



Evaluation of peripartal calcium status, energetic profile, and neutrophil function in dairy cows at low or high risk of developing uterine disease

N. Martinez,* C. A. Risco,† F. S. Lima,* R. S. Bisinotto,* L. F. Greco,* E. S. Ribeiro,* F. Maunsell,† K. Galvão,† and J. E. P. Santos*¹

*Department of Animal Sciences, University of Florida, Gainesville 32611

†College of Veterinary Medicine, University of Florida, Gainesville 32610

ABSTRACT

In this prospective cohort study, Holstein cows considered to be at high risk of developing metritis (dystocia, twins, stillbirth, retained placenta, or their combination) were matched with herd mates at low risk of developing metritis (normal calving) and monitored daily for rectal temperature and uterine discharge during the first 12 d in milk (DIM). Blood was sampled on d 0, 1, and 3 postpartum for assessment of neutrophil phagocytic and oxidative burst activities. Blood was also sampled at 0, 1, 2, 3, 4, 7, and 12 DIM for determination of serum concentrations of Ca, K, Mg, nonesterified fatty acids, β -hydroxybutyrate, and glucose. On the basis of receiver operator characteristic curves, subclinical hypocalcemia (SCH) was defined as a serum Ca concentration ≤ 8.59 mg/dL in at least 1 sample in the first 3 DIM. The overall incidences of metritis and puerperal metritis were 47.3 and 30%, respectively. Concentration in blood and percentages of neutrophils undergoing phagocytosis and oxidative burst were all reduced in cows with SCH compared with normocalcemic cows. Cows with SCH were at a greater risk of developing fever, metritis, and puerperal metritis compared with normocalcemic cows. Among cows at low risk of developing metritis, those with SCH had a greater incidence of metritis (40.7%) compared with normocalcemic cows (14.3%). Similarly, among cows at high risk of developing metritis, cows with SCH had a greater incidence of metritis (77.8%) compared with normocalcemic cows (20.0%). Cows with SCH had elevated concentrations of nonesterified fatty acids and β -hydroxybutyrate compared with normocalcemic cows. The relative risk of developing metritis decreased by 22% for every 1 mg/dL increase in serum Ca. Neither metritis nor SCH influenced the resumption of estrous cyclicity by 38 DIM, but cows with SCH had a reduced pregnancy rate and a longer interval to preg-

nancy compared with normocalcemic cows. Finally, the population risk to develop uterine diseases attributable to SCH was 66.6% for metritis and 91.3% for puerperal metritis in the present study.

Key words: dairy cow, metritis, neutrophil function, subclinical hypocalcemia

INTRODUCTION

The synthesis and secretion of colostrum by dairy cows imposes a large drain in Ca in the first days postpartum. Because Ca loss in colostrum and milk exceeds the plasma pool by several fold, homeostatic mechanisms must be in place to maintain blood concentrations during this period (Goff, 2008). The combined increased nutrient needs for synthesis of colostrum and milk with the decline in feed intake during late gestation generally results in dairy cows undergoing a state of negative nutrient balance during the first 4 to 6 wk of lactation. This negative nutrient balance induces cows to mobilize body tissues, often characterized by increases in serum concentrations of NEFA and BHBA, which have been used to predict the risk of postparturient diseases (Ospina et al., 2010; Chapinal et al., 2011). The ability of cows to overcome these nutrient shortages may influence health and performance during the subsequent lactation.

The incidence of milk fever in US dairy herds averaged 5.2% based on a survey conducted in 2002 (NAHMS, 2002). More recent data from 55 dairy herds from 10 different states in the United States and Canada indicated that the incidence of milk fever decreased and averaged 2.4%, probably because of the increased adoption of feeding acidogenic salts in prepartum diets. Nevertheless, despite the application of acidogenic diet formulations prepartum, the prevalence of subclinical hypocalcemia (SCH) remains at 25 and 47% in primiparous and multiparous cows, respectively (Reinhardt et al., 2011). The importance of hypocalcemia extends beyond its clinical symptoms; preliminary data from cows induced to have SCH indicated that feed intake and rumination were depressed (Hansen et

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¹Corresponding author: jepsantos@ufl.edu

al., 2003). Cows with hypocalcemia are more likely to develop a displaced abomasum (Chapinal et al., 2011; Seifi et al., 2011), ketosis, dystocia (Curtis et al., 1983), and uterine prolapse (Risco et al., 1984), likely mediated by the effects of low ionized Ca (Ca^{2+}) suppressing smooth muscle contractions (Hansen et al., 2003). Furthermore, hypocalcemic cows have increased plasma concentrations of cortisol (Horst and Jorgensen, 1982), a reduced proportion of neutrophils with phagocytic activity (Ducusin et al., 2003), and reduced concentrations of cytosolic Ca^{2+} in mononuclear cells (Kimura et al., 2006). This reduction in measures of immune cell function has linked hypocalcemia to periparturient diseases such as retained placenta (Melendez et al., 2004) and mastitis (Curtis et al., 1983). Although the consequences of SCH have not been fully established, early-lactation cows with low serum Ca in the first 7 DIM had an increased incidence of subclinical ketosis (Ribeiro et al., 2011). Energy status in late gestation and early lactation has been linked to immune function in transition cows (Hammon et al., 2006). In fact, cows that developed metritis and later subclinical endometritis had increased concentrations of NEFA and BHBA early postpartum and had neutrophils with less intracellular glycogen (Galvão et al., 2010). Collectively, these data suggest that the impairment of peripartum energy and Ca metabolism increases the risk of diseases in early lactation, presumably by limiting the supply of glucose and Ca^{2+} for adequate immune function.

The hypothesis of the current study was that SCH or a reduction in serum Ca concentration after calving is associated with an impairment of neutrophil function and with an increased risk of uterine diseases. Therefore, the objectives of the present study were to establish the relationships between serum Ca concentrations and energy metabolites, neutrophil function, and the incidence of uterine diseases in early-postpartum dairy cows considered to be at low or high risk of developing metritis.

MATERIALS AND METHODS

Animals, Housing, and Diets

The study was approved by the animal care and use committee of the University of Florida. One-hundred and ten Holstein cows between 1 and 5 lactations from a commercial dairy in Central Florida milking 4,400 cows and with a rolling herd average of 10,400 kg of milk were used in this study. Primiparous and multiparous cows were housed together during the last 3 wk of gestation and first 2 wk postpartum, and separately thereafter. Cows were milked 3 times daily, and milk yield was measured once monthly. Pre- and postpartum

cows were fed their respective diets as TMR offered twice daily for ad libitum intake. The prepartum diet was formulated to have a negative calculated DCAD by limiting the supply of Na and K and increasing the amount of supplemental Cl. The ingredient composition and nutrient content of the diets offered to pre- and early-postpartum cows are depicted in Table 1.

Study Design

This study followed a prospective observational design. A cow with one or more calving disorders, such as dystocia (assistance >15 min), twins, stillbirth, and retained placenta (>12 h after delivery), was enrolled in the study and considered to be at high risk of developing metritis (**HRM**). Each HRM cow was matched, based on day of calving and parity, to a cow with normal calving, which was defined as being at low risk of developing metritis (**LRM**). None of the cows enrolled in the study received any product containing supplemental Ca or gluconeogenic precursors, or any antimicrobial therapy for the prevention of uterine diseases.

Definition and Diagnosis of Metritis and Puerperal Metritis

The rectal temperature of cows was measured daily in the first 12 DIM, using an electronic thermometer (GLA Agricultural Products, San Luis Obispo, CA), immediately after the morning milking. Cows with a rectal temperature $\geq 39.5^{\circ}\text{C}$ were considered febrile. All cows were evaluated for vaginal discharge at 4, 7, and 12 DIM for the diagnosis of metritis. Additionally, vaginal discharge was evaluated at any day during the first 12 DIM in which rectal temperature was $>39.1^{\circ}\text{C}$ or cows presented an abnormal attitude (inappetent or dull, sunken eyes). Vaginal discharge was obtained by transrectal palpation by retracting the cervix and uterus to obtain fluid of uterine origin. When no discharge was retrieved, vaginal discharge was obtained by using a Metrichick device (Metrichick, Simcro, New Zealand). Metritis was diagnosed based on the presence of an enlarged uterus and a watery, fetid, reddish-brownish uterine discharge. The presence of fever concurrent with the diagnosis of metritis was indicative of puerperal metritis.

Cows with an abnormal attitude, fever, or both, were subjected to a complete physical exam. Cows diagnosed with metritis only were treated with 2 uterine boluses containing 3 g of tetracycline hydrochloride each (Tetracycline Soluble Powder 324; Teva Animal Health, St. Joseph, MO). The treatment was repeated once 2 d after the first treatment. Cows with puerperal metritis received a ceftiofur hydrochloride sterile suspension

Table 1. Ingredient and nutrient composition of pre- and postpartum diets (DM basis)

Item	Diet	
	Prepartum	Early postpartum
Ingredient, %		
Oat hay	17.2	—
Sorghum silage	31.1	—
Corn silage	26.7	40.0
Rye silage	—	15.0
Citrus pulp	—	5.4
Corn grain	6.7	10.8
Distillers dried grains	3.4	2.4
Molasses	—	0.5
Whole cottonseed	—	4.8
Soybean meal, 48% CP	3.4	5.0
Protein supplement ¹	3.4	9.0
Brewers' grains	3.8	5.0
Prepartum mineral supplement ²	1.9	—
Postpartum mineral supplement ³	—	1.9
Bovachlor ⁴	2.3	—
Nutrient profile		
NE _L , ⁵ Mcal/kg	1.49	1.67
CP, %	14.6	19.3
NDF, %	43.5	35.8
ADF, %	26.7	21.2
Starch, %	19.7	20.2
Ether extract, %	3.6	4.7
Ca, %	0.78	0.85
P, %	0.33	0.43
Mg, %	0.31	0.33
K, %	1.28	1.49
Na, %	0.17	0.40
Cl, %	1.25	0.66
S, %	0.23	0.27
DCAD, ⁶ mEq/kg	−94	+202

¹Protein supplement contains the following (DM basis): 23.8% Pro-Lak (blend of marine and animal by-products; H. J. Baker & Bro. Inc., Stamford, CT), 52.2% Amino Plus (cooker-processing soybean meal; Ag Processing Inc., Emmetsburg, IA), and Mepron (carbohydrate-protected DL-methionine; Evonik Degussa Corporation, Kennesaw, GA).

²Each kilogram of prepartum mineral supplement contains the following (DM basis): 22.5% Ca, 10.3% NaCl, 4% Mg, 5,000 mg of Zn, 3,000 mg of Mn, 850 mg of Cu, 39 mg of I, 12 mg of Co, 30 mg of Se, 363,000 IU of vitamin A, 108,000 IU of vitamin D₃, and 2,180 IU of vitamin E.

³Each kilogram of postpartum mineral supplement contains the following (DM basis): 25.0% distillers grains, 2.0% molasses, 29.1% CaCO₃, 20.0% NaHCO₃, 10.5% NaCl, 6.3% MgO, 4.3% Dynamate (mixture of K₂SO₄ and MgSO₄; The Mosaic Company, Plymouth, MN), 2.2% vitamin-trace mineral premix (mixture of Co, Se, I, Cu, Mn, Zn, and vitamins A, D, and E), 1,200 mg of monensin (Rumensin 90; Elanco Animal Health, Greenfield, IN), 60 mg of biotin (Rovimix Biotin; DSM Nutritional Products LCC, Parsippany, NJ), and 0.1% Availa 4 (Zinpro Corporation, Eden Prairie, MN).

⁴Bovachlor (Westway Feed Products LLC, Clewiston, FL) contains the following (DM basis): 60% CP, 0.4% Ca, 0.7% Mg, 2.2% K, 2.2% Na, 0.4% S, 24.4% Cl, and 22.2% inverted sugars.

⁵Calculated at 11 and 19 kg of DM/d for the pre- and postpartum diets (CPM-Dairy version 3.0.10; <http://cahpwww.vet.upenn.edu/node/77>).

⁶Dietary cation-anion difference calculated as follows: DCAD = (mEq of Na + mEq of K) − (mEq of S + mEq of Cl).

(Excenel RTU EZ; Pfizer Animal Health, Madison, NJ) for 3 consecutive days at a dose of 2.2 mg/kg of BW. Supportive therapy with oral and intravenous fluids was administered to cows with puerperal metritis according to farm operational procedures. Incidences of mastitis, digestive problems, and respiratory disease in the first 12 DIM were also recorded for individual cows.

Neutrophil Function

Blood was collected from the coccygeal vein or artery into evacuated tubes containing heparin at 0, 1, and 3 DIM. Whole blood was analyzed for total and differential leukocyte counts using an automated hematology analyzer (ProCyte Dx Hematology Analyzer; Idexx Laboratories, Westbrook, ME).

Flow cytometry was used to gate granulocytes and evaluate phagocytic and oxidative burst activities. Because neutrophils represented >90% of the granulocyte population in the blood of cows in the study, we inferred that the leukocyte activities measured referred primarily to neutrophils.

For the in vitro neutrophil function analysis, phagocytic and oxidative burst activities were measured by using a dual-color flow cytometry (Silvestre et al., 2011). *Escherichia coli* isolated from bovine mastitic milk was used to challenge the neutrophils. The inoculant was maintained in tryptic soy broth media covered with glycerol and kept frozen at −80°C. After thawing, a cotton swab was dipped into the inoculant and transferred to another vial with broth media and kept at 37°C until it reached a concentration of approximately 5×10^5 bacteria/μL, after which the bacteria were kept at 5°C. The intact bacteria were then heat-inactivated at 56°C for 30 min, carefully homogenized, and split into 10-mL vials for labeling. Each tube was centrifuged at 2,200 rpm for 30 min. After centrifugation, the supernatant was discarded and the bacteria were resuspended with 9 mL of PBS + 1 mL of propidium iodide. The vial was wrapped in aluminum foil to protect it from light and maintained on continuous rotation overnight at room temperature for labeling. Twelve hours later, the vials were centrifuged at $960 \times g$ for 30 min at 23°C and resuspended in 10 mL of PBS twice to eliminate excess propidium iodide in the media. The propidium iodide-labeled bacteria were protected from light and stored at 5°C until use. Leukocytes in 100-μL aliquots of blood from each cow were loaded with 10 μL of a 50 μM dihydrorhodamine 123 solution for 10 min at 37°C with continuous mixing. Aliquots of propidium iodide-labeled *E. coli* were added to achieve a bacteria-to-neutrophil ratio of 40:1. Each sample had a negative control tube containing dihydrorhodamine 123-loaded leukocytes in blood without bacteria and a

positive control tube containing phorbol 12-miristate 13-acetate, which chemically induces neutrophil oxidative burst. After incubation at 37°C for 30 min with continuous mixing, the tubes were placed on ice to stop neutrophil phagocytosis and oxidative burst activities. An automated lysing system (Q-Prep Epics Immunology Workstation; Coulter Corp., Miami, FL) was used to add reagents, followed by the addition of 500 μ L of water to each tube for completion of hemolysis. Extracellular fluorescence was suppressed with 10 μ L of 0.4% trypan blue solution. The samples were taken to the flow cytometer for reading within 2 h of preparation.

The samples for neutrophil function were acquired on a flow cytometer equipped with a 488-nm argon-ion laser for excitation at 15 mW (FACSort; Becton Dickinson Immunocytometry Systems, San Jose, CA). Density cytograms were generated by linear amplification of the signals in the forward scatter and side scatter channels. Fluorescence cytograms were based on a logarithmic amplification of the fluorescence signals of each cell as it passed through the argon-ion laser beam. Neutrophils were selectively analyzed on the basis of their size and complexity in the density cytogram, and data from the acquisition of 10,000 cells per sample were processed by computer software (CellQuest, version 3.3; Becton Dickinson Immunocytometry Systems, San Jose, CA). Parameters quantified from the density and fluorescence cytograms included the percentage of neutrophils that contained red fluorescence, indicating phagocytosis of propidium iodide-labeled *E. coli*, and the percentage of neutrophils with red and green fluorescence, indicating oxidative burst. Additional analysis included the mean fluorescence intensity of the red wavelength, indicating the mean number of bacteria phagocytized by neutrophils, as well as the mean fluorescence intensity of the green wavelength, an indicator of the mean amount of reactive oxygen intermediates produced per neutrophil. The samples were run in duplicate with an intraassay CV of 5.4%.

Serum Concentrations of Ca, Mg, K, NEFA, BHBA, and Glucose

Blood was collected from the coccygeal blood vessels into evacuated tubes without an anticoagulant at 0, 1, 2, 3, 4, 7, and 12 DIM. Samples were allowed to clot and were then placed on ice until processing. Within 6 h of collection, samples were centrifuged, and serum was harvested and frozen at -20°C until analysis.

Serum samples were analyzed for concentrations of Ca, Mg, and K by using an atomic absorption spectrophotometer (AAnalyst 200; Perkin-Elmer Inc., Waltham, MA) with the flame technique and acetylene gas. A solution of 0.1% lanthanum (1.766 g of lantha-

num chloride heptahydrate diluted in 1 L of deionized water) was used to dilute serum samples and as a blank solution for measurement of Ca, Mg, and K. After dilution 1:50 of serum in lanthanum, the sample was mixed thoroughly and aspirated by the machine. A hollow cathode lamp was used to detect Ca and Mg with wavelengths of 422.7 and 285.2, respectively, and another hollow cathode lamp was used for K, using a wavelength of 766.5. Inter- and intraassay CV were 1.47 and 1.61% for Ca, 4.45 and 1.84% for Mg, and 5.13 and 1.42% for K, respectively.

Commercial kits were used to determine serum concentrations of NEFA (NEFA-C Kit; Wako Diagnostics Inc., Richmond, VA, as modified by Johnson and Peters, 1993) and BHBA (Wako Autokit 3-HB; Wako Diagnostics Inc.). Inter- and intraassay CV were 6.2 and 4.2% for NEFA, and 8.6 and 3.3% for BHBA, respectively. Concentrations of glucose in serum were determined by colorimetric continuous flow analysis (Autoanalyzer II; SEAL Analytical, Segensworth, Fareham Hampshire, UK) by using a modification of the method described by Gochman and Schmitz (1972).

Ovarian Cyclicity, Clinical Endometritis, and Subclinical Endometritis

Resumption of ovarian cyclicity postpartum was evaluated at 24, 31, and 38 DIM by ultrasonography (Easi-Scan equipped with a 7.5-MHz linear transducer; BCF Technology, Livingston, UK), and cows with at least 1 corpus luteum recorded on these days were considered to be estrous cyclic, whereas those without a visible corpus luteum were considered anovular.

Clinical endometritis was assessed at 31 \pm 1 DIM using the Metrichick tool and was defined as a vaginal discharge score \geq 2, based on the scoring system established by Sheldon et al. (2006; 0 = clear or translucent mucus; 1 = mucus containing flecks of white or off-white pus; 2 = discharge containing \leq 50% white or off-white mucopurulent material; and score 3 = discharge containing $>$ 50% purulent material). Subclinical endometritis was diagnosed at 38 \pm 1 DIM by endometrial cytology using a cytobrush. Endometrial cytology slides were air-dried and fixed with fixative solution for 30 s, and then immersed 25 times at 1-s intervals in solution I and 25 times at 1-s intervals in solution II, using a modified Wright-Giemsa stain (Camco Quick Stain II; Cambridge Diagnostic Products Inc., Fort Lauderdale, FL). Each slide was examined at 400 \times magnification, and a differential count of 100 cells in 2 different locations on the slide was performed by a single observer. The observer was blinded to the animal identification or enrollment group. When 2 results had a difference greater than 10%, a third count was performed and

the average was calculated from the 2 closest results. Subclinical endometritis was defined as the presence of more than 10% of neutrophils in an endometrial smear.

BCS, Milk Yield, and Culling Data

The body condition of all cows was scored on the day of calving and at 38 ± 3 DIM. For purposes of statistical analysis, BCS was categorized as low (≤ 3.00) or high (≥ 3.25). Milk yield was recorded at monthly intervals during 120 DIM. In addition, culling and DIM at the time of culling were recorded up to 230 DIM.

Reproductive Management and Fertility Responses

All cows had their estrous cycles presynchronized with 2 i.m. injections of 25 mg of PGF_{2α} (Lutalyse, 5 mg/mL dinoprost tromethamine sterile solution; Pfizer Animal Health, Madison, NJ) given 14 d apart, at 46 ± 3 and 60 ± 3 DIM. After the second injection of PGF_{2α}, cows were painted with chalk on their tailheads daily, and removal of chalk was used as an indication of estrus. Cows detected in estrus were inseminated on the same day. Cows not inseminated by 72 ± 3 DIM were enrolled in a 5-d timed AI program (Santos et al., 2010). Nonpregnant cows and those not reinseminated were resynchronized with the 5-d timed AI program. Pregnancy was diagnosed on d 34 ± 3 after the first AI based on the presence of an amniotic vesicle with a live embryo (i.e., with a heartbeat) by transrectal ultrasonography. Pregnant cows were reexamined for pregnancy by transrectal palpation 4 wk later, on d 62 ± 3 d of gestation. Pregnancy loss between d 34 and 62 of gestation was recorded. The interval from postpartum to pregnancy was also recorded in the first 230 DIM.

Statistical Analysis

The cow was considered the experimental unit. A cut-point of serum Ca concentration with optimal sensitivity and specificity to predict metritis was established to define SCH by using the receiver operator characteristic (ROC). Cows were categorized as normocalcemic or as having SCH based on the ROC.

Statistical models included the effects of risk group (HRM vs. LRM), which was forced in all final multivariate models, and parity (primiparous vs. multiparous). In addition, SCH (yes vs. no), the change in serum Ca in the first 3 DIM (difference between serum Ca concentration on d 0 and the lowest serum Ca concentration between 1 and 3 DIM), and the reduction in Ca (decline in Ca vs. no decline in Ca during the first 3 DIM) were analyzed. For some analyses, the effect of

metritis and the interaction between Ca status and risk group were also included in the statistical models.

Continuous data were tested for the normality of residuals, and transformation was performed to achieve normality as needed. Continuous data with repeated measures over time were analyzed using the generalized linear mixed-model GLIMMIX procedure of SAS (SAS/STAT version 9.2; SAS Institute Inc., Cary, NC). The covariance structure with the smallest Akaike information criterion was chosen, and most analyses had the first-order autoregressive structure selected. Cow nested within risk group was the random error term. Additional variables in the repeated measures models included day, the interaction between risk group and day, and the interaction between Ca status and day.

Binary data were analyzed using a modified Poisson regression model with the GENMOD procedure of SAS, including a log link function and correction for data dispersion (Spiegelman and Hertzmark, 2005; Fang, 2011). This method was selected to model binary responses to estimate the adjusted risk ratios (ARR) and respective 95% CI. Time to an event was analyzed with Cox's proportional hazard models by using the PHREG procedure of SAS. The final models were attained by removing variables using a stepwise backward elimination procedure based on the Wald statistic criterion when $P > 0.10$. Risk group was included in all the final models.

Population-attributable risk (a measure of effect used in epidemiology to calculate the reduction in incidence of metritis and puerperal metritis that would be achieved if the population had been entirely unexposed to SCH) compared with the actual exposure pattern was used, according to the following formula (Benichou, 2001):

$$\begin{aligned} \text{population-attributable risk} = & \\ & [(\text{incidence of disease in the population}) \\ & - (\text{incidence of disease in the unexposed population})] / \\ & (\text{incidence of disease in the population}). \end{aligned}$$

For all statistical analyses, effects were considered significant at $P \leq 0.05$, and a tendency was considered when $0.05 < P \leq 0.10$.

RESULTS

A total of 110 cows, 74 multiparous and 36 primiparous, were evaluated for the different analyses within the first 12 DIM. Because of premature culling, only 107 cows were available for the analysis of clinical endometritis, 104 cows were included in the analysis of subclinical endometritis, and 99 cows were included in the

analysis of pregnancy to first AI and days open. A total of 26 cows left before the end of the study follow-up period designated at 230 DIM. Among cows of HRM ($n = 55$), the incidences of dystocia, twins, stillbirth, and retained placenta were 61.8% (34/55), 23.6% (13/55), 12.7% (7/55), and 30.9% (17/55), respectively.

Concentrations of Ca in Serum

The concentrations of Ca in serum did not differ between LRM and HRM cows on the day of calving (9.02 ± 0.08 vs. 8.86 ± 0.08 mg/dL); however, an evident reduction in serum Ca occurred starting the day after calving, and this decline was greater for HRM than LRM cows. The nadir concentration was observed at 2 DIM, and it was lower ($P < 0.05$) for HRM than LRM (LRM = 8.75 ± 0.09 ; HRM = 8.40 ± 0.09 mg/dL). Overall, concentrations of Ca were lower ($P < 0.001$) for cows at HRM compared with those at LRM during the first 12 DIM (Figure 1A). Similarly, cows that developed metritis had lower ($P < 0.001$) concentrations of Ca in serum starting on the day after calving until 12 DIM compared with cows that did not develop metritis (Figure 1B). Except for the day of calving, in which primiparous cows had a greater ($P < 0.05$) serum Ca concentration than multiparous cows (9.05 ± 0.10 vs. 8.79 ± 0.07 mg/dL), Ca concentrations did not differ between parities from 1 to 12 DIM.

The lowest serum Ca concentration within the first 3 DIM was used to predict metritis by ROC analysis. The blood Ca value that predicted metritis with the best combined sensitivity (88.5%) and specificity (55.2%) was 8.59 mg/dL. In other words, by using 8.59 mg/dL as a cutoff value, it was possible to truly predict 88.5% of the cows that developed metritis and 55.2% of the cows that did not develop metritis later in lactation (area under the curve = 0.77; 95% CI = 0.68 to 0.84, $P < 0.001$). On the basis of this analysis, SCH was defined as serum Ca concentration ≤ 8.59 mg/dL in at least 1 d within the first 3 DIM. Therefore, 65.5% (72/110) of the cows in the study developed SCH and 34.5% (38/110) remained normocalcemic. As expected, cows classified as having SCH had lower ($P < 0.001$) concentrations of Ca in serum during the first 12 DIM compared with normocalcemic cows (Figure 1C).

Attitude and Rectal Temperature During Early Postpartum

Cows classified as HRM had an increased ($P < 0.01$) number of days with an abnormal attitude (2.50 ± 0.34 vs. 1.41 ± 0.23 d) compared with LRM cows, but the mean rectal temperature did not differ between HRM or LRM cows and averaged 38.7°C . An interaction (P

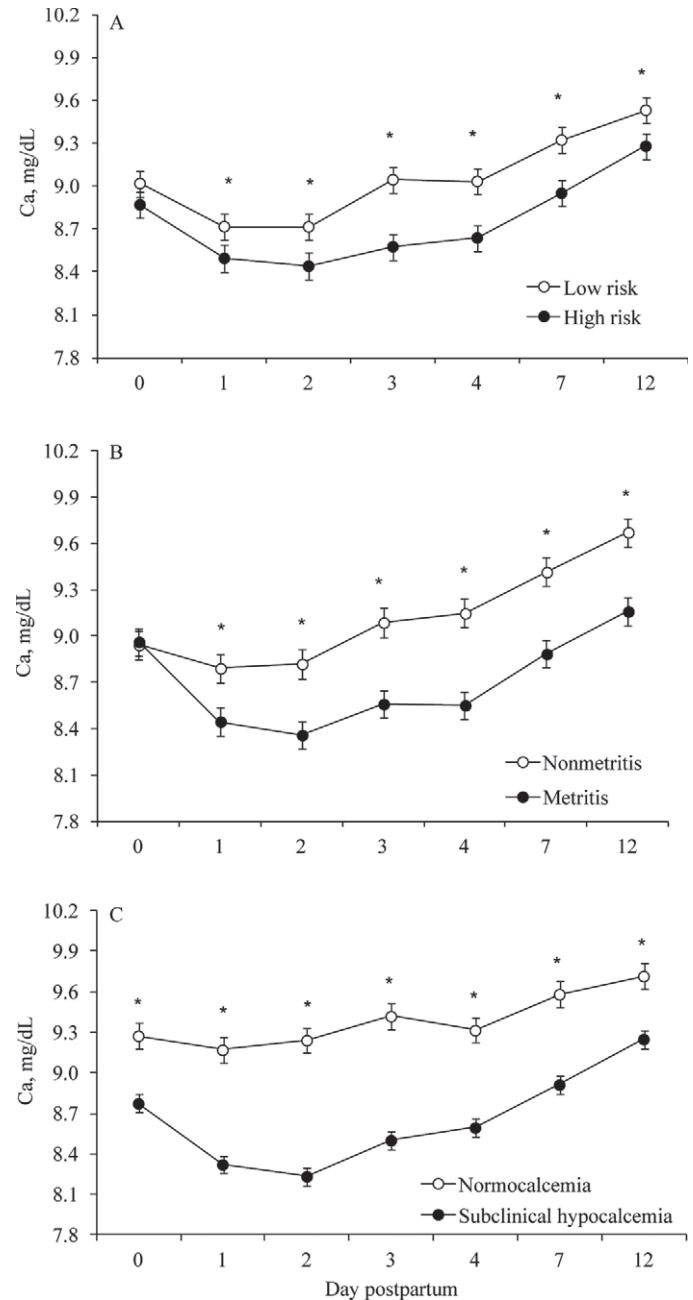


Figure 1. Serum Ca concentrations (mg/dL) in the first 12 DIM according to (A) risk group, (B) diagnosis of metritis, and (C) serum Ca status in the first 3 DIM. For panel A, cows were classified as at low (normal calving) or high risk (dystocia, twins, stillbirth, retained placenta, or their combination) of developing metritis. For panel B, cows were considered as nonmetritis or as being diagnosed with metritis. For panel C, cows were classified as having normocalcemia (Ca > 8.59 mg/dL) or subclinical hypocalcemia (Ca ≤ 8.59 mg/dL) in the first 3 DIM. For panel A, effect of risk group ($P < 0.01$), day ($P < 0.01$), and the interaction of risk group \times day ($P = 0.46$). For panel B, effect of metritis ($P < 0.01$), day ($P < 0.01$), and the interaction of metritis \times day ($P = 0.01$). For panel C, effect of Ca status ($P < 0.01$), day ($P < 0.01$), and the interaction of Ca status \times day ($P < 0.01$). An asterisk (*) indicates daily means differ ($P < 0.05$).

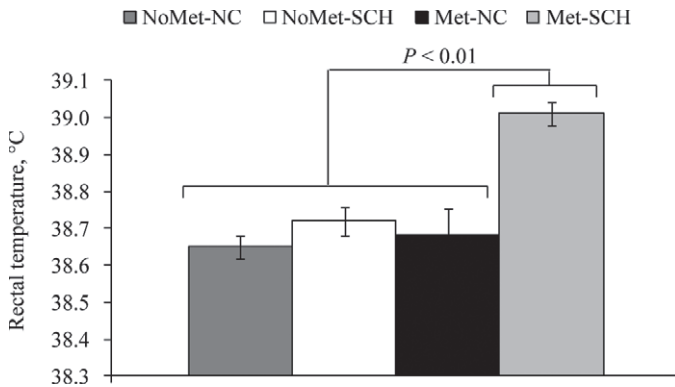


Figure 2. Mean rectal temperature in the first 12 DIM according to a diagnosis of metritis (NoMet = nonmetritis; Met = metritis) and Ca status (NC = normocalcemia, Ca >8.59 mg/dL; SCH = subclinical hypocalcemia, Ca ≤8.59 mg/dL). Effect of metritis ($P < 0.01$), Ca status ($P < 0.01$), and the interaction of metritis \times Ca status ($P < 0.01$).

< 0.01) between metritis and SCH was observed for rectal temperature because mean rectal temperature increased only when cows had both metritis and SCH (Figure 2). Cows with metritis that were able to maintain their serum Ca concentrations above 8.59 mg/dL had rectal temperatures similar to those of cows that did not develop metritis. Cows with SCH had a greater ($P < 0.01$) number of days with an abnormal attitude compared with normocalcemic cows (2.87 ± 0.31 vs. 1.20 ± 0.30 d). Furthermore, cows with SCH had a 2.42-fold increased ($P = 0.001$) risk (ARR = 2.42; 95% CI = 1.16 to 5.03) of developing fever early postpartum compared with normocalcemic cows.

Neutrophil Function

An interaction ($P = 0.02$) between Ca status and DIM was observed for the number of leukocytes in blood. On the day of calving, the number of leukocytes did not differ with Ca status and averaged 12.5×10^3 leukocytes/ μ L; however, cows with SCH had fewer ($P < 0.01$) leukocytes in blood on d 1 (7.84 ± 0.48 vs. $10.19 \pm 0.68 \times 10^3$ leukocytes/ μ L) and d 3 (8.54 ± 0.46 vs. $9.90 \pm 0.63 \times 10^3$ leukocytes/ μ L) postpartum. The reduction in leukocytes was caused primarily because of a more pronounced decline in neutrophil population in the blood, with no change in lymphocytes (Figure 3).

Neutrophil function was reduced for cows with SCH during the first 3 DIM. A decrease in the proportion of neutrophils undergoing phagocytosis was observed for cows with SCH compared with normocalcemic cows (Ca status \times day effect, $P = 0.03$). This effect was observed because, on d 3 postpartum, normocalcemic cows had neutrophils with recovered phagocytic activity, whereas SCH cows still had neutrophils with depressed phago-

cytosis (61.3 ± 3 vs. $73.1 \pm 5\%$; Figure 4A). In addition, an interaction ($P < 0.01$) between Ca status and day postpartum was observed for mean fluorescence intensity for phagocytosis, a measure of the number of bacteria phagocytized per neutrophil. Cows with SCH had a lower mean fluorescence intensity compared with normocalcemic cows at 3 DIM (12.9 ± 1.1 vs. 18.2 ± 1.6 units of fluorescence intensity).

A reduction ($P = 0.05$) in the percentage of neutrophils with oxidative burst was observed in cows with SCH compared with normocalcemic cows in the first 3 DIM (38.7 ± 2.0 vs. $45.4 \pm 2.7\%$; Figure 4B). Nevertheless, no difference ($P = 0.61$) in the mean fluorescence intensity, a proxy for the abundance of reactive oxygen species generated by neutrophils, was observed with Ca status (SCH = 56.2 ± 7.9 vs. normocalcemia = 63.2 ± 10.9 units of fluorescence intensity) during the first 3 DIM. The number of circulating leukocytes

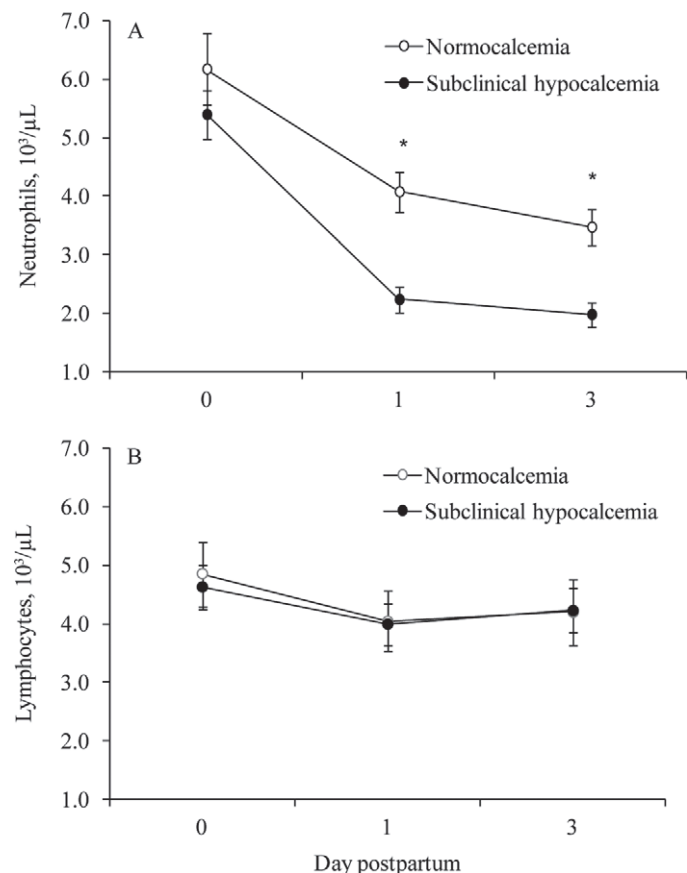


Figure 3. Concentrations of (A) neutrophils ($\times 10^3/\mu$ L) and (B) lymphocytes ($\times 1,000/\mu$ L) in the blood of cows considered to have normocalcemia (Ca >8.59 mg/dL) or subclinical hypocalcemia (Ca ≤8.59 mg/dL) in the first 3 DIM. For panel A, effect of Ca status ($P < 0.01$), day ($P < 0.01$), and the interaction of Ca status \times day ($P = 0.03$). For panel B, effect of Ca status ($P = 0.89$), day ($P < 0.01$), and the interaction of Ca status \times day ($P = 0.91$). An asterisk (*) indicates daily means differ ($P < 0.01$).

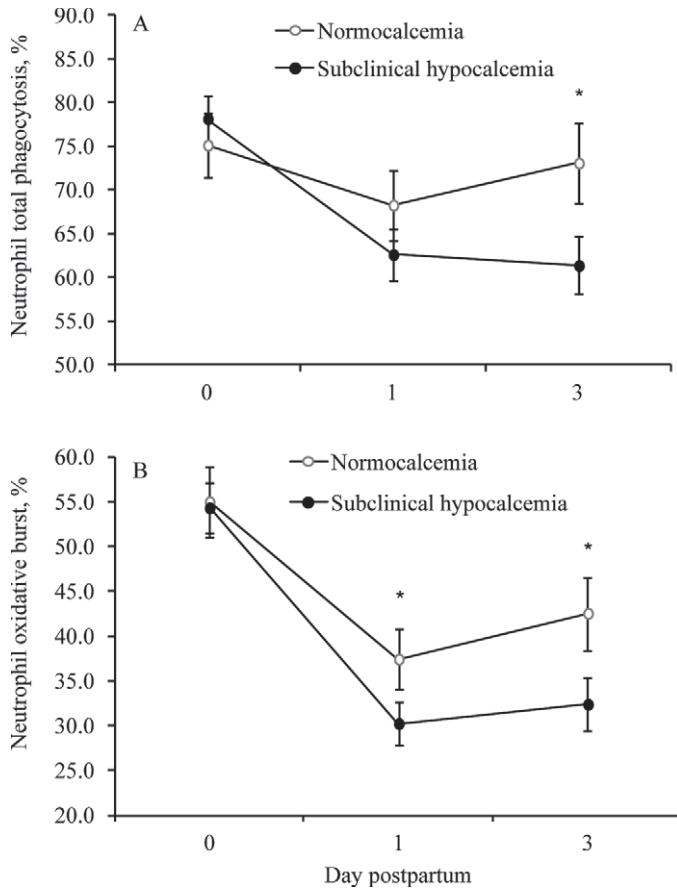


Figure 4. In vitro (A) phagocytosis (% of neutrophils) and (B) oxidative burst (% of neutrophils) in neutrophils of cows considered to have normocalcemia ($\text{Ca} > 8.59 \text{ mg/dL}$) or subclinical hypocalcemia ($\text{Ca} \leq 8.59 \text{ mg/dL}$) in the first 3 DIM. For panel A, effect of Ca status ($P = 0.21$), day ($P < 0.01$), and the interaction of Ca status \times day ($P = 0.03$). For panel B, effect of Ca status ($P = 0.05$), day ($P < 0.01$), and the interaction of Ca status \times day ($P = 0.12$). An asterisk (*) indicates daily means differ ($P < 0.05$).

and neutrophils during early postpartum, as well as the indicators of neutrophil function, did not differ ($P > 0.30$) between HRM and LRM cows.

Incidence of Metritis, Puerperal Metritis, and Other Early Postpartum Diseases

The incidence of metritis for the cows in the study was 47.3% (52/110). Cows with SCH had 3.24 times greater ($P < 0.01$) risk of developing metritis compared with normocalcemic cows (Table 2). The change in serum Ca from calving to the lowest value in the first 3 DIM ranged from a decline of 2.4 mg/dL (-2.4) to an increase of 1.5 mg/dL (Figure 5). This change in serum Ca influenced ($P < 0.05$) the incidence of metritis. For each 1 unit (mg/dL) of change in serum Ca (from -2.5 to $+1.5$), the relative risk of developing metritis de-

creased by 22% ($\text{ARR} = 0.78$, 95% CI = 0.67 to 0.92; Figure 5). As expected, HRM cows had an increased ($P < 0.01$) incidence of metritis compared LRM cows. Primiparous cows had a tendency ($P = 0.09$) for a greater incidence of metritis compared with multiparous cows.

The incidence of puerperal metritis for the cows in the study was 30% (33/110). Cows with SCH had an 11-fold increase ($P < 0.02$) in the risk of developing puerperal metritis compared with normocalcemic cows (Table 2). Cows at HRM showed a tendency ($P = 0.08$) for an increased incidence of puerperal metritis, but parity did not influence the risk of puerperal metritis.

Table 3 shows the incidence of metritis and puerperal metritis stratified by risk group. It is interesting to note that HRM cows that were able to maintain serum Ca concentrations $> 8.59 \text{ mg/dL}$ had one-half and one-third the incidence of metritis and puerperal metritis, respectively, compared with LRM cows that developed SCH. The reduction in incidences of metritis and puerperal metritis that would be achieved if the population had been entirely unexposed to SCH was estimated as 66.6 and 91.3%, respectively. In other words, by eliminating SCH from the population study, the incidence of metritis would potentially be reduced from 47.3 to 15.8% and that of puerperal metritis would be reduced from 30.0 to 2.6%.

The incidence of diseases other than metritis during the first 12 DIM was 28% (31/110), with 13% (14/110) of respiratory disease, 11% (12/110) of mastitis, 3% (3/110) of digestive conditions, and 1% (1/110) of lameness. Cows with a reduction in serum Ca concentration after calving had a greater ($P < 0.05$) risk ($\text{ARR} = 2.2$; 95% CI = 1.1 to 4.5) of developing other diseases compared with cows that were able to maintain or increase Ca concentrations in the first 3 DIM [39.7% (23/58) vs. 15.4% (8/52)]. Cows at HRM had a tendency ($P = 0.08$) for a greater incidence of other diseases ($\text{ARR} = 1.79$; 95% CI = 0.93 to 3.94) compared with LRM cows.

Mg, K, Glucose, NEFA, and BHBA

Serum Mg concentrations were lower ($P < 0.01$) for cows with SCH compared with normocalcemic cows during the first 12 DIM (2.01 ± 0.02 vs. $2.13 \pm 0.03 \text{ mg/dL}$). Similarly, HRM cows had reduced ($P < 0.01$) serum Mg compared with LRM cows (2.02 ± 0.03 vs. $2.13 \pm 0.03 \text{ mg/dL}$) during the first 12 DIM. Concentrations of K in serum did not differ between normocalcemic and SCH cows and between HRM and LRM cows, and it averaged 4.71 mM in the first 12 DIM.

Concentrations of glucose averaged $53.4 \pm 1.5 \text{ mg/dL}$ in the first 12 DIM, and they were not influenced by risk group or Ca status. For NEFA and BHBA, the

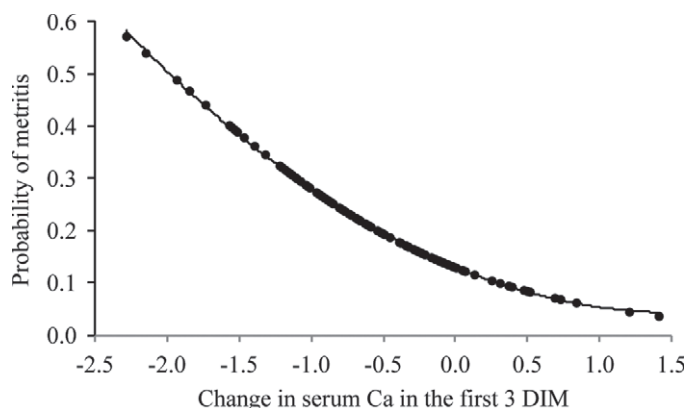


Figure 5. Probability of metritis relative to the change in serum Ca concentrations between the day of calving and the lowest serum Ca concentration within the first 3 DIM. Cows are represented by dots in the figure. Negative values on the x-axis indicate a decline in Ca after calving; positive values indicate an increase in serum Ca after calving. Effect of change in serum Ca on the probability of developing metritis ($P < 0.05$).

main predictor that influenced the concentrations was Ca status; cows with SCH had greater ($P < 0.001$) concentrations of NEFA (0.705 ± 0.04 vs. 0.427 ± 0.04 mM; Figure 6A) and BHBA (9.9 ± 0.4 vs. 7.7 ± 0.5 mg/dL; Figure 6B) compared with normocalcemic cows. It is interesting that concentrations of NEFA (0.559 ± 0.04 vs. 0.536 ± 0.04 mM) and BHBA (8.48 ± 0.50 vs.

8.66 ± 0.50 mg/dL) did not differ between cows with or without metritis. In addition, NEFA (0.539 ± 0.04 vs. 0.558 ± 0.04 mM) and BHBA (8.29 ± 0.5 vs. 9.30 ± 0.4 mg/dL) did not differ between HRM and LRM cows.

Clinical and Subclinical Endometritis

The overall incidence of clinical endometritis was 77.6% (83/107). Cows with SCH had a greater ($P = 0.05$) risk (ARR = 1.30; 95% CI = 1.00 to 1.64) of clinical endometritis compared with normocalcemic cows [82.6% (57/69) vs. 68.4% (26/38)]. Risk group or parity did not influence the incidence of clinical endometritis. The incidence of subclinical endometritis was 36.5% (38/104). Cows with SCH tended ($P = 0.07$) to have an increased risk (ARR = 1.84; 95% CI = 0.92 to 3.70) of subclinical endometritis compared with normocalcemic cows [46.2% (30/65) vs. 21.1% (8/38)]. Subclinical endometritis was not influenced by risk group or parity.

Fertility Responses

Fifty-two percent (56/107) of the cows in the study were found to be cyclic at 38 DIM. Estrous cyclicity was less ($P < 0.05$) for HRM than LRM cows [43.4% (23/53) vs. 63.0% (34/54)]. The percentage of estrous cyclic cows was not influenced by Ca status, but more

Table 2. Incidence and adjusted risk ratio (ARR) of metritis and puerperal metritis¹

Item	Incidence, % (no./no.)	ARR (95% CI)	P-value
Metritis			
Ca status ²			
Normocalcemia	15.8 (6/38)	Referent	
Subclinical hypocalcemia	63.9 (46/72)	3.24 (1.51–6.95)	<0.01
Parity			
Multiparous	40.5 (30/74)	Referent	
Primiparous	61.1 (22/36)	1.28 (0.96–1.69)	0.09
Risk group ³			
Low risk	27.3 (15/55)	Referent	
High risk	67.3 (37/55)	1.80 (1.15–2.81)	<0.01
Puerperal metritis			
Ca status			
Normocalcemia	2.5 (1/38)	Referent	
Subclinical hypocalcemia	44.4 (32/72)	11.5 (1.57–83.6)	<0.02
Parity			
Multiparous	25.7 (19/74)	Referent	
Primiparous	38.9 (14/36)	1.32 (0.82–2.11)	0.24
Risk group			
Low risk	14.5 (8/55)	Referent	
High risk	45.4 (25/55)	1.79 (0.92–3.47)	0.08

¹Metritis = cows with watery fetid vaginal discharge within the first 12 DIM; puerperal metritis = metritis concurrent with a rectal temperature $\geq 39.5^{\circ}\text{C}$.

²Normocalcemia = cows with serum Ca concentrations >8.59 mg/dL in the first 3 DIM; subclinical hypocalcemia = cows with at least 1 d with serum Ca concentration ≤ 8.59 mg/dL in the first 3 DIM.

³Low risk = normal calving; high risk = cows diagnosed with dystocia, twins, stillbirth, or retained fetal membranes.

Table 3. Incidences of metritis and puerperal metritis according to risk group¹ and serum Ca status²

Ca status	Low-risk group		High-risk group	
	Normocalcemia	SCH	Normocalcemia	SCH
Metritis, ^{3,4,5} % (no./no.)	14.3 (4/28)	40.7 (11/27)	20.0 (2/10)	77.8 (35/45)
Puerperal metritis, ^{4,6} % (no./no.)	0.0 (0/28)	29.6 (8/27)	10.0 (1/10)	53.5 (24/45)

¹Low risk = normal calving; high risk = cows diagnosed with dystocia, twins, stillbirth, or retained fetal membranes.

²Normocalcemia = cows with serum Ca concentrations >8.59 mg/dL in the first 3 DIM; subclinical hypocalcemia (SCH) = cows with at least 1 d with serum Ca concentration ≤ 8.59 mg/dL in the first 3 DIM.

³Cows with watery fetid vaginal discharge within the first 12 DIM regardless of rectal temperature.

⁴Effect of Ca status ($P < 0.02$).

⁵Effect of risk group ($P < 0.05$).

⁶Cows with watery fetid vaginal discharge within the first 12 DIM concurrent with rectal temperature $\geq 39.5^{\circ}\text{C}$.

($P < 0.01$; ARR = 2.60; 95% CI = 1.43 to 4.80) multiparous than primiparous cows were cyclic [64.8 (46/71) vs. 30.6% (11/36)].

Thirty-three percent of the cows became pregnant to first AI (33/99), and no pregnancy loss was observed from 34 to 62 d of gestation. The pregnancy to first AI was not affected by risk group [HRM = 31.9% (35/47)

vs. LRM = 34.6% (18/52)], Ca status [SCH = 31.3% (20/64) vs. normocalcemia 37.1% (13/35)], or incidence of metritis [metritis = 28.9% (13/45) vs. nonmetritis = 37.0% (20/54)]. Despite the lack of differences in pregnancy to first AI, normocalcemic cows tended ($P = 0.06$) to have a greater rate of pregnancy than SCH cows, which reduced the median days open by 15 d (Figure 7). The interval to pregnancy was not influenced by risk group or parity.

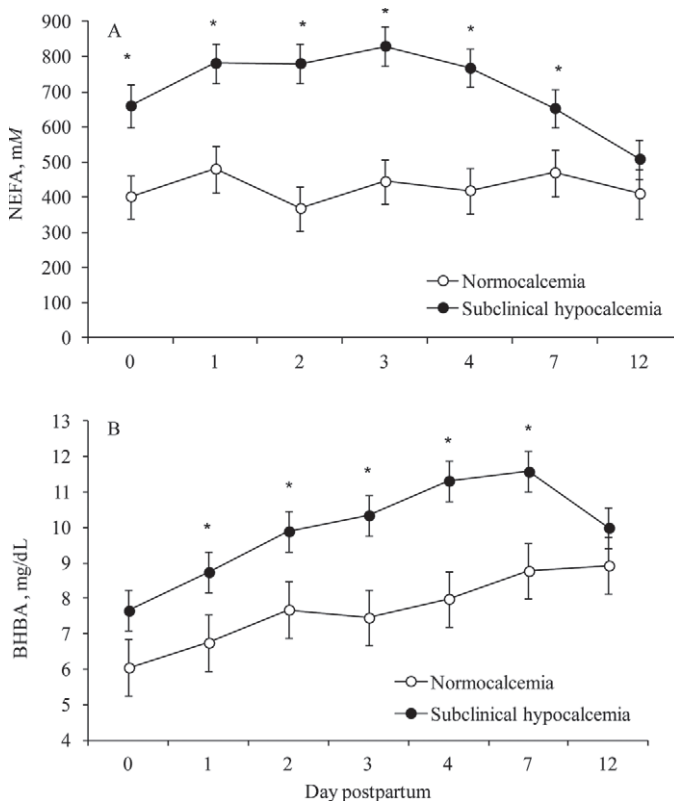


Figure 6. Serum concentrations of (A) NEFA (mM) and (B) BHBA (mg/dL) during the first 12 DIM. For panel A, effect of Ca status ($P < 0.01$), day ($P < 0.01$), and the interaction of Ca status \times day ($P = 0.26$). For panel B, effect of Ca status ($P < 0.01$), day ($P < 0.01$), and the interaction of Ca status \times day ($P = 0.38$). An asterisk (*) indicates daily means differ ($P < 0.05$).

Milk Yield, BCS, and Culling

Milk yield in the first month of lactation did not differ between SCH and normocalcemic multiparous (42.8 ± 1.8 vs. 40.0 ± 3.2 kg/d) or primiparous cows (35.4 ± 2.6 vs. 28.6 ± 3.3 kg/d). During the first 4 mo postpartum, milk yield averaged 37.5 kg/d and it was not influenced by Ca status or risk group.

The BCS of cows at calving did not differ between risk groups or Ca status, and the average BCS at calving was 2.96 ± 0.03 . However, at 38 DIM BCS differed ($P < 0.01$) between normocalcemic and SCH cows (3.06 ± 0.07 vs. 2.78 ± 0.05) because a greater ($P < 0.01$) proportion of SCH cows lost BCS compared with normocalcemic cows (55.2 vs. 32.4%).

Twenty-six of the 110 cows enrolled left the study before 230 DIM. None of the factors evaluated influenced the rate of culling.

DISCUSSION

Subclinical hypocalcemia is a condition commonly observed during the first days after calving (Reinhardt et al., 2011), and it is usually caused by the large quantities of Ca secreted in colostrum associated with an inadequate ability of the cow to mobilize bone to reestablish blood concentrations (Goff, 2008). Different ranges have been published for normal reference values for Ca concentrations in the serum of cattle in the

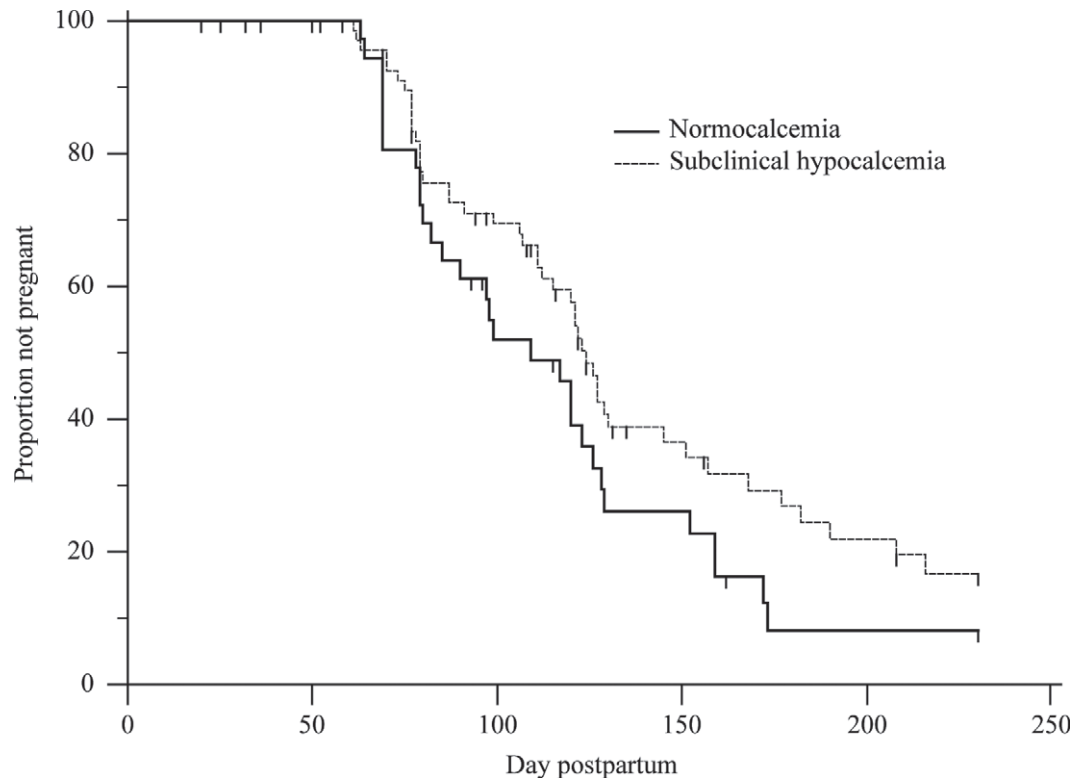


Figure 7. Survival curves for the interval from calving to pregnancy according to Ca status. Cows with normocalcemia tended ($P = 0.06$) to have a greater rate of pregnancy (adjusted hazard ratio = 1.61; 95% CI = 0.97 to 2.65) and fewer median days open (109 d; 95% CI = 82 to 126) compared with cows that developed subclinical hypocalcemia (124 d; 95% CI = 111 to 145).

periparturient period, such as 8.5 to 10 mg/dL (Goff, 2008) or >8.8 mg/dL (Chapinal et al., 2011). In the current study, ROC analysis was used to establish a cutoff value of Ca concentration in serum in the first 3 DIM that best predicted postpartum uterine diseases to objectively define SCH. It was our assumption that a reduction in concentrations of Ca in serum would reduce cytosolic Ca^{2+} (Kimura et al., 2006) and compromise cellular functions critical to the immune response (Jaconi et al., 1990; Ducusin et al., 2003; Br  chard and Tschirhart, 2008).

The observed decrease in serum Ca concentrations after calving found in the present study has been reported previously (Kimura et al., 2006). However, not every cow experienced a reduction in serum Ca concentrations between the day of calving and the first 3 DIM. In fact, 47.3% of the cows either maintained or increased their serum Ca concentrations in the first days postpartum. This ability to avoid a reduction in serum Ca was more pronounced in cows classified as normocalcemic (89.5% of them maintained or increased serum Ca) than in those with SCH, of which only 25% were able to avoid a reduction in serum Ca.

The mean and median DIM when serum Ca was <8.59 mg/dL were 1.6 and 1.0 d, respectively. Cows

that developed metritis were diagnosed on average 2.9 d later, at 4.5 DIM. Therefore, because SCH was defined based on the lowest serum Ca concentration in the first 3 DIM, it occurred in almost all cows before the diagnosis of metritis. Only 1 of the 52 cows with metritis had serum Ca greater than 8.59 mg/dL before the diagnosis of metritis, and the lowest serum Ca for this cow was from a sample collected the day after the diagnosis of metritis. In all other 51 cows, the diagnosis occurred either on the same day or after the blood sample was collected to define SCH. The fact that SCH preceded the metritis diagnosis allowed us to postulate SCH as a risk factor for metritis. However, causality is yet to be established.

In the present study, 65.5% of the cows had serum Ca below 8.59 mg/dL in at least 1 d between calving and 3 DIM, and this prevalence was similar between primiparous and multiparous cows. Others have reported the prevalence of SCH to be 25% in primiparous cows and 47% in multiparous cows (Reinhardt et al., 2011). In fact, Reinhardt et al. (2011) reported an increased prevalence of SCH as the lactation number increased. A few aspects might have influenced the discrepancies between our findings and those of Reinhardt et al. (2011). First, the cows in the current study originated

from a single herd, and it is known that the prevalence of postparturient problems varies with the herd. In addition, cows were sampled daily and the lowest value from 4 samples (0 to 3 DIM) was selected. Most studies evaluating the prevalence of SCH have used 1 or 2 samples postpartum (Chapinal et al., 2011; Reinhardt et al., 2011) and sampling schemes were usually weekly, not daily. Finally, one-half of the cows in the current study were a select population at high risk of developing postpartum disease, and these cows typically have lower serum Ca concentrations.

After parturition, contamination of the uterus with bacteria is inevitable; however, not all cows develop metritis or puerperal metritis. The persistence of uterine infection and the development of disease depend greatly of the bacterial load, pathogenicity of the microorganism, and defense mechanisms of the uterus (Sheldon et al., 2006). The reduction of immune function during the peripartum period has been documented (Hammon et al., 2006; Silvestre et al., 2011), and this is suggested as one of the major reasons for the high incidence of bacterial diseases such as mastitis and metritis in early-lactation cows. Furthermore, the existence of metabolic conditions such as clinical hypocalcemia and exacerbated negative energy balance have been shown to further suppress immune function (Hammon et al., 2006; Kimura et al., 2006; Galvão et al., 2010). Hypocalcemia can increase concentrations of cortisol in blood (Horst and Jorgensen, 1982), and elevated cortisol compromises chemotaxis and the bactericidal activity of neutrophils (Roth et al., 1982; Salak-Johnson and McGlone, 2007).

Results from the present study indicate that cows with SCH have neutrophils in the blood that are less capable of phagocytizing and killing pathogenic bacteria *in vitro*. It is suggested that the neutrophil function in cows with SCH is compromised by reducing the cytosolic Ca^{2+} required for the initiation of phagocytosis, although this process is not exclusively Ca dependent (Sayeed, 2000). In addition, Ca^{2+} is necessary to control the fusion of secondary granules with the phagosomal membrane (Jaconi et al., 1990) during bactericidal activity. Inadequate concentrations of Ca in blood are likely to influence the availability of Ca^{2+} for cellular function. Blood mononuclear cell cytosolic Ca^{2+} was reduced around parturition, and the reduction was greater in cows with hypocalcemia compared with those that were capable of restoring blood Ca concentrations quickly after calving (Kimura et al., 2006). Neutrophil activation involves the binding of soluble inflammatory mediators to receptors on the cell neutrophil surface, followed by activation of cytosolic components such as phospholipase C, protein kinase C, and inositol 1,4,5-triphosphate. This trans-

duction mechanism releases Ca^{2+} from the endoplasmic reticulum to increase cytosolic Ca^{2+} up to 10-fold of its basal concentration (Brécard and Tschirhart, 2008). A high cytosolic Ca^{2+} is critical for the activation of NADPH oxidase to produce reactive oxygen species to effectively kill phagocytized pathogens (Brécard and Tschirhart, 2008). In addition, once Ca^{2+} is released from the endoplasmic reticulum, receptors localized in the endoplasmic reticulum signal the plasma membrane to open Ca^{2+} membrane channels in a retrograde process called store-operated Ca^{2+} entry. This additional Ca^{2+} entry from the extracellular space helps replenish Ca^{2+} stores in the endoplasmic reticulum (Burgos et al., 2011). It is hypothesized, based on work by others (Ducusin et al., 2003), that cows with SCH have less endoplasmic reticulum Ca^{2+} to increase cytosolic concentrations and are unable to replenish the intracellular Ca^{2+} because of the reduced concentrations in blood. Impairing the increase in cytosolic Ca^{2+} reduces the activation of neutrophils and the generation of reactive oxygen species, which could be reflected in decreased phagocytosis and killing activities, as observed in the current study in cows with SCH.

Cows with SCH not only had neutrophils with reduced *in vitro* phagocytic and killing activities, but they also had a smaller leukocyte population in the blood because of a sharp decline in neutrophils compared with the level in normocalcemic cows. Cows with SCH had a 3.2-fold increase in the incidence of metritis, and it is known that inflammation of the reproductive tract increases the influx of leukocytes, primarily neutrophils, to the tissue (Bondurant, 1999). Therefore, it is possible that the exacerbated neutropenia was caused by a greater migration of cells to inflamed tissues, although less bone marrow neutrophil proliferation cannot be ruled out.

The incidence of uterine disease in the study population was high and was greater than typically reported in the literature for dairy cows (Sheldon et al., 2006; Ospina et al., 2010; Chapinal et al., 2011). This high incidence is explained by the select population of cows in the HRM group, which are known to be more susceptible to periparturient diseases. In addition, the rigorous methodology and daily monitoring of the cows likely allowed for better detection of the condition than in most epidemiological studies that rely on farm records. In this context, it is important to consider that the diagnosis of metritis in dairies might be overlooked on some farms, particularly when the diagnosis is dependent primarily on the detection of fever for the evaluation of uterine discharge. In the current study, puerperal metritis occurred in 33 of the 52 cows with metritis. In other words, 37% of the cows diagnosed with metritis did not display fever. Finally, the study

population was represented by a single farm, and it is clear that large variations in disease incidence, ability to diagnose them, or both occur among farms and investigators (Chapinal et al., 2011).

The result that LRM cows that developed SCH had a greater incidence of metritis and puerperal metritis than HRM normocalcemic cows provides compelling evidence that Ca status might play an important role in the development of uterine diseases during early postpartum. In fact, almost all cows that developed puerperal metritis were subclinically hypocalcemic (97%), and the mean rectal temperature increased only when cows were diagnosed with metritis and SCH. It is possible that cows with metritis that were not able to regulate serum Ca concentrations were less capable of containing the uterine bacterial infection. Gram-negative bacteria make up a large portion of the uterine flora of cows with metritis (Sheldon et al., 2006). Lipopolysaccharide endotoxins released from these bacteria can gain access to the vasculature and influence the production of PGE₂ in endothelial cells, which can activate thermoregulatory neurons in the preoptic area of the hypothalamus inducing fever (Nakamura, 2011).

The association between negative energy status during the periparturient period, represented by elevated NEFA concentrations, on neutrophil function and uterine diseases has been supported and documented in some studies (Hammon et al., 2006; Ospina et al., 2010; Chapinal et al., 2011) and refuted by others (Scalia et al. 2006; Melendez et al., 2009). As opposed to the findings by others (Hammon et al., 2006; Galvão et al., 2010), cows that developed metritis in the current study did not have greater concentrations of NEFA and BHBA; however, our findings suggest a relationship between SCH and serum concentrations of NEFA or BHBA. The increased concentrations of these energy metabolites during the first 12 DIM in cows with SCH suggest that both mineral and energetic statuses are interrelated, and they influence the risk of periparturient diseases. In fact, increased concentrations of some FA can induce the activation of neutrophils *in vitro* (Hidalgo et al., 2011), and altering the FA intake can influence neutrophil function in lactating dairy cows (Silvestre et al., 2011). Because most studies involving periparturient energetic status and risk of postpartum diseases have been of an epidemiological nature (Ospina et al., 2010; Chapinal et al., 2011), the established cause-effect relationship remains to be elucidated.

Although DMI was not measured in the present study, one could hypothesize the SCH could compromise DMI, which in turn would lead to greater lipomobilization and increased concentrations of serum NEFA and BHBA. Contrary to the latter hypothesis are the data from Jawor et al. (2012), in which cows with SCH (Ca

<7.2 mg/dL) had greater DMI prepartum and a tendency for greater intake in the first weeks postpartum compared with normocalcemic cows. It is interesting that Jawor et al. (2012) observed that cows with SCH produced 5.7 kg/d more milk than their normocalcemic counterparts in the first month postpartum, a numerical difference in production that was only observed in primiparous cows in the first month postpartum in the current study, but did not perpetuate for the first 120 DIM. In the work by Jawor et al. (2012), every postpartum cow received 500 mL of a Ca borogluconate solution after the blood was sampled to diagnose SCH. Cows in the present study received no supplemental Ca postpartum. It is possible that cows with SCH are better producing cows that, when receiving Ca supplementation to minimize inadequate serum concentrations, do not have other diseases in greater incidence than do their normocalcemic counterparts, and they are then capable of expressing this increased milk potential.

Magnesium is an important mineral involved in Ca homeostasis, and a decrease in Mg concentrations could induce hypocalcemia (Goff, 2008). Although Mg concentrations were significantly lower for cows with SCH compared with normocalcemic cows, the biological significance is questionable because the differences were small, and Mg concentrations were maintained within the normal range, with the lowest concentration manifested at 7 DIM, after the diagnosis of SCH and metritis. Thus, it is likely that Mg did not contribute to the development of SCH in the present study.

The rate of pregnancy was reduced and the interval to pregnancy was extended by 15 d when cows developed SCH. Because SCH markedly increased the risks of acute uterine diseases such as metritis and puerperal metritis that perpetuated into the more chronic states of endometrial inflammation with clinical and subclinical endometritis, it is not a surprise that reproduction was compromised. Furthermore, cows with SCH had marked increases in serum concentrations of NEFA and BHBA, both markers of energetic status. Increases in concentrations of NEFA and BHBA in the periparturient period have been linked with a delay in pregnancy in dairy cows. Therefore, the combination of increased uterine diseases and a less favorable energetic profile in cows with SCH likely explains the extended interval to pregnancy.

It is possible, based on the calculated population-attributable risk, to reduce the incidences of metritis and puerperal metritis by 66.6 and 91.3%, respectively, by completely eliminating SCH. To fully confirm these observations, future intervention studies are needed with populations of cows in which serum Ca concentrations from 0 to 3 DIM are maintained above 8.59 mg/dL.

CONCLUSIONS

Cows with SCH, as defined by serum Ca ≤ 8.59 mg/dL in at least 1 d from 0 to 3 DIM, had reduced concentrations of neutrophils in the blood, impaired neutrophil function, and increased incidences of metritis and puerperal metritis compared with normocalcemic cows. This increased incidence of uterine diseases was observed regardless of the risk group for metritis at calving. The probability of metritis increased parallel to the reduction in concentrations of Ca in the serum during the first 3 DIM. Cows with SCH had elevated concentrations of NEFA and BHBA in the serum in the first 12 DIM compared with normocalcemic cows. Approximately 66.6% of the incidence of metritis and 91.3% of the incidence of puerperal metritis observed in the study population could be attributed to SCH. Calcium status did not influence milk yield, survivability, and pregnancy to first AI, but cows with SCH had a reduced rate of pregnancy and extended days open. These data suggest that SCH increases lipomobilization and compromises neutrophil function, which increases the risk of uterine diseases in dairy cows. It is suggested that maintaining serum Ca concentrations above 8.59 mg/dL from calving to 3 DIM might reduce the incidences of metritis and puerperal metritis in lactating dairy cows. Further studies are warranted to elucidate the exact mechanism by which SCH might compromise innate immunity and increase periparturient diseases in dairy cows.

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