant at the first insemination. This classification, initially based on cytology results, was confirmed by retrospective histological analysis. Details for the characterization of these cows were previously published (Pereira et al., 2022).

Blood Sampling and Small RNA Extraction

Blood samples were aseptically collected by venipuncture of the coccygeal vein into 10 mL dry tubes with K3 EDTA (13060, Vacutest KIMA, Arzegrande, Italy). Tubes were immediately centrifuged (2,000 × *g* for 15 min), and the resulting cell-free plasma was aliquoted into 1.5-mL tubes and stored at −20°C until further processing. Cell-free circulating small RNAs were extracted from the plasma aliquots (n = 34) using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The miRNA percentage and concentration were analyzed using a Bioanalyzer 2100 and a small RNA chip kit (Agilent Technologies) according to the manufacturer's instructions and stored at −80°C until further processing (Supplemental File S1, https://figshare.com/articles/dataset/Supplemental_File_1_xlsx/24082338; Pereira et al., 2023a).

RNA-Seq Analysis

The RNA-seq analyses were performed by the GenomEast platform (Strasbourg, France). Briefly, small RNA-seq libraries from both stages (H samples = 12, 6 cows, 2 samples per cow; SCE samples = 22, 11 cows, 2 samples per cow), were generated from 10 to 20 ng of purified small RNA fraction (acrylamide gel purification) using the TruSeq Small RNA Library Prep Kit (Illumina, San Diego, CA) according to the manufacturer's instructions. Specific miRNA and small RNA adapters were added to each end of the miRNA, followed by reverse transcription and PCR amplification to obtain cDNA, and the final cDNA libraries were sequenced on HiSeq 4000 (Illumina) as single-end 50 base reads according to the manufacturer's instructions. Adaptor sequences and reads with undetected bases were removed (FASTX-Toolkit, http://hannonlab.cshl.edu/fastx_toolkit/index.html), and all remaining reads were filtered according to their size. Read quantification and annotation were performed using the ncPRO-seq pipeline (Chen et al., 2012). The sequence reads were aligned against the *Bos taurus* btau5.0.1 genome as miRBase_v22.1 using the bowtie v1.0.0 software (Langmead et al., 2009). The normalization and differential expression analyses of miRNAs were conducted using the DESeq2 R package v1.18.1. The following terms for main effects and interaction were added in the GLM model (stage (21DPP, 44DPP) + cow_group (H, SCE) + stage:cow_group). Significance was considered at Padj ≤0.05, and only known mature miRNAs with a mean read count ≥100 normalized reads were considered to increase confidence in subsequent analysis and reduce technical noise. Power analysis was performed using the method described by Bi and Liu (2016) and compiled in the R package ssizeRNA (version 1.3.2). Calculated at a false discovery rate (FDR) of 0.05, power was 32%, 71%, and 93% to detect 1.5, 2, and 3 log2 fold change respectively. Data were deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE225913 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE225913).

Bioinformatic Analyses

Principal component analysis (**PCA**) was performed with FactoMineR (R package, version 1.4.1) using the variance stabilizing transformation output files from DESeq2. In silico prediction of miRNA-interacting genes on a sequence complementary basis was conducted using the miRWalk database (http://mirwalk.umm.uni-heidelberg.de/). Only possibly interacting genes possessing sequence complementary sites with miRNAs within the 3UTR region with a binding probability >0.95 were kept in the analysis. The lists of DEG identified in the 3 main endometrial cell types, luminal epithelial (**LE**), glandular epithelial (**GE**), and stromal (**ST**) cells were retrieved from Pereira et al. (2022), and the Venn diagrams of common genes between miRNA-interacting genes and DEG identified were obtained with Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/).

Significant gene ontology (**GO**) terms of the GO-slim Biological Process domain enriched in the lists of common genes between miRNA-interacting genes and DEG were found using the PANTHER classification system (Protein ANalysis THrough Evolutionary Relationships version 16.0, http://pantherdb.org). Identified GO terms were summarized by removing redundant GO terms with dispensability at a tiny level, using REVIGO (http://revigo.irb.hr/; Supek et al., 2011).

The miRNA-mRNA interaction subnetworks were created with Cytoscape (version 3.8.2) comprising differentially abundant miRNA and common genes between miRNA-interacting genes and DEG identified in LE, GE and stromal cells.

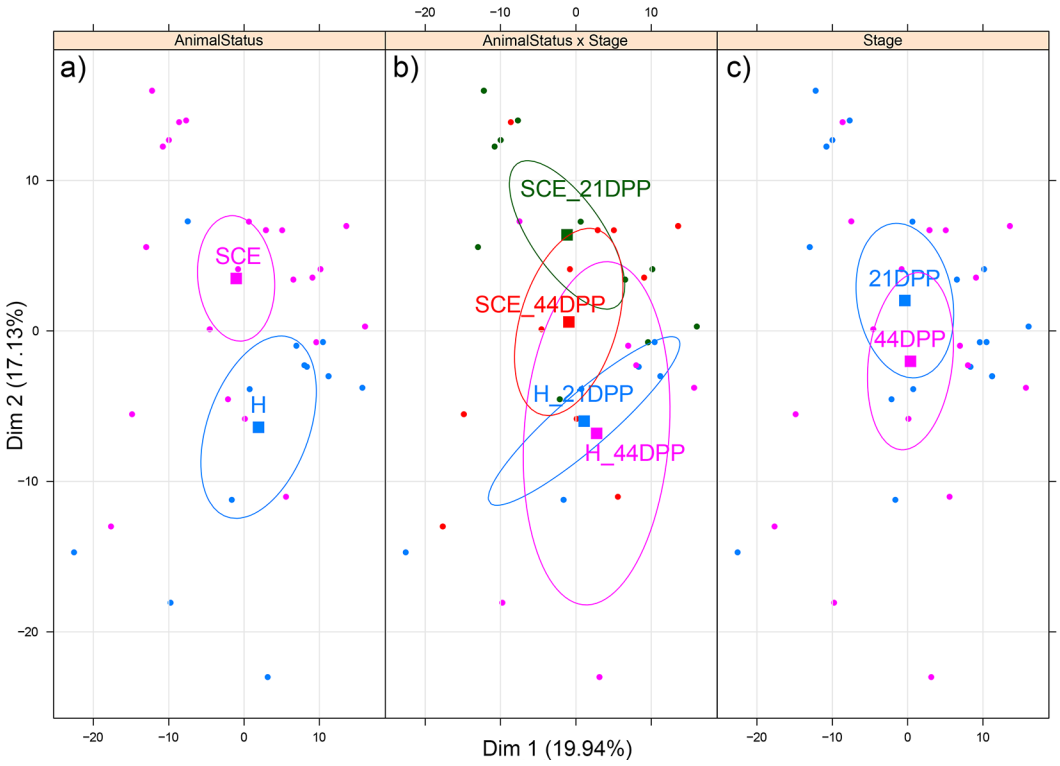

Figure 1. Principal component analysis of circulating miRNA data from postpartum dairy cows classified as Healthy (H) or Persistent Subclinical Endometritis (SCE) at 21 and 44 d postpartum (DPP). (a) Main effect of endometrial health status; (b) interaction between endometrial health status and postpartum stage; (c) main effect of postpartum stage. The projection of data on the first 2 principal components explains 37% of the variance.

Approach for Biomarker Selection

To select the most predictive miRNA sets to discriminate H versus SCE cows we performed a sparse partial least squares–discriminant analysis (**sPLS-DA**) using the mixOmics R package (version 6.10.8; Lê Cao et al., 2011). The sPLS-DA included only miRNAs with mean of normalized read counts above 100. The number of components and variables to select were tuned at 2 and 9, respectively, by using the perf() and tune.splsda() functions to get the better performance of the sPLS-DA model.

RESULTS

Cell-Free Plasma Circulating miRNAs

From the 34 cow libraries (2 postpartum stages for each cow of the H and SCE groups), 799 known circulating miRNAs were identified (Supplemental File S2, https://figshare.com/articles/dataset/Supplemental_File_2_xlsx/24082356; Pereira et al., 2023b). The mean of normalized read counts of these 799 miRNAs, were ≥1,000 for 129 (16.1%), <1,000, and ≥100 for 90 (11.3%), and <100 for 580 (72.6%). The top5 most abundant miRNAs, averaging more than 300,000 reads, were miR-486, miR-191, miR-3600, miR-22–3p, and miR-192.

Differential Abundance of Circulating miRNAs in SCE Versus H Cows

The generalized linear model used in DESEq2 for differential expression analysis of individual miRNAs indicated that the effect of group (H vs. SCE) was significant ($P < 0.05$) whereas the effect of stage and the interaction between group and stage were not significant. This is illustrated by the PCA, which showed a clear clustering of individuals associated with group (H vs. SCE; Figure 1a), whereas there was no clear distinction for the effect of stage (21 vs. 44 DPP; Figure 1c). However, despite differences remaining at 44 DPP, the miRNA profiles of H and SCE cows became more similar than at 21 DPP (Figure 1b).

Due to the absence of a significant effect of stage, the following part of the results focuses on the main effect of group (H vs. SCE). The analysis identified 46 circulating known mature miRNAs with differential abundance between H and SCE cows (Supplemental File S3, https:

//figshare.com/articles/dataset/Supplemental_File_3_xlsx/24082371; Pereira et al., 2023c). However, 12 of these miRNAs had an average number of normalized read counts under 100 and were excluded from further analysis and discussion, due to their probable low biological relevance. The remaining 34 circulating miRNAs with differential abundance in SCE compared with H cows are represented in Figure 2. From these, 12 were less abundant (miR-10b; miR-148a; miR-148d; miR-151–3p; miR-103; miR-107; miR-30a-5p; miR-99b; miR-1839; miR-10225a; miR-182; miR-11971) and 22 were more abundant (miR-26a; miR-26c; miR-27a-3p; miR-150; miR-30b-5p; miR-30c; miR-16a; miR-361; miR-29c; miR-339a; miR-28; miR-222; miR-425–3p; miR-145; miR-374b; miR-497; miR-138; miR-505; miR-195; miR-6119–3p; let-7a-3p; miR-345–5p) in SCE cows when compared with H cows. The 2 miRNAs with the lowest adjusted p-values (6.05×10^{-9} and 2.99×10^{-5}) and the highest log2 fold changes (1.93 and 2.16) were miR-425–3p and miR-30b-5p, respectively, both more abundant in SCE cows. Among SCE less abundant miRNAs, miR-10b had the highest mean of normalized read counts and the lowest adjusted p-value (2.8×10^{-3}) and log2 fold change (−1.76). Among the 10 miRNAs with the lowest adjusted p-values, 9 had a positive log2 fold change, and only miR-10b had a negative log2 fold change.

Comparison of Circulating miRNA-Associated Genes with DEG identified in Endometrial Cells

The 22 more abundant miRNAs in SCE cows are predicted to interact with 8,143 transcripts, whereas the 12 less abundant miRNAs are predicted to interact with 6,905 transcripts. Overall, the 34 miRNAs with differential abundance are predicted to interact with 10,104 transcripts, and a high proportion of these transcripts (4,944/10,104; 48.9%) relates to both more and less abundant miRNAs. In previous work (Pereira et al., 2022), the comparison of the transcriptome of LE, GE, and ST endometrial cells of SCE and H cows identified 84, 194, and 251 DEG, respectively. From these DEG, 43/84 (51.2%), 81/194 (41.8%), and 147/251 (58.6%) in LE, GE, and ST cells, respectively, are predicted to interact on a sequence complementary basis with the circulating miRNAs with differential abundance found here (Table 1).

miRNA-mRNA Interaction Networks

Full miRNA-mRNA interaction networks were constructed based on the comparison between the lists of predicted interacting genes of miRNAs with differential abundance and the DEG previously identified in the LE, GE, and ST endometrial cells of the same cows; see Supplemental Figure S1 (https://figshare.com/articles/figure/Supplemental_Fig_S1/24082377; Pereira et al., 2023d), Supplemental Figure S2 (https://figshare.com/articles/figure/Supplemental_Fig_S2/24082383; Pereira et al., 2023e), and Supplemental Figure S3 (https://figshare.com/articles/figure/Supplemental_Fig_S3/24082389; Pereira et al., 2023f), respectively.

Due to the large size of these interaction networks (66 nodes in LE cells, 110 nodes in GE cells, and 180 nodes in ST cells), subnetworks from enriched GO terms were selected for discussion of the miRNA-mRNA regulatory associations. The enriched GO terms identified in the lists of common genes between miRNA-interacting genes and DEG identified in each cell type (LE, GE, ST) are presented in Supplemental Figure S4 (https://figshare.com/articles/figure/Supplemental_Fig_S4/24082395; Pereira et al., 2023g), Supplemental Figure S5 (https://figshare.com/articles/figure/Supplemental_Fig_S5/24082401; Pereira et al., 2023h), and Supplemental Figure S6 (https://figshare.com/articles/figure/Supplemental_Fig_S6/24082407; Pereira et al., 2023i), respectively.

In LE cells, the over-represented biological processes of cell population proliferation and negative regulation of epithelial to mesenchymal transition included DEG forming 2 subnetworks (Figure 3A). In GE cells, a subnetwork included DEG involved in the processes of angiogenesis, extracellular matrix organization, and positive regulation of cell migration (Figure 3B) and another subnetwork included DEG involved in defense response to virus and negative regulation of viral genome replication (Figure 3C). In ST cells, one large subnetwork comprised DEG involved in immune response (Figure 3D).

The Ability of Different miRNAs to Serve as SCE Biomarkers

The MixOmics′ PLS-DA approach was used to identify the miRNAs that were specifically associated with SCE and H cows. In the first component, miR-425–3p showed a significant association to SCE cows. In the second component, miR-2285z, miR-2285q, miR-6529b, miR-6529a, miR-1468, miR-2419–5p, miR-182, miR-107, and miR-455–5p, in this descending order, were significantly associated with H cows (Figure 4). Following receiver operating characteristic curve analysis the first component displayed an area under the curve (**AUC**) of 0.8182 whereas the second component exhibited an AUC of 0.9394. The first component miR-425–3p and the second component miR-2285z were used

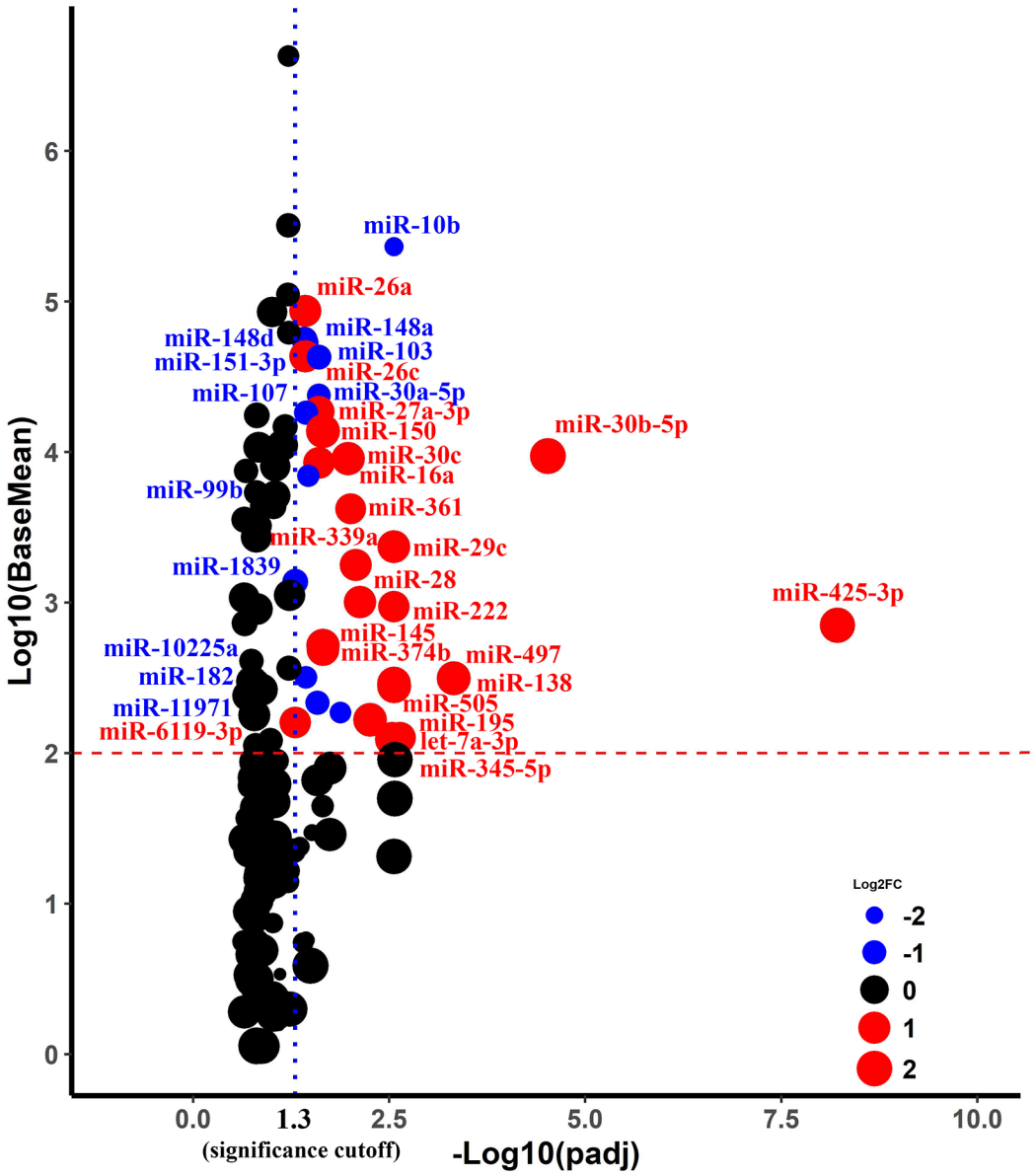

Figure 2. Volcano plot representing the data distribution of circulating miRNAs with differential abundance between healthy (H) and persistent subclinical endometritis (SCE) cows. The miRNAs more abundant in SCE cows are represented in red, and miRNAs less abundant in SCE cows are represented in blue.

to construct a 2-miRNA model. In this model, the first dimension AUC remained unchanged, while the second dimension AUC only slightly decreased to 0.9318.

DISCUSSION

Following the role played by miRNAs in bovine immune responses (Lawless et al., 2014; Do et al., 2021), and the evidence that altered circulating miRNA profiles are associated with various inflammatory diseases in dairy cattle (Kasimanickam and Kastelic, 2016; Gupta et al., 2018; Miretti et al., 2020; Luoreng et al., 2021), the present study aimed to describe the circulating miRNA profiles associated with persistent SCE in postpartum dairy cows. Primarily, the present study substantiated which miRNAs are circulating in higher abundance in postpartum dairy cows (both H and SCE cows), with miR-486, miR-191, miR-3600, miR-22–3p, miR-192, miR-423–5p, miR-10b, miR-21–5p, miR-27b, and miR-142–5p entering the list of top10 most abundant. In 3 previous studies, 8 (Ioannidis and Donadeu, 2018), 6 (Webb et al., 2020), and 5 (Veshkini et al.,

Table 1. Percentages of common genes between the lists of predicted interacting genes from miRNAs with differential abundance and the list of differentially expressed genes (DEG) identified in luminal epithelial (LE), glandular epithelial (GE), and stromal (ST) endometrial cells between persistent subclinical endometritis (SCE) and healthy (H) cows

Item	Differentially abundant miRNAs	Interacting genes for differentially abundant miRNAs	DEG identified in endometrial cells[1]								
			LE			GE			ST		
			84 DEG total, n (%)	65 over[2], n (%)	19 under[3], n (%)	194 DEG total, n (%)	134 over, n (%)	60 under, n (%)	251 DEG total, n (%)	197 over, n (%)	54 under, n (%)
SCE vs. H	34	10,104	43 (51.2)	29 (44.6)	14 (73.7)	81 (41.8)	43 (32.1)	38 (63.3)	147 (58.6)	112 (56.9)	35 (64.8)
SCE vs. H <0[4]	12	6,905	29 (34.5)	18 (27.7)	11 (57.9)	54 (27.8)	26 (19.4)	28 (46.7)	105 (41.8)	80 (40.6)	25 (46.3)
SCE vs. H >0[5]	22	8,143	35 (41.7)	22 (33.8)	13 (68.4)	62 (32.0)	34 (25.4)	28 (46.7)	118 (47.0)	95 (48.2)	23 (42.6)

[1]Lists of DEG retrieved from Pereira et al. (2022).

[2]Genes overexpressed in SCE compared with H cows.

[3]Genes underexpressed in SCE compared with H cows.

[4]Differentially abundant miRNAs with decreased abundance in SCE compared with H cows.

[5]Differentially abundant miRNAs with increased abundance in SCE compared with H cows.

2022) of these, were identified also as the most abundant miRNAs. The postpartum stage (21 vs. 44 DPP) had no significant effect on the differential abundance of circulating miRNAs. This was especially true for H cows, for which miRNA profiles from the 2 stages appeared remarkably stable, whereas miRNA profiles from SCE cows approached those from H cows at the second stage.

There was a limited correspondence between the differentially abundant circulating miRNAs of the present study and those previously identified in endometrial samples of dairy cows with SCE (Hailemariam et al., 2014; Salilew-Wondim et al., 2016). From the 34 differentially abundant circulating miRNAs found here, 11 were also dysregulated in endometrial samples in the study by Salilew-Wondim et al. (2016), and of these, only miR-361, miR-99b, miR-148a, and miR-151–3p exhibited the same sense of variation. In the study by Hailemariam et al. (2014), the low correspondence was more evident, as only 3 miRNAs were also dysregulated in endometrial samples, and only miR-16a and miR-27a-3p displayed the same sense of variation. Although not fully inconsistent when compared with former studies, the differences in the amount and type of differentially abundant miRNAs may result from the advantage, in this study, of sampling the same cows at 2 different postpartum stages, thus increasing the power of analysis. In addition, results from the above studies are based on endometrial samples, with miRNAs arising mainly from stromal, epithelial and immune cells. While it cannot be ruled out that endometrial-produced miRNAs may reach the bloodstream, we consider it is more likely that the circulating miRNAs in this study originated from other sources, considering the subclinical and localized nature of the condition. Effectively, contradictory to the initial belief that circulating cell-free miRNAs originate from local tissue injury, it has been shown that massive amounts of extracellular circulating miRNAs conveyed within microvesicles, exosomes, or bound to proteins, originate also from other organs such as the liver, muscle, heart, or brain (Turchinovich et al., 2012; Benmoussa and Provost, 2019), and blood cells (Turchinovich et al., 2012; Sunderland et al., 2017).

The present study demonstrates that, despite the subclinical character of the disease and the apparent confinement to the endometrium, SCE is associated with distinct circulating miRNA profiles. These may represent a link between the systemic and endometrial compartments and function as a noninvasive diagnostic biomarker. The circulating miRNAs with differential abundance between SCE and H cows were predicted to interact with half of the DEG identified in the endometrial cells of the same cows (Pereira et al., 2022).

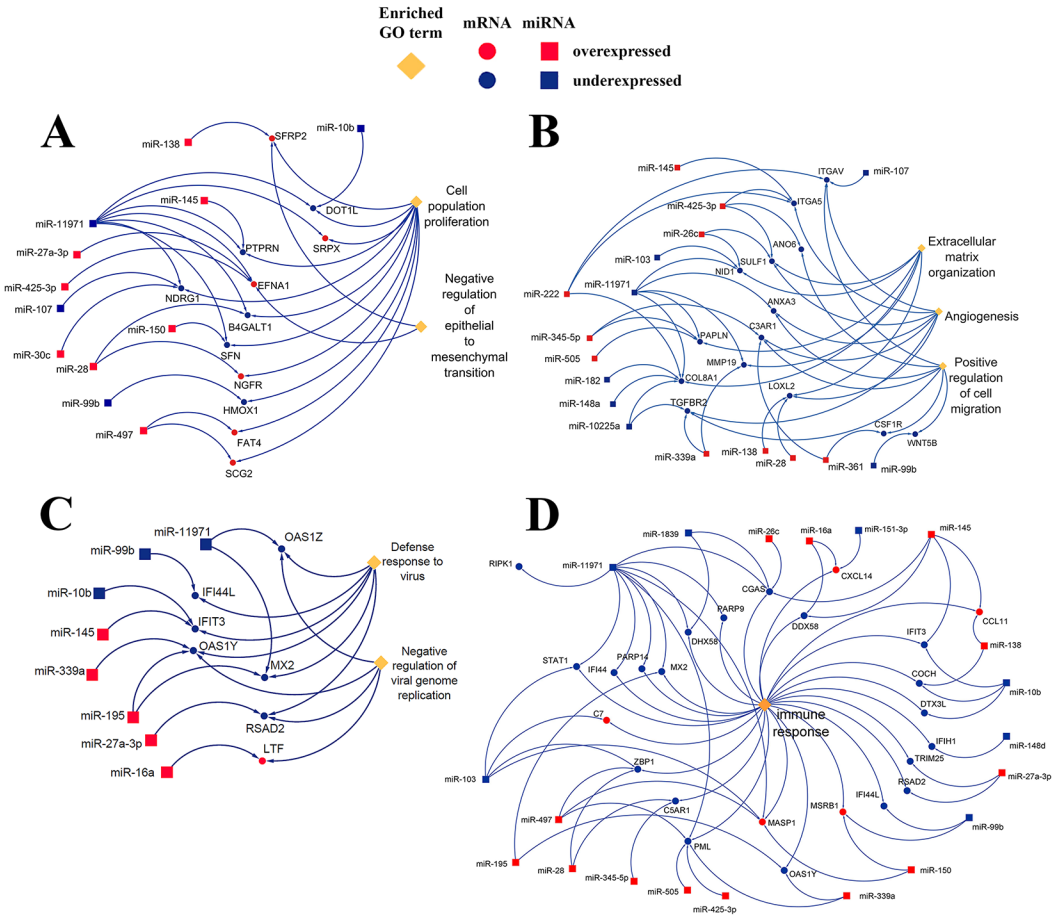

Figure 3. The miRNA-mRNA interaction subnetworks comprising enriched gene ontology (GO) terms in the lists of common genes between miRNA-interacting genes and differentially expressed genes (DEG) identified in luminal epithelial (A), glandular epithelial (B and C), and stromal cells (D).

Therefore, it is hypothesized that if these circulating miRNAs reach endometrial cells, they may regulate post-transcriptional gene expression at the endometrial level. However, how extracellular vesicle-free miRNAs can be delivered into recipient cells and exert cell-cell communication effects remains unknown (Turchinovich et al., 2016; Park, 2017; Zhao et al., 2019). Unraveling these mechanisms would be of great relevance, as in plasma >90% of extracellular miRNAs are vesicle-free (Turchinovich et al., 2016; Park, 2017). In the present work, the extracellular miRNAs' packaging was not characterized. Therefore, the discussion below will only address the functional roles of differentially abundant miRNAs, based on sequence complementarity and published data.

Persistent SCE cows were mainly characterized by an overabundance of known miRNAs compared with H cows. Among the overabundant miRNAs in SCE cows, miR-26a was the most abundant. This miRNA is upregulated by IFN-τ and inhibits the inflammatory damage to bovine endometrial epithelial cells by reducing the secretion of IL-1β, IL-6, and TNF-α (Liu et al., 2022). In mice models, miR-26a is a determinant of M1/M2 macrophage balance (Sahu et al., 2017; Xu et al., 2019), with increased abundance of miR-26a tilting the polarization toward M1 phenotype and production of proinflammatory cytokines. However, miR-26a was also suggested to function as a negative regulator of the hyperinflammatory response via inhibition of PGE2 production (Yu et al., 2022). Although in need of clarification, the role of increased abundance of miR-26a in SCE cows is probably linked to the regulation of inflammation, acting as an anti- or proinflammatory agent.

Persistent SCE cows had increased abundance of miR-145. In dairy cows, miR-145 is upregulated at calving and downregulated in the following 28 d (Veshkini et al., 2022), also being found in metritic cows (Kasimanickam and Kastelic, 2016). This miRNA is known to regulate the proliferation of bovine epithelial cells (Li

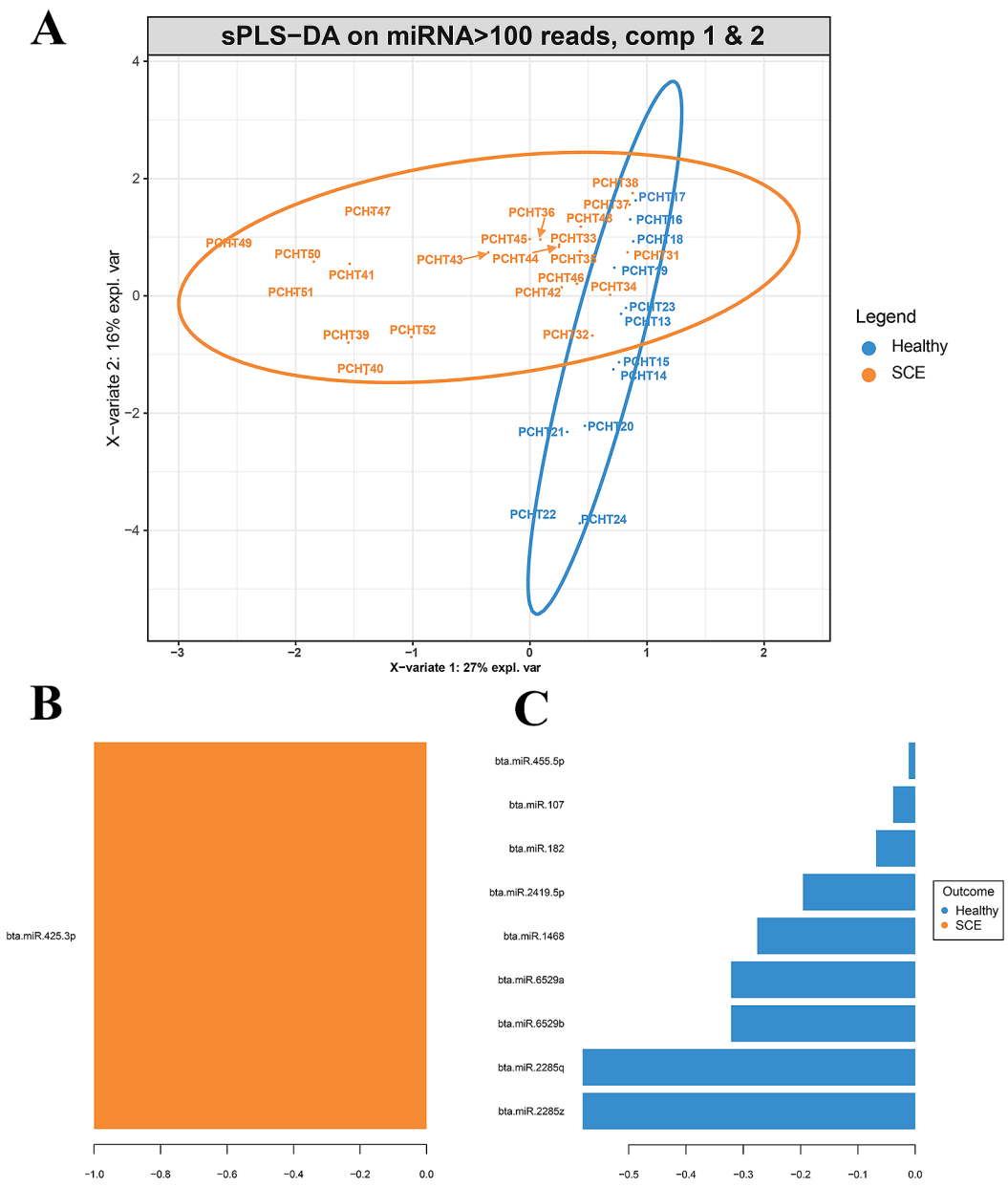

Figure 4. The sPLS-DA performed on the miRNAs data set associated with healthy and subclinical endometritis (SCE) groups. (A) Sample plot including 95% confidence ellipses. (B) The miRNA contribution to component 1. (C) The miRNA contribution to component 2. Colors indicate the animal group in which the median is maximum for each miRNA, blue for healthy and orange for SCE.

et al., 2020) and the inflammatory response of human endometrial cells (Wang et al., 2021), and to interact with insulin receptor substrate 1 (IRS1) in human hepatocytes contributing to insulin resistance (Wang et al., 2014; Wen et al., 2014). Also, miR-195 is highly abundant before calving in dairy cows (Veshkini et al., 2022), and interacts with the insulin receptor (INSR) to contribute to a state of insulin resistance (Nigi et al., 2018). The fact that both miR-145 and miR-195 had increased abundance in SCE cows supports the concept that these cows, may also be affected by a metabolic imbalance, as evidenced by a state of increased insulin resistance and further mobilization of body fat. The above information may be consistent with results from former work showing differences in the levels of some of the markers of fat mobilization (Pereira et al., 2020).

Persistent SCE cows had an increased abundance of miR-30b-5p, which contrasts with the decreased abundance identified by Kasimanickam and Kastelic (2016) in metritic cows. A clue for this discrepancy

may arise from the work of Luoreng et al. (2021), who showed that circulating miR-30b-5p was decreased at 3 d postinduced mammary infection but increased at 5 d postinduced infection. This profile may indicate that circulating miR-30b-5p is decreased in the acute inflammatory response but increased on the way to a subacute or chronic inflammatory response, such as in persistent SCE. Interestingly, miR-30b-5p was shown to suppress lysosomal biogenesis and autophagy (Guo et al., 2021), suggesting that SCE cows may have compromised autophagy processes. Active autophagy can be protective by preventing an excessive inflammatory response through inflammasome activation, the clearance of DAMPs and damaged mitochondria, and degrading inflammatory mediators (Gong et al., 2020).

The abundance of miR-138 was higher in SCE than in H cows. In clinically healthy dairy cows, Veshkini et al. (2022) showed that circulating miR-138 is less abundant at calving but returns to antepartum levels by 28 DPP, regulating key signal transduction pathways associated with energy homeostasis and immune response, contributing to the metabolic adaptation to lactation. In SCE cows, the increased abundance of miR-138 may reflect an impaired or late adaptation of those processes, as miR-138 also stimulates the macrophage inflammatory response, possibly contributing to tissue damage (Bai et al., 2018).

Both miR-26a and miR-150 were in higher abundance in SCE than in H cows. These 2 miRNAs are downregulated upon T-cell activation (Rodríguez-Galán et al., 2018). In particular, miR-150 reduces proliferation, increases apoptosis, and lowers T-cell activation in humans (Sang et al., 2016), suggesting that the peripheral T-cell function may be impaired in SCE cows, contributing to the persistence of inflammation.

The pattern of higher abundance in SCE cows was also observed for miR-222. This miRNA was upregulated by TNF-α and negatively correlated with adiponectin, while dysregulated in adipose and vascular tissues (reviewed by Hulsmans et al., 2011). These authors summarize how miR-222 is associated with endothelial dysfunction, adhesion and infiltration of inflammatory cells into the endothelial space, monocytic differentiation, and regulation of angiogenesis. The abundance of miR-222 was related to body condition, being upregulated during obesity in mice (Xie et al., 2009). Therefore, differences in the abundance of miR-222 between SCE and H cows may reflect TNF-α stimulation levels or adipose tissue metabolism, including the previously reported (Pereira et al., 2020) differences in blood and uterine fluid concentrations of adiponectin.

Several other miRNAs were significantly more abundant in SCE than in H cows. Circulating miR-505 is an anti-inflammatory regulator of LPS-mediated endometritis due to the suppression of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) and the inhibition of HMGB1/NF-κB signaling (Liu et al., 2020). Its higher abundance in SCE cows may represent a response to limit persistent endometrial inflammation. Increased in SCE cows, miR-425–3p was also increased in bovine infected mammary glands (Li et al., 2015). Despite its relatively lower base level, the higher abundance of miR-193a-3p is consistent with its reported role in bovine endometritis, markedly increasing the expression of proinflammatory cytokines induced by LPS, such as IL-1β, IL-6, and TNF-α (Yin et al., 2021).

Although to a lesser extent, some miRNAs had a significantly lower abundance in SCE than H cows. This was the case of miR-148a, contrary to that observed in metritic cows (Kasimanickam and Kastelic, 2016). This miRNA, a member of the miR-148/-152 family, was proposed as a potential therapeutic target for human chronic inflammatory diseases due to its regulatory role in T and B lymphocyte function (Friedrich et al., 2017). Although there is limited knowledge on the participation of miR-148a in the cow uterus immune system, Jiang et al. (2020) and Umar et al. (2021) suggested its potential value as a therapeutic target due to effects on NF-κB activation and decreased production of proinflammatory cytokines such as IL1β and TNFα. Also, Oladejo et al. (2020) hypothesized that miR-148a, through its regulatory effects on tumor growth and immunity, may play a significant role in the pathogenesis of dairy cow endometritis. This may also occur through miR-148a influence in adipose metabolism. In mouse pre-adipocyte cell lines miR-148a promotes adipogenesis by repressing Wnt signaling (Qin et al., 2010). In this context, by displaying a decreased abundance of miR-148a, SCE cows may exhibit impaired adipogenesis, thus extending the negative effects of lipid mobilization in the postpartum period.

In the mice model, the downregulation of proinflammatory cytokines such as IL-6, IL-12, IL-1β, and TNF-α, which are responsible for the activation of dendritic cells to clear the engulfed bacteria, was reported to be modulated by miR-99b (Singh et al., 2013). In persistent SCE cows the underabundance of miR-99b may be associated with the upregulation of these cytokines whose concentrations correlate with the adipokine chemerin, reported earlier to be increased in these animals (Pereira et al., 2020). Thus, it may be speculated that the decreased abundance of miR-99b exerts a positive regulatory effect on the secretion of chemerin and proinflammatory cytokines during the course of endometritis pathogenesis.

The subnetworks of miRNA-mRNA interactions reveal that the disrupted profiles of circulating miRNAs in SCE cows can potentially dysregulate specific endo-

metrial functions, such as cell proliferation, angiogenesis, and immune response, which are also affected in the endometrium of these cows (Pereira et al., 2022). In LE cells, dysregulation of DEG involved in processes of cell proliferation may impair epithelial repair, because signals triggered by tissue damage promote epithelial cell migration and proliferation to replace damaged epithelial cells and restore tissue homeostasis (Lara et al., 2017; Brazil et al., 2019). In particular, *EFNA1*, which can interact with miR-11971, miR-27a-3p, and miR-425–3p, regulates ephrin signaling which is involved in proliferation, protection against endoplasmic reticulum stress, and inflammatory responses in bovine endometrial and mammary epithelial cells (Kang et al., 2018; Lim et al., 2019).

In GE cells, among DEG related to angiogenesis, *ITGAV* can interact with miR-222, *ITGA5* can interact with miR-222, miR-145 and miR-425–3p, and *TGFBR2* can interact with miR-339a. These DEG are involved in the regulation of inflammation in epithelial cells (Mu et al., 2002), suggesting another miRNA-driven mechanism of endometrial function dysregulation in SCE cows. This latter miRNA (miR-339a) can also interact with *MMP19*, which encodes a basement membrane-degrading protease responsible for maintaining epithelial barrier function and for regulating immune response (Brauer et al., 2016).

In both GE and ST cells, DEG included in biological processes of defense response to virus and immune response include IFN-dependent and IFN-stimulated genes such as *RSAD2*, *MX2*, *OAS1Y* and *IFIT3*, which are involved in the maternal recognition of pregnancy (Forde et al., 2011). These genes have the potential to interact with the overabundant miR-339a, miR-195, miR-145, and miR-27a-3p identified in SCE cows, leading to the hypothesis that this dysregulation is detrimental for embryo–maternal crosstalk and contributes to the known lower fertility of SCE cows.

In addition to explaining how disturbed circulating miRNA profiles of persistent SCE cows have the potential to dysregulate specific endometrial functions, this study also demonstrates the possible use of convenient samples (blood) as a source of biomarkers to diagnose SCE and evaluate the likelihood of persistency of the inflammation. Once the emerged biomarker panel with miR-425–3p and miR-2285z is validated/improved in a larger data set, it is feasible to design a diagnostic tool for SCE based on circulating miRNAs. Given the stability of circulating miRNAs (Mi et al., 2013), it is reasonable to assume that a blood sample collected at the farm can be sent to a laboratory in time to accurately analyze miRNAs and identify animals at greater risk of persistent SCE.

CONCLUSIONS

In conclusion, this study first characterized the circulating miRNA profiles of postpartum dairy cows with persistent SCE. Despite the subclinical pattern and apparent confinement to the endometrium, SCE is associated with a distinct circulating miRNA profile, and these distinct miRNA profiles may represent a link between the systemic and endometrial compartments. Cows with SCE show 22 overabundant and 12 less abundant known circulating miRNAs compared with H cows. These dysregulated circulating miRNAs are predicted to interact with transcripts which are differentially expressed in the endometrium of SCE cows. The identified miRNA-mRNA networks have the potential to dysregulate endometrial function and be related to the known negative effect on pregnancy establishment in SCE dairy cows. The emerged miRNA panel, once validated, has the potential to function as noninvasive diagnostic biomarker, a valuable tool to improve the ability to diagnose and predict the persistence of SCE and initiate appropriate early therapy and management policies.

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