Epidemiology of subclinical hypocalcemia in early-lactation Holstein dairy cows: The temporal associations of plasma calcium concentration in the first 4 days in milk with disease and milk production

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ABSTRACT

The objectives of this study were to characterize the epidemiology of subclinical hypocalcemia (SCH) in Holstein dairy cows by assessing the temporal associations of plasma Ca concentrations in the first 4 d in milk (DIM) with the risk of cows being diagnosed with metritis or displaced abomasum (or both), and milk production across the first 15 wk of lactation. A prospective cohort study was conducted in 2 dairy herds in New York State, in which cows had a blood sample collected daily for the first 4 DIM. A total of 396 Holstein cows (137 primiparous and 259 multiparous) were enrolled. Multivariable Poisson regression models were built to evaluate the associations of plasma Ca concentration at each of the 4 d following parturition with the risk of primiparous cows being diagnosed with metritis and multiparous cows being diagnosed with metritis, displaced abomasum, or both. Similarly, generalized linear mixed models were built to evaluate the associations of plasma Ca concentration with milk production across the first 15 wk of lactation. Plasma Ca concentration was assessed on a continuous scale in all models; dichotomization and SCH classification only occurred in the final models if the Ca concentration variable was meaningful by creating an optimized threshold based on receiver operating characteristic curve analysis. Plasma Ca concentration assessed at 1 DIM was not associated with the risk of metritis in primiparous cows, but an association was observed at 2, 3, and 4 DIM (critical thresholds were plasma Ca concentration ≤ 2.15 , 2.10, and 2.15 mmol/L, respectively). Plasma Ca concentration was associated with the risk of metritis or displaced abomasum diagnosis (or both) for 2nd parity animals at 2 DIM (threshold ≤ 1.97 mmol/L), and at 4 DIM for 3rd and greater lactations (threshold ≤ 2.20 mmol/L).

Reduced plasma Ca concentration was associated with higher milk production when assessed at 1 DIM in primiparous and multiparous cows, and lower milk production when assessed at 4 DIM in multiparous cows only. For primiparous cows, plasma Ca concentration was not associated with lower milk production at any of the DIM assessed. In conclusion, assessments of SCH at the individual cow level must take into account the DIM of Ca concentration measurement and parity of the cow, as the epidemiology of the disorder was demonstrated to be highly dependent on these variables. This study advances the knowledge of the epidemiology of SCH and better establishes thresholds for optimizing SCH diagnosis.

Key words: calcium, subclinical hypocalcemia, epidemiology, dairy cow

INTRODUCTION

Hypocalcemia is one of the most common periparturient abnormalities afflicting dairy cows. The disorder is frequently divided into a clinical (i.e., parturient paresis) and a subclinical state, with the latter receiving much recent research attention. For classification purposes, subclinical hypocalcemia (**SCH**) should be defined as low blood Ca concentrations that are associated (i.e., no proven distinction as to cause or consequence of other underlying issues) with periparturient health disorders, poorer production and reproduction outcomes, or both, without associated signs of postpartum paresis.

The first study that initiated categorization of blood Ca concentrations in the early postpartum period was done in an attempt to more quantitatively assess a dietary preventative strategy to decrease clinical hypocalcemia by addition of ammonium chloride and sulfate to decrease the dietary DCAD level (Oetzel et al., 1988). That study instituted a classification of parturient hypocalcemia based on a serum ionized Ca < 4.0 mg/dL (1.0 mmol/L) on the day of calving, which included

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cows with and without signs of paresis. Later, Oetzel (1996) in an effort to evaluate the efficacy of a Ca chloride gel product in decreasing parturient hypocalcemia (with or without signs of paresis), used a classification scheme based on a serum Ca concentration <8.0 mg/dL (2.0 mmol/L) collected 18 to 24 h after calving. Descriptive data published on the potential prevalence of SCH obtained from 1,462 dairy cows in 480 dairy herds participating in the 2002 Dairy National Animal Health Monitoring Survey across 21 states in the United States revealed a high prevalence of the disorder (Reinhardt et al., 2011); the study used a cut point of serum Ca concentration <2.0 mmol/L collected within 48 h of parturition, which was chosen based on 1 unit below the normal serum biochemical range described by the Merck Veterinary Manual. This threshold resulted in 25% of primiparous cows and an average of 50% of multiparous cows being classified as having suboptimal serum Ca concentrations. The aforementioned studies made an effort to demonstrate the challenges of the dairy cow to maintain optimum Ca balance in the early postpartum period. However, those studies did not perform any analyses to associate blood Ca concentration with postpartum outcomes (e.g., disease and milk production), and extrapolation of those cut points for SCH classification could have resulted in misclassification (i.e., under- or overrepresentation) of the disorder.

More recently, various research groups are working on improving the characterization of SCH by associating reduced blood Ca concentration in the postpartum period with detrimental health and production outcomes (Chapinal et al., 2011; Rodríguez et al., 2017; Wilhelm et al., 2017). Differences in the day relative to parturition of blood collection for Ca concentration assessments could influence the comparability of results between studies, as Ca dynamics in the first few DIM are quite variable. In an innovative block-randomized study, Martinez et al. (2012) has shown that cows reaching a Ca nadir concentration ≤ 2.14 mmol/L at one of the first 3 d after calving had an increased risk of being diagnosed with metritis. To date, there is no consensus in the literature as to how (i.e., what cut point to use) and when to classify SCH in relation to parturition, as blood Ca testing is more likely to be applied by dairy producers and consultants if a specific time point relative to parturition can be better identified; this is further complicated by the fact that the appropriate cut point or DIM of blood Ca assessment could differ based on the outcome measured. In an earlier study, our group was able to demonstrate that the risk factors associated with a suboptimal Ca concentration state in the early postpartum period are different depending on the time of blood Ca assessment (i.e., within 4 h of parturition and at 2 DIM; Neves et al., 2017). This sug-

gests that timing of blood sample collection relative to parturition for blood Ca assessment may be an important factor to take into account when determining the association between SCH and downstream outcomes.

The objectives of this study were to describe the temporal associations of plasma total Ca concentrations in the first 4 DIM with (1) the risk of cows being diagnosed with metritis, displaced abomasum (DA), or both, and (2) milk production in the first 15 wk of lactation. We hypothesized that the associations of plasma Ca concentration with those outcomes would vary depending on the DIM of Ca concentration measurement. Our goal was to describe the epidemiology of SCH, and to elucidate whether one or more of the time points analyzed would offer superior association with the outcomes of interest, and ultimately, allow for improved cow- and herd-level SCH assessments.

MATERIALS AND METHODS

Experimental Design

A prospective cohort study was conducted in 2 dairy herds in New York State from February until November 2015. This study was part of a larger randomized clinical trial in which cows were randomly allocated to either receive or not receive a Ca bolus within 24 h of parturition (Leno et al., 2018); only control cows (i.e., no Ca supplementation) were eligible to be included in the analyses. To detect a 2.0 times greater risk of early-lactation clinical disease (i.e., metritis, DA, or both) among SCH cows versus normocalcemic cows, while assuming a 40% incidence of SCH in the cohort under study, enrollment of 400 cows was necessary for a study with 80% power and type I error rate of 5% (OpenEpi version 3.01; OpenEpi, Atlanta, GA).

All animal procedures were reviewed and approved by the Cornell University Institutional Animal Care and Use Committee (protocol 2014-0171). Farms were visited once a day at the same time of day, and cows had a blood sample collected at 1, 2, 3, and 4 DIM by the research group. Of note, the classification scheme of DIM adopted in this study followed the same format used by the herd management's software used at both studied dairies (DairyComp 305; Valley Agricultural Software, Tulare, CA), in which classifies 1 DIM as the day after the inputted calving date. Therefore, 1 DIM more closely resembles a time point closer to 24 h after parturition. As sampling occurred at the same time of the day within a herd, the mean (SD) and median of the 1 DIM sampling relative to parturition was calculated to occur at 2152 h (0647 h) and 2150 h, respectively. Blood samples were collected by coccygeal venipuncture using a 20-gauge $\times$ 2.54 cm needle

into 10-mL vacutainer lithium heparin tubes (Greiner Bio-One, Monroe, NC). Samples were transported to Cornell University (Ithaca, NY) within 2 h of collection for processing. At arrival, BHB was measured in whole blood for the samples obtained at 3 DIM using the Precision Xtra handheld device (Abbott Laboratories, Lake Bluff, IL). Samples were then centrifuged at $1,000 \times g$ for 10 min at 22°C and plasma stored at -80°C until further analysis. Plasma samples were submitted to the University of Illinois Veterinary Diagnostic Laboratory (Urbana, IL) for total Ca analysis using a high-throughput chemistry analyzer (AU680, Beckman Coulter, Brea, CA; intra- and interassay CV <1.0%).

Farm personnel recorded disease event data in DairyComp 305 using standard disease definition protocols discussed before the start of the study. Retained placenta (**RP**) was defined as the failure to expel fetal membranes after 12 h of parturition; metritis was defined as a fetid reddish to brownish uterine discharge often accompanied by systemic signs of illness (fever; rectal temperature $\geq 39.5^\circ\text{C}$) within 14 DIM; and DA was defined as a classical resonant sound, commonly referred to as a “ping,” during simultaneous auscultation and percussion of the left flank region. No right-sided DA was diagnosed for the cohort under study. At 3 DIM, BCS following a 5-point scale with quarter-point increments (Ferguson et al., 1994) and locomotion score (**LS**) following a 5-point scale (Sprecher et al., 1997) were performed by the first author. Description of the herds and rations fed in the prepartum and postpartum periods can be found in Neves et al. (2017).

One multiparous cow was diagnosed with a DA on the day of calving and was not enrolled in the cohort. Additionally, 7 cows were diagnosed as having clinical hypocalcemia by farm personnel within 1 DIM, and were therefore, not included in any of the analyses. Health events and milk production records were extracted from DairyComp 305 into Microsoft Excel (Microsoft Corp., Redmond, WA) before statistical analyses. Daily milk yield was averaged on a weekly basis to represent the milk production for the first 15 wk of the lactation (i.e., up to 105 DIM).

Statistical Analyses

A series of multivariable models were built to analyze the temporal associations of plasma total Ca concentration from DIM 1 to 4 with the risk of early-lactation clinical diseases (i.e., whether the cow was diagnosed with metritis, DA, or both), and milk production for the first 15 wk of lactation. Of note, no primiparous cow had a DA event; therefore, metritis was the only clinical disease outcome analyzed for that parity group. Because the main objective of this study was to singu-

larize and measure the association of plasma total Ca concentration at each of the first 4 DIM with different outcomes, separate models were built for each time point.

In an attempt to maintain the temporal associations between plasma Ca concentration and the diagnosis of the clinical diseases analyzed, an additional number of animals were excluded from the data sets at different time points, as follows: 5, 3, and 1 multiparous cow(s) that received oral Ca supplementation at 1, 2, and 3 DIM, respectively (i.e., Ca supplementation occurring at the same day that plasma Ca concentration was being assessed). Additionally, 7 cows (4 multiparous and 3 primiparous) were excluded from the DIM 4 data set as they had a diagnosis of metritis at 3 DIM. All statistical analyses were performed in SAS version 9.4 (SAS Institute Inc., Cary, NC). Descriptive statistics were performed using the UNIVARIATE, FREQ, CORR, and MEANS procedures. The calving ease score variable was dichotomized to represent cows that required small to no assistance or suffered from dystocia; BCS was considered as a 3-level variable (thin: $\text{BCS} \leq 2.75$; normal: $3.0 \leq \text{BCS} \leq 3.5$; overconditioned: $\text{BCS} \geq 3.75$); LS was considered as a dichotomous variable to denote cows that were lame ($\text{LS} \geq 3$) or not lame ($\text{LS} \leq 2$). Calving season was dichotomized to represent cows calving in the months of June to August (warm season), and cows calving from February to May and September to November (cool season).

Potential collinearity among explanatory variables were investigated using the Pearson correlation coefficient, Cohen's kappa coefficient, and principal component regression (PLS and FACTOR procedures; Yu, 2011). Due to collinearity concerns between the variables parity (i.e., a 3-level variable representing 1st, 2nd, and ≥ 3 rd lactations) and the plasma Ca concentration at 1 DIM, as evidenced on principal component regression, separate models were constructed for primiparous and multiparous cows. For the multiparous cow models, parity was dichotomized to represent 2nd, and 3rd and greater lactations.

Potential associations among explanatory variables with the outcomes under evaluation were first screened by performing univariable analysis using chi-squared statistics. Variables associated with the outcome at $P \leq 0.2$ were offered to the multivariable model. A manual backward stepwise selection procedure was used to build the final models, with variables at $P \leq 0.05$ being retained as main effects. If a variable caused more than a 20% difference in one or more estimates, it was maintained as a confounder. Potential 2-way interactions were tested in the final models between significant variables and plasma Ca concentration (i.e., predictor of interest). Plasma Ca concentration was

handled in the continuous scale throughout the model building process; if significant at $P \leq 0.05$ in the final model, dichotomization was performed based on receiver operating characteristic (ROC) curves. This was conducted to obtain an optimized Ca concentration cut point for easier model interpretation, and more applicable characterization of SCH. For the milk production models, if applicable, cut points were tested based on 0.01 mmol/L increments of the Ca concentration variable for the specific DIM under analysis, within the 10th to 90th percentiles of the variable distribution.

For the clinical disease outcome models, Poisson regression was fitted using the GENMOD procedure with a log link and Poisson distribution. Generalized linear mixed models were fitted for the milk production models using the MIXED procedure, while accounting for the repeated measurements within cow and the nested effect of cows within herd. Three covariance structures were tested in the final milk production models (the first order autoregressive, Toeplitz, and Toeplitz heterogeneous), and the one yielding the lowest Akaike's information criterion was chosen; Toeplitz heterogeneous was the covariance structure used for all final milk models. The effect of BHB concentration was accounted for in the models evaluating the associations of plasma Ca concentration sampled at DIM 3 and 4 (i.e., measurements performed at the same day or after the predictor of interest); inclusion of BHB in the DIM 1 and 2 models was avoided due to concerns of its potential effect of acting as an intervening variable and, therefore, distorting the relationship of the predictor of interest with the outcomes of interest (Dohoo et al., 2009). Goodness of fit statistics based on Pearson and deviance chi-squared statistics for the Poisson models and residual plots for the milk production models were evaluated for all final models. The effect of herd was accounted for as a fixed effect in every model.

Risk of Diagnosis with Metritis or DA. The association of plasma Ca concentration across the first 4 DIM with the risk of primiparous and multiparous cows being diagnosed with metritis, DA, or both, was analyzed. Potential explanatory variables included in these models were calving-related disorders (a single input variable representing whether the cow had dystocia, twins, stillbirth, RP, or a combination of these), BCS, LS, and calving season.

Milk Production During the First 15 Wk of Lactation. The association of plasma Ca concentration across the first 4 DIM with milk production during the first 15 wk of lactation was analyzed. Potential explanatory variables included in these models were calving-related events (a single input variable representing whether the cow had dystocia, stillbirth, twins,

or a combination of these), whether the cow was diagnosed with one or more clinical diseases (primiparous: single input variable representing RP, metritis, clinical mastitis, or a combination of these; multiparous: single input variable representing whether the cow had RP, metritis, DA, clinical mastitis, or a combination of these), week of lactation, previous 305-d milk mature-equivalent (multiparous cow models only), and all previous variables described for the clinical disease outcome models.

RESULTS

A total of 396 cows were included in the cohort across the 2 herds (herd A = 196; herd B = 200), consisting of 35% for 1st, 26% for 2nd, and 39% for 3rd parity and greater cows. Summary data detailing the incidences of calving-related events and clinical diseases for the cohort under study can be found in Table 1. Average plasma Ca concentrations for primiparous and multiparous cows by herd at each of the first 4 DIM are summarized in Table 2. Pearson correlation coefficients for plasma Ca concentration at 2, 3, and 4 DIM in relation to 1 DIM are presented in Table 3. Most notably, no correlation between plasma Ca concentration at 1 versus 4 DIM ($P = 0.24$) were found for multiparous cows, suggesting an important effect of DIM when assessing Ca status in fresh multiparous cows.

Risk of Metritis for Primiparous Cows

Plasma Ca concentration sampled at 1 DIM was not associated with the risk of primiparous cows being diagnosed with metritis ($P = 0.22$). Other variables included in that model were calving-related disorders ($P \leq 0.001$) and herd ($P = 0.001$). Plasma Ca concentration sampled at 2, 3, and 4 DIM was associated with the risk of metritis ($P = 0.001$, <0.001 , and <0.001 , respectively). The effect of calving-related events ($P < 0.001$, 0.002 , and <0.001) and herd ($P = 0.004$, 0.01 , and 0.04) were the other covariates included in the models evaluating the association of plasma Ca concentration at 2, 3, and 4 DIM with the risk of metritis. Optimized cut points based on the association of plasma Ca concentration with metritis across DIM 2 to 4, their respective area under the curve (AUC), sensitivity, and specificity are presented in Table 4.

Risk of Metritis, DA, or Both for Multiparous Cows

The association of plasma Ca concentration across the first 4 DIM with the risk of multiparous cows being diagnosed with metritis or DA (or both) was analyzed.

Table 1. Incidence (%) of calving-related events, early-lactation clinical diseases, and median days to diagnosis (in parentheses where applicable) in a cohort study across 2 herds in New York State

Item	Herd A		Herd B	
	Primiparous (n = 75)	Multiparous (n = 121)	Primiparous (n = 62)	Multiparous (n = 138)
Dystocia	12.0	5.8	12.9	10.9
Stillbirth	9.3	3.3	9.7	2.2
Twins	0	5.8	1.6	1.4
Retained placenta	1.3	2.5	3.2	0.7
Metritis ¹	17.3 (5)	12.4 (5)	9.7 (10)	5.1 (7)
Displaced abomasum ²	0	4.1 (9)	0	5.1 (10)

¹Range of metritis diagnosis was as follows: herd A, 3 to 11 DIM for primiparous and 3 to 7 DIM in multiparous; and herd B, 7 to 12 DIM for primiparous, and 3 to 10 DIM for multiparous.

²Ranges of displaced abomasum diagnosis were as follows: herd A, 5 to 11 DIM, and herd B, 7 to 11 DIM.

Table 2. Average plasma Ca concentration (mmol/L; SEM in parentheses) measured at DIM 1 through 4 by herd and parity group in a cohort study across 2 herds in New York State

DIM of plasma Ca concentration	Herd A		Herd B	
	Primiparous (n = 75)	Multiparous (n = 121)	Primiparous (n = 62)	Multiparous (n = 138)
1	2.19 (0.11)	1.98 (0.27)	2.19 (0.09)	1.87 (0.26)
2	2.18 (0.18)	2.05 (0.32)	2.17 (0.13)	2.03 (0.25)
3	2.18 (0.15)	2.15 (0.24)	2.18 (0.11)	2.19 (0.20)
4	2.21 (0.18)	2.20 (0.21)	2.22 (0.12)	2.27 (0.17)

Table 3. Pearson correlation coefficients of plasma Ca concentrations measured at DIM 2, 3, and 4, in relationship to DIM 1 by parity group in a cohort study across 2 herds in New York State

Variable	DIM			
	1	2	3	4
Plasma Ca concentration at 1 DIM in primiparous (n = 137)				
r	1.00	0.52	0.48	0.32
P-value	—	<0.001	<0.001	<0.001
Plasma Ca concentration at 1 DIM in multiparous (n = 259)				
r	1.00	0.49	0.24	0.07
P-value	—	<0.001	<0.001	0.24

Table 4. Receiver operator characteristic curve determination of critical Ca thresholds associated with the risk of primiparous cows being diagnosed with metritis in a cohort study conducted in 2 herds in New York State

DIM of plasma Ca concentration	n	AUC ¹	Cut point (mmol/L)	Se ²	Sp ³	% of cows below cut point	RR ⁴	95% CI
2	137	0.78	≤2.15	72.2	68.6	36.5 ⁵	4.0	2.0 to 8.0
3	137	0.80	≤2.10	70.6	79.7	26.3 ⁶	5.2	2.6 to 10.3
4	134	0.80	≤2.15	71.4	79.1	25.4 ⁷	6.1	3.0 to 12.2

¹Area under the curve.

²Sensitivity.

³Specificity.

⁴Relative risk.

⁵Herd A had 31% (23/75) incidence of subclinical hypocalcemia (SCH)-classified cows at 2 DIM and herd B had 43.5% (27/62).

⁶Herd A had 31% (23/75) incidence of SCH-classified cows at 3 DIM and herd B had 21% (13/62).

⁷Herd A had 25% (18/72) incidence of SCH-classified cows at 4 DIM and herd B had 26% (16/62).

For the multiparous cow models, plasma Ca concentration at 1 DIM was not associated with the risk of metritis, DA diagnosis, or both ($P = 0.17$). Other variables included in the model were calving-related events ($P < 0.001$), parity ($P = 0.23$), herd ($P = 0.33$), and BCS ($P = 0.009$).

A parity by Ca concentration interaction was found for the model evaluating the plasma Ca concentration at 2 DIM ($P = 0.007$). As differential effects were observed to be dependent on parity, the models for plasma Ca concentrations at 3 and 4 DIM were also kept stratum specific. Briefly, following model stratification, plasma Ca concentration at 2 DIM was associated with the risk of 2nd parity cows being diagnosed with metritis, DA, or both; however, it was not associated for parity ≥ 3 . Plasma Ca concentration at 3 DIM was not associated with the risk of metritis or DA diagnosis (or both) for parity 2 or ≥ 3 . Plasma Ca concentration at 4 DIM was not associated with the risk of metritis or DA (or both) for parity 2, though it was associated for parity ≥ 3 . Table 5 describes the association of plasma Ca concentration at 2, 3, and 4 DIM by parity (2 versus ≥ 3) with the risk of cows being diagnosed with metritis, DA, or both.

Milk Production During the First 15 Wk of Lactation for Primiparous Cows

Plasma Ca concentration at 1 DIM was associated with milk production across the first 15 wk of lactation for primiparous cows ($P = 0.01$). After ROC curve analyses, the cut point that maximized the AUC (0.57) was plasma Ca concentration ≤ 2.15 mmol/L. Primiparous cows with plasma Ca concentration ≤ 2.15 mmol/L (40% of that population; 55/137) produced on average, 2.9 kg (± 0.80) more milk per week than their counterparts with plasma Ca concentration > 2.15 mmol/L across the first 15 wk of lactation. Other explanatory variables included in that model were the effect of herd ($P = 0.02$), wk of lactation ($P < 0.001$), and clinical diseases ($P = 0.20$). Plasma Ca concentration sampled at 2, 3, and 4 DIM was not associated with milk production during the first 15 wk of lactation ($P = 0.32$, 0.65, and 0.94, respectively). Other variables included in the 2, 3, and 4 DIM models were the effect of herd ($P \leq 0.04$ for all models), week of lactation ($P < 0.001$ for all models), and clinical diseases ($P \geq 0.21$ for all models). Additionally, the models at 3 and 4 DIM included the effect of BHB at 3 DIM ($P \leq 0.001$ for both instances).

Table 5. Association of plasma Ca concentration at DIM 2 to 4 assessed on a continuous scale, and receiver operator characteristic curve determination, if applicable, of critical Ca thresholds associated with the risk of multiparous cows being diagnosed with metritis, displaced abomasum, or both in a cohort study conducted in 2 herds in New York State

Parity	DIM of plasma Ca concentration	n	P-value	AUC ¹	Cut point (mmol/L)	Se ²	Sp ³	% of cows below cut point	RR ⁴	95% CI
2	2 ⁵	105	<0.001	0.67	≤ 1.97	44.4	88.8	20.0 ⁶	4.1	1.8 to 9.5
	3 ⁷	104	0.24	—	—	—	—	—	—	—
	4 ⁸	103	0.25	—	—	—	—	—	—	—
3	2 ⁹	151	0.50	—	—	—	—	—	—	—
	3 ¹⁰	151	0.60	—	—	—	—	—	—	—
	4 ¹¹	148	0.04	0.70	≤ 2.20	72.7	60.5	43.2 ¹²	3.1	1.4 to 6.8

¹Area under the curve.
²Sensitivity.
³Specificity.
⁴Relative risk.
⁵Other variables included in the model were calving-related disorders ($P = 0.07$), herd ($P = 0.69$), and BCS ($P = 0.04$).
⁶Herd A had 11% (5/44) incidence of 2nd parity cows being classified with subclinical hypocalcemia (SCH) at 2 DIM and herd B had 26% (16/61).
⁷Other variables included in the model were calving-related disorders ($P = 0.02$), herd ($P = 0.45$), BCS ($P = 0.03$), and BHB at 3 DIM ($P = 0.01$).
⁸Other variables included in the model were calving-related disorders ($P = 0.53$), herd ($P = 0.40$), BCS ($P = 0.005$), and BHB at 3 DIM ($P = 0.006$).
⁹Other variables included in the model were calving-related disorders ($P = 0.006$), herd ($P = 0.33$), and BCS ($P = 0.18$).
¹⁰Other variables included in the model were calving-related disorders ($P = 0.08$), herd ($P = 0.39$), BCS ($P = 0.21$), and BHB at 3 DIM ($P = 0.005$).
¹¹Other variables included in the model were calving-related disorders ($P = 0.07$), herd ($P = 0.82$), BCS ($P = 0.48$), and BHB at 3 DIM ($P = 0.01$).
¹²Herd A had 51% (38/74) incidence of ≥ 3 rd parity cows being classified with SCH at 4 DIM and herd B had 35% (26/74).

Milk Production During the First 15 Wk of Lactation for Multiparous Cows

Plasma Ca concentration at 1 and 4 DIM were associated with milk production across the first 15 wk of lactation for multiparous cows (Table 6); however, the direction of the associations were opposite depending on the DIM of assessment. Reduced plasma Ca concentration at 1 DIM was associated with greater milk production, whereas it was associated with reduced milk production at 4 DIM. After ROC curve assessments, plasma Ca concentration for SCH classification was dichotomized at ≤ 1.77 mmol/L (AUC = 0.61) and 2.20 mmol/L (AUC = 0.52) at 1 and 4 DIM, respectively. Of note, dichotomization of the Ca concentration variable at 4 DIM for the various cut points tested did not provide AUC ≥ 0.60 ; therefore, the same cut point used to classify SCH for the metritis/DA outcome model at 4 DIM was used (i.e., Ca concentration ≤ 2.20 mmol/L). Plasma Ca concentration at 2 and 3 DIM were not associated with milk production ($P = 0.67$ and 0.38 , respectively). Other variables controlled for in these models were the effect of parity ($P = 0.07$ and 0.01 , respectively), herd ($P = < 0.0001$ for both models), week of lactation ($P < 0.001$ for both models), clinical diseases ($P < 0.001$ for both models), calving season ($P = 0.01$ for both models), and previous 305-d mature

equivalent ($P < 0.001$ for both models). The model at 3 DIM also included the confounding effect of BHB sampled concomitantly to plasma Ca concentration ($P = 0.15$).

DISCUSSION

The objectives of this study were to characterize the temporal associations of plasma Ca concentration within the first 4 DIM with the risk of early-lactation clinical diseases and milk production. As Ca concentration can be quite variable within the first few days following parturition, we hypothesized that SCH classification would be dependent on the DIM of assessment (i.e., different DIM would show different associations). Analogous to that, thresholds to classify SCH were hypothesized to be variable depending on the DIM of assessment, as well in that lower thresholds would be obtained during time points closer to the blood Ca concentration nadir at 1 and 2 DIM. However, SCH thresholds showed to be variable depending on parity, and not solely DIM of assessment. As we initially did not expect a strong effect of parity in the analyses, stratification of the data set has impaired the study power and may have influenced the temporal associations between Ca concentration with the outcomes analyzed (i.e., lack of

Table 6. Association of plasma Ca concentration dichotomized in critical thresholds for classification of subclinical hypocalcemia with milk production across the first 15 wk of lactation for multiparous cows enrolled in a cohort study of 2 farms in New York State

Item	DIM 1 ¹			DIM 4 ¹		
	Estimate	SE	P-value	Estimate	SE	P-value
Parity group						
2	Referent	—	—	—	—	—
3	1.22	0.75	0.10	1.77	0.73	0.02
Herd						
A	Referent	—	—	—	—	—
B	3.45	0.80	<0.001	3.59	0.81	<0.001
Calving season ²						
0	Referent	—	—	—	—	—
1	−1.78	0.71	0.01	−1.84	0.73	0.01
Clinical diseases						
Healthy	Referent	—	—	—	—	—
Retained placenta, metritis, displaced abomasum, clinical mastitis, or a combination of these	−3.58	0.90	<0.001	−4.12	1.03	<0.001
Subclinical hypocalcemia ³						
No	Referent	—	—	—	—	—
Yes	2.57	0.83	0.002	−1.75	0.84	0.04
BHB at 3 DIM	—	—	—	1.57	1.00	0.12

¹Milk production models accounting for the repeated measurements across 15 wk of lactation and the nested effect of cow within herd, and previous 305-d mature equivalent ($P < 0.001$).

²Calving season was dichotomized to represent cows calving in the months going from February to May and September to November (0; cool season), and June to August (1; warm season).

³Subclinical hypocalcemia was dichotomized using a cut point of plasma Ca concentration ≤ 1.77 mmol/L (23.5% of the multiparous cows; 61/259) for the DIM 1 model and ≤ 2.20 mmol/L (39% of the multiparous cows; 99/251) for the DIM 4 model.

associations being declared when, if adequate animal numbers were present by parity for a specific outcome, differences could have been found; type II error). We caution readers about adoption of the thresholds found in this study for SCH classification based on parity and DIM of assessment until further evidence is demonstrated by other research groups and greater animal numbers are used. However, our study indicates that more strict sampling strategies should be adopted and most likely differentiated based on parity and DIM of Ca concentration assessment if more sensitive and specific approaches are to be applied in the field to quantify the burden of SCH at the cow and herd level. If the thresholds found in this study are to be applied by stakeholders, one must carefully consider the sensitivity (true positives) and specificity (true negatives) associated with the cut point being used for the specific outcome of interest.

To our knowledge, limited work has been done characterizing specific differences in Ca metabolism and Ca curves/dynamics among 1st parity, 2nd parity, and 3rd and greater parity cows in the postpartum period. However, our findings that SCH classification was dependent on parity and that optimal cut points varied by disease and production parameter analyzed are not fully unexpected. It is not uncommon to find a moderate level of correlation between parity and blood Ca concentration in the immediate postpartum period when both independent variables are to be considered in a model (Miltenburg et al., 2016; Neves et al., 2018), which might reflect an underlying biological difference in Ca metabolism by age group. Calcium partitioning in early lactation is different between parities. For instance, Kume et al. (2003) showed that primiparous cows had greater plasma Ca concentration and lower PTH compared with multiparous cows at parturition. In a small study (6 primiparous and 9 multiparous cows), Iwama et al. (2004) demonstrated that milk Ca concentration was lower in primiparous compared with multiparous cows. Additionally, plasma osteocalcin (a marker of bone synthesis) is known to be higher in primiparous than multiparous cows (Iwama et al., 2004; Taylor et al., 2009). Collectively, it is plausible that the Ca turnover rate is different between primiparous and multiparous cows; therefore, characterization of a suboptimal Ca state in early lactation is likely different between parities.

Several epidemiological studies have analyzed the association of plasma Ca concentration in postpartum cows with the risk of diseases and production outcomes. Differences in the timing relative to parturition of Ca concentration assessment and cut point used for SCH definition between studies must be considered when

interpreting different studies, as these variables can majorly affect the comparability between them. Two recent studies that classified SCH within 24 h of parturition have reported no associations between blood Ca concentration with metritis (Chamberlin et al., 2013; Neves et al., 2018). To the best of our knowledge, only one study has found an association between Ca concentration and metritis when SCH assessment was performed in the first few h following calving; Wilhelm et al. (2017) found that organically managed cows with a serum Ca concentration ≤ 2.0 mmol/L within 2 h of parturition had increased odds of being diagnosed with metritis. Rodríguez et al. (2017) demonstrated that cows with serum Ca concentration ≤ 2.14 mmol/L with samples collected between 24 and 48 h postpartum had increased odds of being diagnosed with metritis. Martínez et al. (2012) in a matched cohort analysis (low risk versus high risk group of cows for developing metritis) found that SCH-classified cows had a greater risk of developing metritis; however, no differences in Ca concentration between the high risk versus the low risk groups or the metritic and nonmetritic cows were found at 0 DIM after pairwise comparisons, but differences became evident starting at 1 DIM.

In our study, plasma Ca concentration was not associated with the risk of primiparous cows being diagnosed with metritis if assessments were performed at 1 DIM; however, plasma Ca concentration at 2 DIM was a good predictor of metritis diagnosis for this parity group. This detrimental association of reduced plasma Ca concentration with an increased risk of metritis diagnosis remained true whether assessments were performed at 3 or 4 DIM. Due to minor to negligible gains in test sensitivity and specificity for SCH assessments from 2 to 4 DIM, it seems more prudent to apply SCH assessments at 2 DIM for primiparous cows. For multiparous cows, the association of plasma Ca concentration with an increased risk of metritis or DA (or both) varied by DIM; if cow-level assessments were to be performed, 2nd parity and 3rd and greater parity cows should be screened at 2 and 4 DIM, respectively. However, the threshold obtained for 2nd parity cows should be applied with caution due to the poor sensitivity, which would incur a large percentage of high risk cows being classified as low risk for developing those diseases (i.e., false negatives). Interpretation of the results must be taken into context with the design of the study and presents limitations. Even though disease diagnosis was performed by trained farm personnel, the frequency in which cows were being screened for metritis, for example, was not strictly controlled by the researchers. Therefore, it is more likely that metritis was underdiagnosed in our context. Parker Gaddis et al. (2012) has described

underreporting issues when comparing on-farm health event records versus incidences reported by researchers. Incidence of metritis in the range of 20 to upper 30% can be found in studies that researchers are in control of the diagnosis, and more thorough examinations are performed using consistent scoring of vaginal discharges under routine schedule (Galvao et al., 2009; Martinez et al., 2012). In the United States, producer-reported metritis incidence is about 8% (USDA-NAHMS, 2014). For our data set, herd B had an overall metritis incidence lower than herd A; we hypothesize that this could be an artifact of herd B performing less frequent health assessments in their animals, or most probable, diagnosing the condition at a later stage (as reflected by a greater median day to diagnosis as compared with herd A) and in more severely sick cows. Although the effect of herd was accounted for in all models, one still cannot rule out whether this issue had any effect on the temporal associations of Ca concentration at different DIM. It is unknown whether an earlier association between Ca concentration and the risk of metritis or DA (or both) could have been found if more frequent assessments for metritis diagnosis were being performed or attempted to be more strictly standardized between herds.

Although we attempted to strictly maintain a temporal character of SCH presence before the diagnosis of a clinical disorder, one cannot rule out the fact that some cows could have been afflicted by the disorder of interest (i.e., metritis or DA) before SCH classification. The peak incidence of metritis is known to occur at 5 to 7 DIM (Galvao, 2012). In a large study, LeBlanc et al. (2005) reported that the median time to DA diagnosis was 11 d, which is near the median time of 9 to 10 d obtained in this study. By restricting the statistical analyses to up to 4 DIM, we attempted to better control for the chronological progression of a risk factor presence and the occurrence of early-lactation diseases and, therefore, reduce misclassification problems.

Primiparous and multiparous cows with reduced plasma Ca concentration at 1 DIM produced more milk when compared with their counterparts with greater Ca concentration. In an earlier study, we found that plasma Ca concentration within 12 h of parturition was only associated with greater milk production in multiparous cows but not primiparous cows (Neves et al., 2018). Initiation of lactation is a key period in the adaptation of Ca metabolism for the dam, as the animal must maintain adequate amounts of Ca in the milk to support the growth of the offspring while maintaining its own demands (Anderson and Garner, 1996). Uniquely to lactation, parathyroid hormone-related protein (PTHrP), a peptide that shares homology

with parathyroid hormone on its amino terminal (Suva et al., 1987) and is known to affect bone mineral resorption (Martin et al., 1997; VanHouten et al., 2004), has been shown to be highly expressed by mammary cells solely during lactation (Thiede and Rodan, 1988). In a rat model, Thiede and Rodan (1988) showed that PTHrP mRNA expression was at its highest around 24 h after parturition and varied according to differently imposed suckling frequencies. It is very likely that PTHrP expressed by mammary cells exerts a key function in the homeostatic adaptation to lactation in cows. In fact, plasma PTHrP has been shown to increase after parturition in cows (Filipović et al., 2008). Peripheral serotonin has recently been shown to regulate PTHrP gene expression (Hernandez et al., 2012) and, therefore, is involved (through PTHrP) in mammary epithelial cell Ca transport (Laporta et al., 2014). It is possible that a transient decrease in blood Ca concentration in the immediate postpartum period is associated with higher milk production as a reflection of a more pronounced PTHrP response. In our study, the direction of plasma Ca concentration association with milk production changed in multiparous cows according to the DIM of assessment; at 4 DIM, reduced plasma Ca concentration was associated with decreased milk production. This temporal association of plasma Ca concentration could be related to a reflection of a delayed/impaired adaptation of some cows to lactation. The same pattern, however, was not observed in primiparous cows. It is possible that a more chronic state of SCH is needed for that age group to detect a negative association of plasma Ca concentration with milk production. More data in primiparous cows are needed to ascertain this hypothesis.

Finally, it is possible that herd-level practices could have affected the associations between Ca concentration and the health and production parameters analyzed. This study only included 2 farms, and therefore, was not designed to examine the effect of herd management practices on SCH classification. Miltenburg et al. (2018) has found a tendency for prepartum cows being housed at 80% pen density to have greater Ca concentrations in the postpartum as compared with cows housed at 120% stocking density. Silva et al. (2013) failed to observe any differences in postpartum energy metabolites when prepartum Jersey cows were housed as a closed group (i.e., no animal additions) versus weekly pen-moves (i.e., with addition of new animals) in the close-up pen; that study did not investigate the effect of pen-moves on postpartum Ca concentrations. For most dairies, when cows have calves the cows are moved into a new pen; therefore, new cows are being added to the fresh cow pen on a daily basis, which may

be detrimental to optimal feeding behavior during a critical period of heightened Ca demands. Regrouping has been shown to negatively affect feeding behavior by reducing the time a cow spends eating and by increasing feed bunk displacements (Huzzey et al., 2006; von Keyserlingk et al., 2008). Taken together, one can hypothesize that most herd practices applied to maximize and improve feeding behavior and DMI in a direct or indirect manner (e.g., through appropriate cow comfort and bunk space allowance, more frequent feed push-ups, and so on) and applied more specifically to the early postpartum period could benefit Ca balance. Herd-level studies investigating management practices that can potentially minimize DMI disturbances in the early postpartum cow and its effect in alleviating SCH are needed.

CONCLUSIONS

To our knowledge, this is the first data set that attempts to describe the epidemiology of SCH in the early postpartum period of Holstein dairy cows. The association of plasma Ca concentration with the different outcomes evaluated was demonstrated to be dependent not solely on the DIM of Ca concentration assessment but also parity. In primiparous cows, reduced Ca concentrations at 2, 3, and 4 DIM were associated with an increased risk of metritis. For multiparous cows, reduced Ca concentration was associated with an increased risk of metritis or DA diagnosis (or both) at 2 DIM for 2nd parity, and 4 DIM in parities ≥ 3 . An inverse relationship was found when evaluating plasma Ca concentration in multiparous cows and its association with milk production; reduced Ca concentration at 1 DIM had a positive association with milk production, and was demonstrated to be negative only at 4 DIM. This study highlights the difficulty in describing the epidemiology of SCH in the early-lactation period and draws attention to the importance of considering the DIM and parity of the cow when evaluating blood Ca concentrations of fresh cows for individual-level SCH assessment. More research on this topic are needed with greater cow numbers by parity group to further validate our findings.

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