Circulating and endometrial cell oxidative stress in dairy cows diagnosed with metritis

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Abstract

Dairy cows diagnosed with metritis may experience a greater degree of oxidative stress (OS) and a deficit in the antioxidative capacity compared to healthy cows. We aimed to assess circulating OS markers and endometrial cell mitochondrial function, intracellular reactive oxygen species (ROS) production, and mean endometrial nuclear cell area in postpartum cows diagnosed with metritis or as healthy. From an initial pool of 121 Holstein cows, we retrospectively selected 34 cows and balanced for metritis (n = 17) or healthy (n = 17). Metritis was defined as an enlarged uterus with red-brown watery or thick off-white purulent discharge occurring within 21 days postpartum. Cows with no signs of clinical disease (including dystocia or retained placenta) were referred to as healthy. Blood samples for serum reactive oxygen metabolites (d-ROM), antioxidants (OXY), and oxidative status index (OSI) tests, evaluated via photometric determination of plasma thiols, were performed at 7, 14, 21, 28, and 35 days postpartum. Furthermore, from the initial pool, a random subset of 5 cows diagnosed with metritis and 6 diagnosed as healthy we collected (at the same time points as for the blood samples) endometrial cytology samples using the cytobrush technique. From the uterine samples, we evaluated the endometrial cell mitochondrial function, intracellular ROS levels, and the endometrial cell nuclear area using MitoTracker Orange, dichlorodihydrofluorescein diacetate, and Hoechst 33258, respectively. Mixed linear regression models, accounting for repeated measurements, were fitted to assess the effect of metritis versus healthy on circulating and endometrial cell OS parameters and endometrial cell size. The effect of days postpartum and its interaction with uterine health status were forced into each model. Serum concentrations of d-ROMs and OSI were greater in metritis at 7, 14, and 35 days postpartum than in healthy cows. Interestingly, the mean endometrial cell nuclear area was lower in metritis than healthy cows at 14 and 21 days postpartum. We found no differences between metritis and healthy for endometrial cell mitochondrial function and intracellular ROS production. In conclusion, cows diagnosed with metritis experienced greater systemic OS levels than healthy cows, but their OS was not higher in the uterine milieu.

Keywords: inflammation; uterine disease; reactive oxygen species; mitochondrial function.

1. Introduction

Negative energy balance and systemic inflammation are well-described physiological conditions that most high-yielding dairy cows experience during the transition period [1,2,3]. Though, maladaptation to these conditions may result in metabolic or infectious disease in the postpartum period [4,5]. Most dairy cows also experience a certain degree of oxidative stress (OS) around the time of calving [6,7,8]; however, these phenomena and their eventual link with the health or disease state of the animals, are not well-depicted in the literature. As per definition, OS is characterized by the imbalance between oxidant versus antioxidant substances in the body [9-11]. Concretely, OS occurs due to the overproduction of reactive oxygen species (ROS) [12,13]. Nevertheless, OS can be particularly challenging to study as, per se, no pathognomonic clinical symptoms directly associated with OS are shown [14].

Half of the postpartum dairy cows experience at least one type of reproductive tract inflammatory disease [15]. However, the inflammatory reaction associated with uterine involution and endometrial regeneration after parturition is stated to be essential for eliminating bacterial contamination from the uterus [16,17]. The role of OS in the development of uterine disease is uncertain. Yet, it is well known that the imbalance between oxidant production and the neutralizing capacity of antioxidants leads to cellular damage and is therefore regarded as the nexus between the intermediary metabolism and the immune system [18,14]. Thus, the increase in the prevalence of transition disease (e.g., metritis) in dairy cows, may be indicative of OS because of the rise in ROS production or defensive antioxidant consumption [19,20,21].

In one study, cows with metritis in the early postpartum had a higher degree of OS and a significant decrease in antioxidant concentrations compared to healthy cows [22]. In mares, the *in vitro* exposure of endometrial cells to lipopolysaccharides induced substantial modifications in the oxidative status and provoked an increase in the endometrial cell mitochondrial function and intracellular ROS production [23,24]. In postpartum dairy cows, to the best of our knowledge, no study has assessed the mitochondrial function or the intracellular ROS status of endometrial cells (*in vivo* nor *in vitro*), while only a few studies assessed systemic OS markers in cows diagnosed with metritis. The objective of the present study was to investigate the serum oxidant/antioxidant status and endometrial cell mitochondrial function, intracellular ROS levels, and the mean endometrial cell nuclear area in cows diagnosed with metritis within 35 days postpartum. The main hypothesis was that cows diagnosed with metritis experience greater systemic levels of OS in serum levels of reactive oxygen metabolites (d-ROMs), and Oxidative status Index (OSI) than healthy cows. Whereas antioxidants (OXY) serum concentrations in healthy cows will be higher than in cows diagnosed with metritis. We furthermore tested the hypothesis that cows suffering from metritis have a higher mitochondrial activity,

intracellular ROS levels, and cell nuclear area at the level of the endometrial cells in comparison to healthy cows.

2. Materials and methods

2.1. Ethical statement

The present study obtained the approval of the Ethical Committee of the Department of Veterinary Science, University of Turin (Italy) (66/10/01/2020). Included procedures did not interfere with the clinical management of the animals and were performed in compliance with the EU Directive 2010/63/CE. All conducted procedures were accomplished in consent with the Italian Ministry of Health guidelines for the care and use of animals (D.L. 4 March 2014 n. 26 and D.L. 27 January 1992 n. 116) and with EU Directive 86/609/CEE.

2.2. Animals housing and management

The present study was carried out at the experimental dairy farm of the University of Turin located at None, Turin, between September 2020 and February 2021. The total number enrolled in this study was 250 Holstein-Friesian cows (83 primiparous and 167 multiparous), the average 305 days of milk production was 10,083 kg/cow, with an average of 4.0% fat and 3.4% protein. Prepartum cows were housed in a free-stall barn and were moved to calving pens when indicators of imminent calving were observed (e.g., swelling of the vulva and pelvic ligament relaxation) or 3 days before the expected calving date. Within 5 days after calving, cows were moved to a free stall lactating pen, where they remained until 35 days postpartum and were fed a totally mixed ration (TMR). Cows were milked twice daily at 0500 and 1700 h in a milking parlor and milk yield was automatically recorded for each cow at every milking by an electronic milk meter (Metatron P21, GEA Farm Technologies). The dairy farm used for this study has forced ventilation with fans and water sprinklers and is able to maintain adequate thermal and humidity conditions all year long limiting the potential effects of heat stress [25,26].

To diagnose metritis, transrectal palpation and uterine discharge evaluation via the gloved hand method were weekly performed [27] until 35 days postpartum. To define metritis, we used the definition of Sheldon et al., [28], which defined metritis as an enlarged uterus with red-brown watery or off-white purulent uterine discharge often but not always accompanied by fever (>39.5ºC) and fetid odour within 21 days postpartum. Cows positive for metritis were treated parenterally with Naxcel Bovini Zoetis 200 mg/ml (ceftiofur 6.6 mg/kg of body weight). If clinical symptoms did not improve 48 hours after treatment, diagnosis and treatment were reassessed [29,30]. Cows with no signs of clinical disease (including dystocia or retained placenta) were referred to as healthy. To assess the metabolic condition in the postpartum period, blood β-hydroxybutyric acid (BHBA) and glucose levels

were weekly checked from calving until 35 days postpartum [31]. Initially, for the sake of a larger, comprehensive study on transition dairy cows, 121 cows that calved during the above-mentioned period were included. For the present study, in which we focused on the oxidant/antioxidant status of transition cows, from the initial pool of animals we randomly selected 34 cows evenly divided between clear cases of healthy (n = 17) and metritis (n = 17) cows. Furthermore, a subset of 12 cows (6 healthy and 6 metritis) was randomly selected for harvesting endometrial samples to evaluate the mitochondrial function, intracellular ROS levels, and mean endometrial cell nuclear area. In total, 170 blood samples and 55 endometrial samples were collected at 7 ± 2, 14 ± 2, 21 ± 2, 28 ± 2, and 35 ± 2 days postpartum (Figure 1). Blood and endometrial samples were collected between 0800 and 0900 h (2 hours before daily feeding).

2.3. Blood sampling and analyses

Blood samples were collected by coccygeal venipuncture into vacuum tubes without anticoagulant (BD Vacutainer serum tube, Precision Glide, Becton Dickinson, Plymouth, UK). After collection, blood tubes were placed in ice for transportation and were centrifuged at 1.500 g for 15 min within 2 hours of collection. Serum was stored in aliquots at -20ºC until analysis.

The definable reactive oxygen metabolites (d-ROMs) were assessed as an indicator of ROS with the standardized [6,22,32] d-ROMs Test (Free Radical Elective Evaluator; *Carpe Diem* systems, automatic analyzer, and micro-plate readers, Diacron International, Grosseto, Italy). This test determines hydroperoxides (breakdown products of lipids and other organic substrates generated by the oxidative attack of ROS), through their reaction with the chromogen N, N-diethylparaphenylenediamine. This reaction is based on Fenton's reaction and therefore depends on the iron released from serum proteins [9,18]. The results are expressed in arbitrary 'Carratelli Units' (CarrU), where 1 CarrU is equivalent to the oxidizing power of 0.08 mg H2O2/dL. According to standards, the normal range of d-ROM is 250-300 CarrU with a linear regression coefficient of R^2=0.9981 and a recovery test of 90-100%. Intra (within-run precision) and inter (day-to-day precision) assay coefficients of variation were 2.07 and 1.79%, respectively [33,34]. As described by Trotti et al., [35], antioxidants were measured using the OXY-Adsorbent Test (Free Radical Elective Evaluator; *Carpe Diem* systems, automatic analyzer, and micro-plate readers, Diacron International, Grosseto, Italy). This test exploits the capacity of a solution of hypochlorous acid (HClO) to oxidize the complete pool of antioxidants in serum, and thus OXY is a measure of the cumulative action of all the antioxidants present in serum rather than simply the sum of measurable antioxidants [11,22,36]. The results are expressed as μmol HClO/mL. The normal range of OXY according to standards is 440-600 μmol HClO/mL of sample and the linear regression coefficient is R^2= 0.9895 and the recovery test is 91-100%. Intra (within-run precision) and inter (day-to-day precision) assay coefficients of variation

were 1.90 and 2.05 %, respectively [37,38]. The degree of Oxidative Stress index (OSi) was calculated as d-ROMs/OXY ×100 [18,16]. The ratio of increase in OSi indicates a higher risk for OS due to an increase in ROS production and/or defensive antioxidant consumption [39].

For the analysis of blood concentrations of BHBA and glucose, we used the FreeStyle Precision Neo™ (FSP; Abbot Diabetes Care Inc., Mississauga, ON, Canada) portable device as described by Jeong et al., [40,41]. The evaluation of BHBA and glucose had the purpose to reveal the presence of ketosis, defined as BHBA ≥1.2 mmol/L, and hypoglycemia (glucose < 2.5 mmol/L) [42,5]. The intra-assay coefficient of variation (CV) was 1.3% and 1.7% for BHBA and glucose analyses, respectively. The inter-assay CV was 2.9% for low BHBA samples and 2.4% for high BHBA samples, whereas the inter-assay CV was 1.7% for low glucose samples and 1.8% for high glucose samples [43].

2.4. Endometrial sample collection and analysis

The perineum of the cows was cleaned with iodide soap and dried with paper towels. A double-guarded sterile cytobrush device (cytology brush equine, Minitube, Tiefenbach, Germany) was introduced into the vagina and guided through the cervix via transrectal palpation. Once the tip of the device reached the uterine body, the cytobrush was exposed from the inner guard. The cytobrush was rotated 3 times against the wall of the uterine body applying some gentle pressure by the index finger through the rectum. The cytobrush was then retracted and removed from the vagina. Once outside the genital tract, the head of the brush was cut with scissors and placed in a 1.5 mL microcentrifuge tube containing 1 mL of phosphate-buffered saline (PBS; Gibco/Thermo Fisher Scientific, Waltham, MA, USA). Endometrial samples were transported on ice to the laboratory within 2 hours after collection.

First, the tubes (Falcon, Becton Dickinson) containing the endometrial samples were vortexed for 1 min to dislodge cells from the cytobrush. For assessing the mitochondrial function, intracellular ROS levels, and mean nuclear area, endometrial cells were washed three times (centrifugation at 300 g for 10 min) in 15 mL tubes containing 1.5 mL PBS with 0.3% bovine serum albumin (BSA) and incubated for 30 min in the same medium containing 280 nM MitoTracker Orange CMTM Ros (Molecular Probes, OR, USA) at 38.5 °C under 5% CO_2. The MitoTracker Orange probe contained a thiol-reactive chloromethyl moiety that passively enters the cell membrane, and the probe is readily sequestered only by active mitochondria, and it can react with accessible thiol groups on peptides and proteins to form an aldehyde-fixable conjugate. After incubation, endometrial cells were washed three times and incubated for 15 min in the same tubes containing 1.5 mL PBS with 0.3% BSA supplemented with 10 μM 2′,7′- dichlorodihydrofluorescein diacetate (H2DCF-DA). H2DCF-DA is membrane-permeant and can diffuse into cells. Once inside the cell, the acetate groups are hydrolysed by intracellular esterase producing H2DCF, a polar molecule retained inside the cell. H2DCF fluoresces when it is oxidized by H_2O_2 or lipid peroxides to produce 2′,7′-dichlorofluorescein (DCF). The level of DCF is

related linearly to that of peroxides, and thus, its fluorescence provides a measure of peroxide levels. Next, cells were fixed with 2% paraformaldehyde in PBS for 2 h. Then, after centrifugation at 300 g for 10 min, cells were stained with 2.5 mg/mL Hoechst 33258 in 3:1 of glycerol to PBS solution and mounted onto a glass slide. Fluorescence intensities were evaluated using a Zeiss epifluorescence microscope (Axiophot 2, Carl Zeiss, Germany) at ×200 magnification. The aggregate red (MitoTracker Orange for mitochondrial function), green (H2DCF-DA for intracellular ROS), and blue dye (Hoechst 33258 for cell nuclei) have an absorption/emission of 551/576, 495/519, and 346/460, respectively [24,44,45] allowing the evaluation of each parameter in the same slide (Figure 2). For the quantification analysis, 5 randomly selected microscope fields were captured, and the fluorescent intensity was evaluated using ImageJ software (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, MD, USA, https://imagej.nih.gov/ij/, 1997– 2018). For evaluating the area of the endometrial cells, we used the freehand method for each cell in ImageJ software. In each image, endometrial cells were selected to measure the area of the cells. Fluorescence intensities are expressed as arbitrary densitometric unit log-transfer nuclear area of the cells detected with the help of Image J software by adjusting the gray threshold value [46,47].

2.5. Statistical analyses

Statistical analysis was performed using the R language for statistical programming (R Core Team, Vienna, Austria, v3.6.0). The function lme of the package nlme [48] was used to fit mixed linear regression models. The effect of sampling day, reproductive tract inflammatory disease status (healthy vs. metritis), and their interaction were forced into each model (base model) to evaluate their association with blood and endometrial parameters. Covariable selection, BCS at enrolment (≤3.5 or ≥3.75) and parity (primiparous or multiparous), was performed using the stepAIC function from the MASS package [49]. It performs a stepwise selection procedure forward and backwards, in- or exclusion of BCS and parity was based on the Akaike information criterion. All models accounted for both repeated measures as well the cows as a random effect. Model residuals were assessed using a scatterplot of the studentized residuals for homoscedasticity, linear predictor for linearity, and a Shapiro-Wilk test for normality. When the residuals of the models were not normally distributed ($P < 0.05$), the raw data was squarely rooted, or log transformed. For all transformed variables, the residuals were normally distributed (Shapiro-Wilk's $P > 0.05$). Differences between levels of explanatory variables were assessed with Tukey's post hoc test. Results are expressed as least squares means and standard errors with their respectively measured units. The level of significance was set at $P \leq 0.05$.

Mikulková et al. [22] found a difference of 0.3 ± 0.6 μmol/L (mean ± SD) in malondialdehyde (a marker of oxidative stress) and 0.1 ± 0.08 mmol/L in total antioxidative status between healthy and

metritis cows. Based on these results, 17 cows per experimental group are enough to detect differences in pro- and antioxidative markers with significance $\alpha = 0.05$ and power $\beta = 0.20$ between cows diagnosed as healthy or with metritis. No power analysis was done for the endometrial cell parameters since no previous data is published in this aspect. Thus, the endometrial cell outcome of the present manuscript should be considered as a pilot study and therefore interpreted with caution.

3. Results

Of the 34 included cows, 17 were classified as healthy [parity 1.2 ± 1.1 (mean ± SD; 6 primiparous and 11 multiparous) and BCS 3.8 ± 0.2], and 17 as metritis (parity 1.3 ± 1.4 (7 primiparous and 10 multiparous) and BCS 3.7 ± 0.2). Among the 34 included cows, a subset of 12 cows was randomly selected for the collection of endometrial cells. Unfortunately, one cow was culled for other clinical reasons, and 11 cows (n = 6 healthy and n = 5 metritis) were considered for the evaluation of mitochondrial function, intracellular ROS levels, and mean nuclear area of the endometrial cells.

The daily mean milk yield was 32.6 ± 11.1 kg (26.4 ± 7.1 kg for primiparous and 38.1 ± 9.9 kg for multiparous cows). The average milk production was higher ($P < 0.03$) in healthy (38.4 ± 1.8 kg) compared to metritis cows (32.1 ± 2.1 kg) at 14 days postpartum (Figure 3).

3.1 Oxidant/antioxidant status and metabolic profile in blood

Serum concentrations of d-ROMs were greater in metritis than healthy cows at 7 ± 2 (150.3 ± 2.90 vs 79.9 ± 3.12 UCarr; $P < 0.001$), 14 ± 2 (105.6 ± 2.91 vs 71.6 ± 3.12 UCarr; $P < 0.001$), and 35 ± 2 days (98.2 ± 2.91 vs 66.1 ± 3.12 UCarr; $P < 0.001$) postpartum (Figure 4). Serum OSi (log_{10}-scale) was lower in healthy than metritis cows at 7 ± 2 (0.07 ± 0.007 vs 0.13 ± 0.007; $P < 0.001$) and 14 ± 2 (0.06 ± 0.007 vs 0.09 ± 0.007; $P < 0.002$) and 35 days (0.05 ± 0.007 vs 0.09 ± 0.007; $P < 0.008$) postpartum (Figure 5). Blood glucose was higher in healthy than metritis cows at 14 days (57.2 ± 2.71 vs 47.8 ± 2.71 mg/dL; $P < 0.01$) postpartum (Supplemental Figures S1). Blood concentrations of BHBA and OXY did not differ ($P > 0.05$) between healthy and metritis cows (Supplemental Figures S2 and S3).

3.2 Mitochondrial function, intracellular ROS levels, and mean nuclear area of endometrial cells

Mitochondrial function and intracellular ROS levels did not differ ($P > 0.05$) between metritis and healthy cows at any day postpartum (Supplemental Figures S4 and S5). Interestingly, the mean endometrial cell nuclear area was greater in healthy compared to metritis cows at 14 ± 2 (136.3 ± 10.7 vs 90.2 ± 12.2 μm^2; $P = 0.001$) and 21 ± 2 days (138.8 ± 10.7 vs 105.6 ± 11.0 μm^2; $P = 0.004$) postpartum (Figure 6).

4. Discussion

This study aimed to investigate serum oxidant/antioxidant status and endometrial cell mitochondrial function, intracellular ROS levels, and endometrial cell nuclei area in cows diagnosed with metritis within 21 days postpartum. Our results support the hypothesis that cows diagnosed with metritis experience greater systemic levels of OS including higher serum levels of d-ROMs and OSI than healthy cows. Interestingly, the mean endometrial cell nuclear area was lower in metritis cows in the in the second and third week postpartum compared to healthy cows. However, the mitochondrial activity and the intracellular ROS levels did not differ between groups. Oxidative stress markers can be used as a management tool to monitor the early stages of the uterine disease and implement nutritional strategies with antioxidants. However, larger studies should be performed to assess systemic OS markers' cut-off points and their association with reproductive tract disease.

Oxidative stress plays a crucial role in several pathological conditions directly linked with animal production, reproduction, and welfare [50,6]. In this regard, the postpartum period is generally characterized by the depletion of antioxidants resulting in an imbalance between prooxidants (e.g., d-ROM) and antioxidants (e.g., OXY) [18,34,36]. As a marker of ROS, d-ROM is used to detect changes in ROS caused by an increase in oxidants after parturition triggered by the metabolic challenges associated with the commencement of lactation [32,33,38]. Metabolic stress linked to milk production contributes to a high level of ROS production during lactation [37,39]. Therefore, to identify a clear signal associated with metritis, we used clinically healthy cows from calving to 35 days postpartum, excluding cows with other clinical diseases during the study period. Based on the results of the present study, we confirm the association found by Mikulková et al., [22] that OS was greater at multiple days postpartum in cows diagnosed with metritis in comparison to healthy cows. Furthermore, as suggested by other authors [18,22], OSi was greater in the first two weeks postpartum in cows that developed metritis in the present study. This suggests that subjects with an active uterine infection (and inflammation) within the first two weeks postpartum, tend to maintain a high level of OS even later in the postpartum period. Thus, our study using OS reveals their potential as markers for the early identification and hence for an eventual early intervention to cease the development of metritis. However, to use these indices as diagnostic parameters or risk factors, it becomes vital to accurately standardize "basal" values of OS and define if they can be used as cut-offs for uterine disease [17].

Results regarding milk yield and blood BHBA and glucose in cows diagnosed with metritis versus healthy, our data agree with the published literature. Dervishi et al., [51] reported that milk yield was lower in cows with metritis during the postpartum period, with an average decrease in daily milk yield of 7 kg at several d postpartum. Furthermore, our results are in agreement with the study by Barragan et al., [52], in which there were no changes in blood BHBA concentrations between healthy or cows with metritis. However, we found a modest increase in blood glucose in healthy compared to metritis

cows at 14 days postpartum. This may be associated with the glucose dependent neutrophil activation at the peak of metritis occurrence, which often happen in the second week postpartum [27,28].

Mitochondria are responsible for producing ROS and energy, sustaining the normal function of cells and tissues. It has been reported that mitochondrial dysfunction may lead to the increased mammary OS and impaired milk yield [53,54]. However, little is known about how mitochondria regulate ROS generation and energy metabolism when confronted with disease. Mitochondria cannot efficiently operate in high levels of OS, and they rapidly lose their integrity, leading to a failure of the energetic system of the affected cell. We did not find differences in mitochondrial function and intracellular ROS production of endometrial cells in healthy cows or those diagnosed with metritis. Since there is inherent damage after parturition in the endometrium of all postpartum cows, it seems that this damage equally affects the endometrial cells of healthy cows and cows with metritis in the early postpartum period. However, it is unclear why these differences were not evident in endometrial samples collected after 28 d postpartum, a time period in which healthy cows would have completed their normal uterine involution. Probably, subclinical inflammation of the uterine endometrium in 'healthy' cows may have played a role, but, in the present study, we did not check for subclinical endometritis. Moreover, although we sampled for endometrial mitochondrial function and intracellular ROS production at multiple days postpartum, the number of cows included in these analyses was low, making this a limitation of the present study. On the other hand, we found that cows with metritis showed lower mean endometrial cell nuclear area values than healthy cows in the from the second to the third week postpartum. In humans, Fu et al., [55] found the mean nuclear area as the most important parameter for differentiating between various endometrial conditions. The heightened inflammatory profile associated with active uterine neutrophil function and/or bacterial enzymes may have changed the morphological landscape of endometrial cells, so their cell surface is smaller (and sometimes partially destroyed or distorted) in cows with metritis indicating stress on the uterine cells.

5. Conclusion

Elevated blood markers for OS were observed in cows diagnosed with metritis. These findings provide a new avenue for research for potential supportive treatment for metritis. We found no evident differences in OS markers in the endometrial cells of metritis versus healthy cows. In addition, baseline levels of oxidative status biomarkers under field conditions for commercial high-yielding dairy cows should be identified, which will bring them a step forward to their applicability in the field.

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Credit authorship contribution statement

Sanjana Malledevarahalli Chandrappa: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Osvaldo Bogado Pascottini:** Conceptualization, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization, Supervision. **Geert Opsomer:** Conceptualization, Writing - review & editing, Supervision, Acquisition. **Giorgia Meineri:** Investigation, Resources, Funding, Acquisition, Project administration. **Nicola Antonio Martino:** Methodology, Investigation, Validation, Writing - review & editing. **Penelope Banchi:** Writing - review & editing. **Leila Vincenti:** Resources, Data curation, Writing - review & editing, Project administration. **Alessandro Ricci:** Conceptualization, Methodology, Investigation, Resources, Writing - review & editing, Supervision, Funding, Acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at

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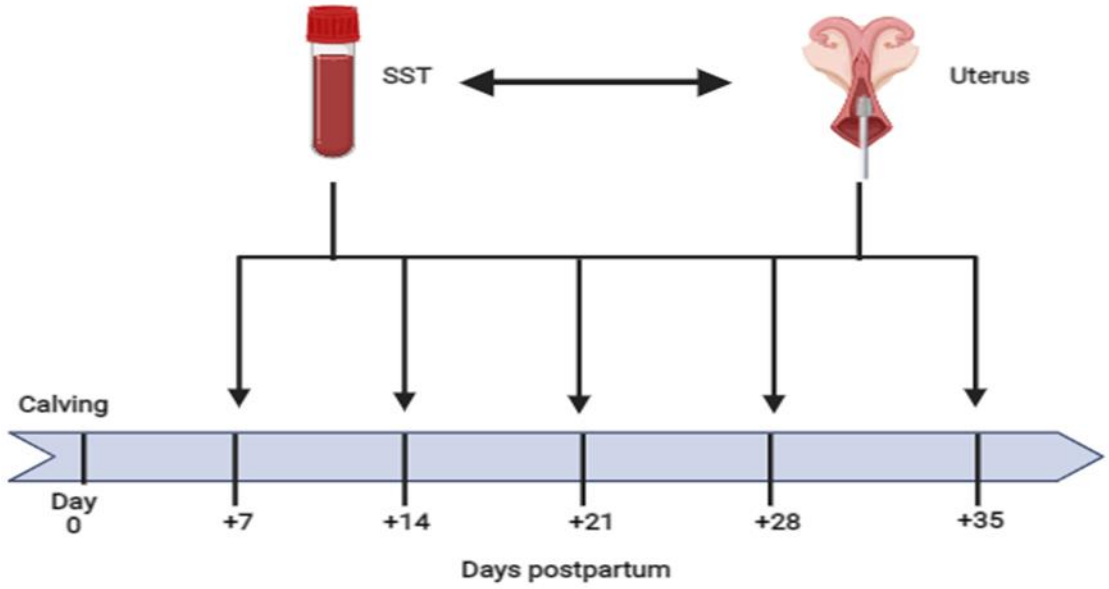
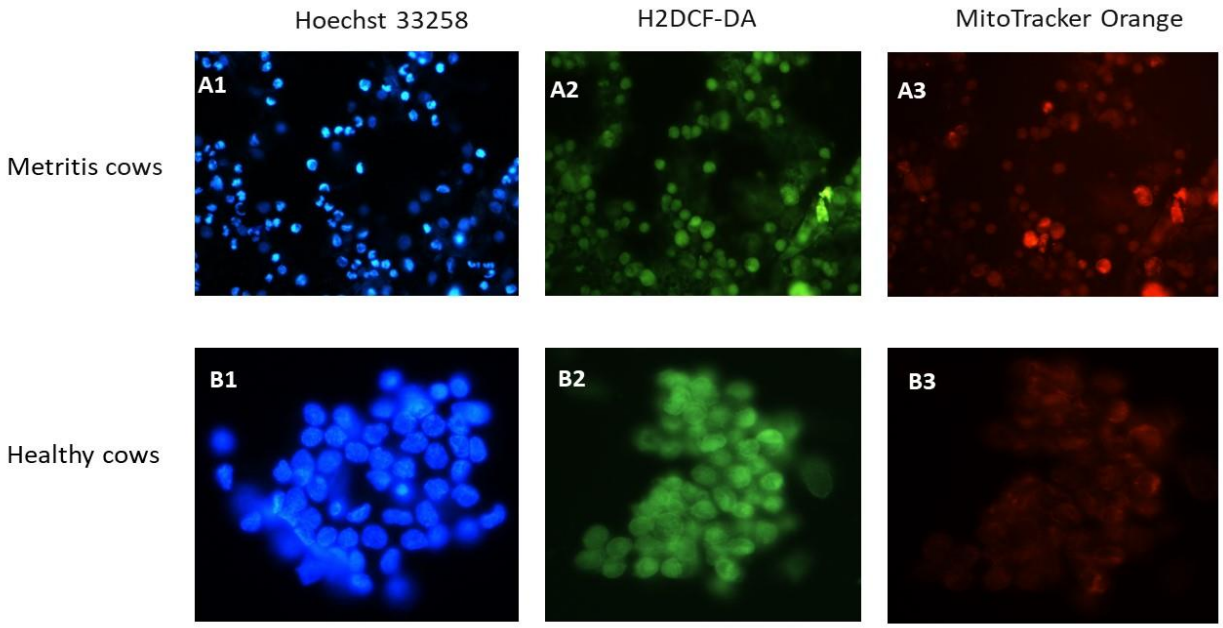
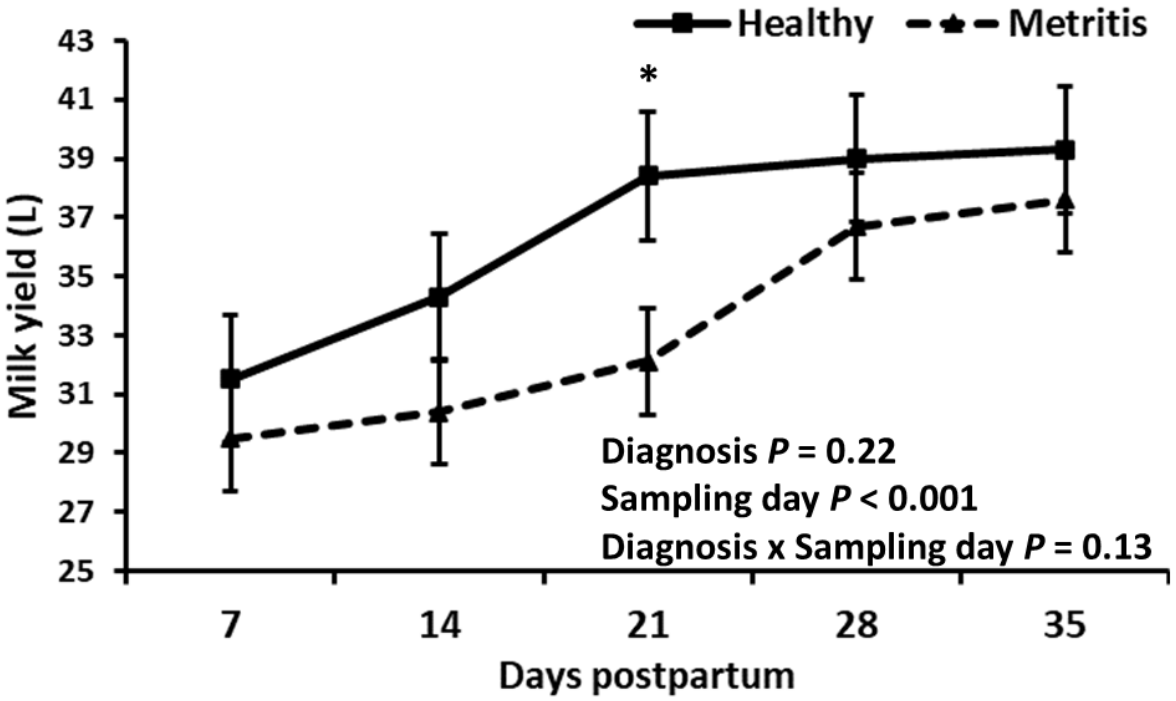


Figure 1. Schematic overview of the experimental design showing the selected time points for sample collection. Blood samples from 34 dairy cows (17 healthy and 17 with metritis) were collected from the coccygeal vessels in blood tubes containing a clot activator (serum separation tube, SST). Endometrial cytobrush samples were collected from 11 dairy cows (6 healthy and 5 with metritis).

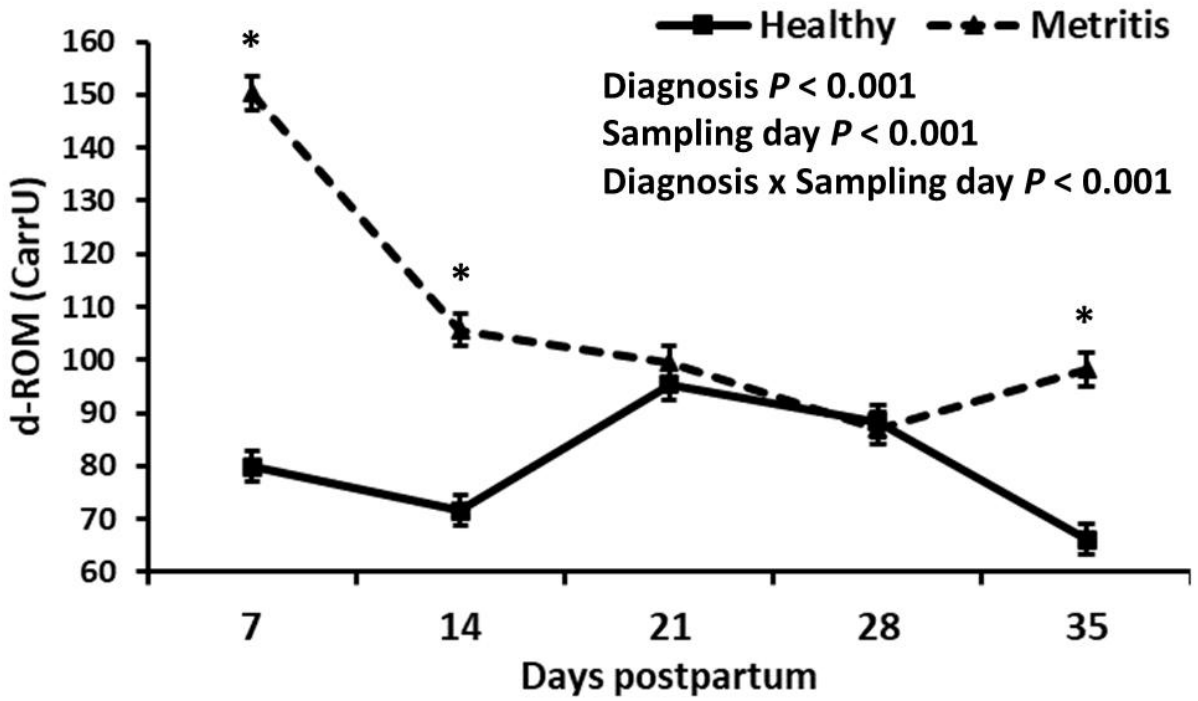


462 **Figure 2.** Representative images of metritis and healthy cows of three different epifluorescence
463 images showing, A1 and B1 cell nuclei (Hoechst 33258), A2 and B2 intracellular reactive oxygen
464 species (H2DCF-DA), A3 and B3 mitochondrial function (Mito Tracker Orange).



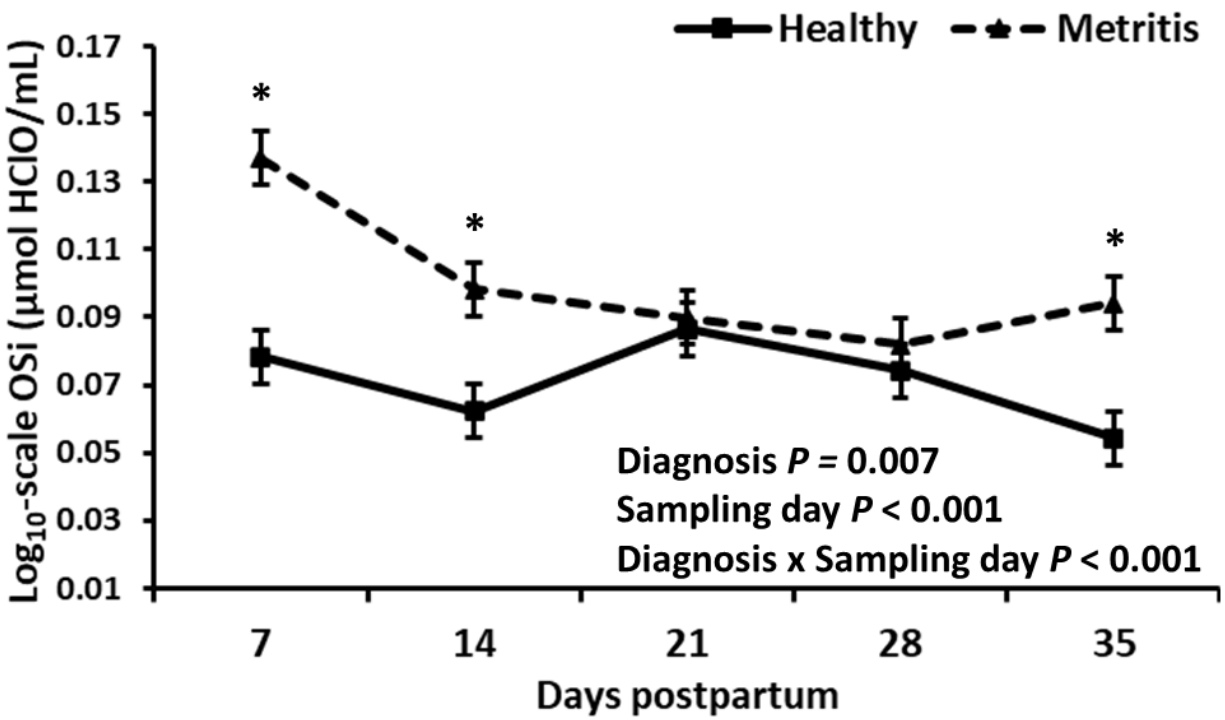
465

466 **Figure 3.** Least square means ± standard errors of milk yields in 34 Holstein cows. Groups consisted
467 of cows diagnosed healthy (n = 17) or metritis (n = 17) with 35 days postpartum. *Milk yield was
468 greater for healthy than metritis cows 21 days postpartum ($P < 0.03$).



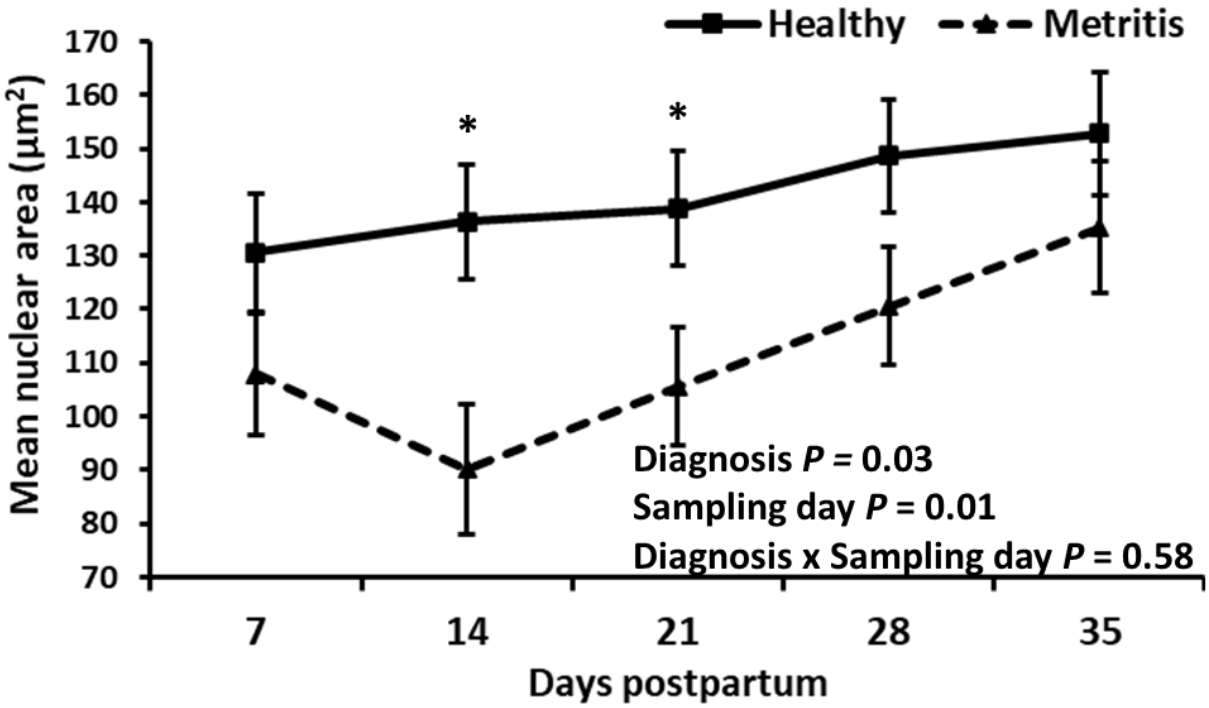
469

470 **Figure 4.** Least square means ± standard errors of serum reactive oxygen metabolites (d-ROM)
471 concentrations in 34 Holstein cows. Groups consisted of cows diagnosed healthy (n = 17) or metritis
472 (n = 17) with 35 days postpartum. *Serum d-ROM was greater for metritis than healthy cows at 7 (P
473 < 0.001), 14 (P < 0.001), and 35 days postpartum (P < 0.001).



474

475 **Figure 5.** Log_{10}-scale least square means ± standard errors of serum oxidative status index (OSi)
476 concentrations in 34 Holstein cows. Groups consisted of cows diagnosed healthy (n = 17) or metritis
477 (n = 17) with 35 d postpartum. *Serum OSi was greater for metritis than healthy cows at 7 ($P <$
478 0.001), 14 ($P < 0.002$), and 35 days postpartum ($P < 0.008$).



479

480 **Figure 6.** Least square means ± standard errors of the mean nuclear area (µm^2) in 11 Holstein cows.
481 Groups consisted of cows diagnosed healthy (n = 6) or metritis (n = 5) with 35 d postpartum. *Mean
482 nuclear area was greater for healthy than metritis cows at 14 (*P* = 0.01) and 14 days postpartum (*P* =
483 0.04).