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Evaluation of the relationship between ionized and total calcium concentrations in blood during the first week of lactation in dairy cows

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Abstract

Background: Diagnosis of subclinical hypocalcemia in cattle is based on concentration of total Ca ([TCa]) in serum or plasma below the reference range, with [TCa] serving as proxy for the concentration of ionized Ca ([iCa]).

Hypothesis/Objectives: To investigate the relation between [iCa] and [TCa] in periparturient cows over time, and its association with various metabolites.

Animals: Thirty periparturient dairy cows.

Methods: Prospective observational study. Blood from periparturient cows was obtained between 4 days before and 7 days after calving. Samples underwent blood gas analysis and blood biochemical analysis. The ratio of [iCa] : [TCa] was computed. Repeated measures linear regression analyses, spearman correlation-, Deming regression- and receiver operating characteristic (ROC) analyses were conducted.

Results: Correlations between [iCa] and [TCa] ranged from $r = 0.55$ to $r = 0.84$. The diagnostic value of [TCa] to identify cows with [iCa] below the arbitrary cut-off of 1.1 mmol/L was weak in particular during the first 24 hours of lactation, but continuously improved toward the end of the study with areas under the ROC curve increasing from 0.64 at d 0 to 0.93 at d +4 after calving. Strongest association with [iCa] : [TCa] were found for albumin ($r^2 = 0.58$, $P < .0001$), pCO_2 ($r^2 = 0.45$, $P = .0003$), the standard [HCO_3] ($r^2 = 0.22$, $P = .01$), lactate ($r^2 = 0.16$, $P = .04$) and [NEFA] ($r^2 = 0.15$, $P = .05$).

Conclusion and Clinical Importance: The [TCa] is of limited value to identify cows with subnormal [iCa] in the first hours and days of lactation, a finding apparently attributable to the increased variation of a number of metabolic variables that affect the ratio of [iCa] : [TCa].

KEYWORDS
calcium balance, diagnosis, subclinical hypocalcemia, transition cow

Abbreviations: [Alb], plasma concentration of albumin; AP, treatment group "adequate phosphorus"; AUC, area under the curve; [BHBA], plasma concentration of betahydroxybutyric acid; DCAD, dietary cation anion difference; DM, dry matter; [Gluc], plasma concentration of glucose; [HCO3std], plasma concentration of standard bicarbonate; iCa, ionized calcium; [iCa], blood concentration of ionized calcium; [iCa] : [TCa], ratio of ionized calcium to total calcium in plasma; IU, international units; [Lact], plasma concentration of L-lactate; LP, treatment group "low phosphorus"; [NEFA], plasma concentration of nonesterified fatty acids; ROC, receiver operating characteristics; SCH, subclinical hypocalcemia; TCa, total calcium; [TCa], plasma concentration of total calcium; [TP], plasma concentration of total protein.

1 | INTRODUCTION

Periparturient hypocalcemia is an economically important metabolic disturbance in periparturient cows.[1] Subclinical hypocalcemia (SCH) is receiving greater attention because of its potential association with impaired health and productivity. As for other subclinical disturbances, diagnosing SCH can be challenging as it requires a validated diagnostic tool with an unambiguous cut-off value to correctly identify affected animals.[2]

The total amount of Ca (TCa) in blood can be divided into the fractions of ionized Ca (iCa), Ca bound to protein, and Ca complexed to anions such as lactate, phosphate or bicarbonate, comprising approximately 50%, 40% and 10% of TCa, respectively.[3] Although the concentration of iCa is unambiguously recognized as the metabolically relevant fraction, for the purpose of diagnosing calcium balance disturbances the concentration of TCa ([TCa]) is the established reference variable.[2,4] The [TCa] serves as proxy for the concentration of iCa ([iCa]) under the assumption that the ratio of [iCa] : [TCa] is stable.[3] This ratio varies within a range in healthy individuals but is generally treated as a constant for reasons of practicality.[2] Variables related to the acid-base equilibrium, the concentration of protein, phosphate [Pi], NEFA or lactate in plasma affect the ratio of [iCa] : [TCa] in cattle and other species.[5-7] Many of these variables show greatly increased variability in periparturient cows, and thus have the potential to alter the ratio of [iCa] : [TCa], and thereby to affect the diagnostic value of the [TCa] to identify cows with subnormal [iCa] in particular in early lactation.

A considerable body of literature studying and supporting the association between SCH diagnosed by measuring plasma [TCa] and the occurrence of health incidents, impaired fertility or increased cull rates is available, which corroborates the usefulness of TCa to identify cows at risk of developing transition cow problems.[8-10] More recent work, however, revealed that subnormal [TCa] is associated with increased risk for disease only when occurring after the second day of lactation.[11-13] In contrast subnormal [TCa] in the first hours and day of lactation is not predictive for increased disease occurrence.[11-13] Furthermore using [TCa] or [iCa] within the same fresh cow population to diagnose SCH does not identify the same cohort of cows.[11,12,14] A possible explanation for this observation could be a weaker association between [iCa] and [TCa] in the early postparturient period compared to later in lactation.

We investigated the development of the association between [TCa] and [iCa] over time during the first week of lactation, and aimed at identifying most influential factors on the ratio of [iCa] : [TCa] in fresh cows. We hypothesized that [TCa] in plasma in the first days of lactation is of limited value to identify cows with subnormal [iCa], due to interference of a variety of metabolic factors with increased variability in early lactation that may alter the equilibrium between [iCa] and [TCa].

2 | MATERIALS AND METHODS

2.1 | Study design

Results presented here have been obtained from a secondary analysis of an existing data set of a randomized controlled study investigating the effect of dietary P deprivation during the dry period in dairy cows.[15] The design of this randomized controlled study was described in detail in an earlier publication. Briefly, a total of 30 late-pregnant and dry multiparous Holstein-Friesian dairy cows housed in a free stall barn of a research farm, with separate areas for dry and lactating cows were used. Four weeks before expected calving cows were randomly assigned to either a dry cow ration with low P (LP, 0.16% P in DM) or adequate P content (AP, 0.30% P in DM). At calving cows of both treatments were switched to the same lactating cow diet with adequate P content (0.46% P in DM). The number of animals included in the present dataset is based on the sample size calculation conducted for this study.[15]

2.2 | Blood sampling

Blood samples were collected daily between 0800 and 1000 hours from 7 days before expected calving, immediately after calving (d 0), 6, 12 and 24 hours after calving (d +0.25, d +0.5 and d +1, respectively) as well as on day 2, 3, 4 and 7 after calving (d +2, d +3, d +4 and d +7, respectively). Blood samples obtained during the last days of gestation were retrospectively assigned a sampling time in days relative to the day of calving (d −4, d −3, d −2, d −1). Sampling time d −1 was defined as the last regular blood sample obtained in the morning of the day prior to calving. Samples obtained at the sampling times d +2 to d +7 were obtained in the morning between 0800 and 1000 hours. Blood was obtained by puncture of a jugular vein using an anaerobic collection system with a 20-gauge needle (VACUETTE with 10 mL LH Vacuette, Greiner Bio-One, Kremsmünster, Austria).

Cows suspected to have clinical periparturient hypocalcemia based on physical examination as described[16] were checked for the blood [iCa] with a point of care unit as described below and, if confirmed to be hypocalcemic, were treated with Ca salts administered subcutaneously and orally. Blood samples of cows treated with Ca, obtained after treatment, were excluded from the dataset. Of the 30 cows included in the study 4 cows were prematurely removed after having developed clinical hypocalcemia and having received treatment with Ca salts as described above. Specifically, 1 case of clinical hypocalcemia was diagnosed at each of the sampling times d +0.25, d +1, d +4 and at d +7. Samples from cows affected by clinical hypocalcemia were only included until the time of diagnosis and before treatment. One cow became anorectic 2 days after calving, blood samples obtained after d +1 from this animal were excluded from the dataset.

2.3 | Sample processing and analyses

2.3.1 | Blood gas analyses

Blood gas analyses were conducted using a cartridge based point of care unit previously validated for the use in cattle (EPOC Host and Reader, Siemens Healthineers, Erlangen, Germany).[15] The analytical

range for [iCa] provided by the manufacturer is 0.25-4.0 mmol/L; the intra-assay coefficient of variation determined in our lab (all measured on 1 single unit, n = 4 × 10) was 2.3%.

Blood gas analyses were conducted on whole blood collected anaerobically in lithium heparin tubes filled to the exact volume indicated by manufactory instructions. This yielded blood samples with an activity of 17 IU of heparin/mL. One milliliter of blood was aspirated anaerobically from the lithium heparin tube into a microliter syringe (SOFT-JECT, Henke Sass Wolf, Tuttlingen, Germany). Samples were analyzed within 5 minutes of sample collection. The point of care unit measures [iCa], pH and pCO_2 in whole blood by direct potentiometry and blood glucose ([Gluc]) and L-lactate ([Lact]) by amperometry. Reported values for blood pH and pCO_2 were corrected to the rectal temperature determined immediately prior to sample collection in each animal with an algorithm programmed in the POC unit. The standard bicarbonate concentration ([HCO_3std]), defined as the concentration of bicarbonate at 37°C when the pCO_2 is 40 mm Hg, was calculated using the equation $HCO_3std = 0.0307 \times 40 \times 10^{(pH - 6.129)}$, where 0.0307 corresponds to the solubility coefficient for CO_2 in plasma at 37°C, pH is the blood pH at 37°C, 40 is the fixed pCO_2 in mm Hg, and 6.129 is the apparent dissociation constant of carbonic acid at 37°C.[17]

2.3.2 | Sample processing

Blood remaining in the lithium heparin tubes was centrifuged at room temperature at 1730*g* for 15 minutes (Jouan CR422, Thermo Fisher Scientific, Waltham, MA) within 20 minutes of collection; plasma was harvested and stored at −21°C until further analysis.

2.3.3 | Plasma biochemical analysis

Plasma samples were assayed for the concentrations of [TCa] (Arsenazo III method), inorganic phosphorus ([Pi], ammonium molybdate method), nonesterified fatty acids ([NEFA], acetyl-CoA-synthetase-acetyl-CoA-oxydase method), betahydroxybutyric acid ([BHBA], UV-method, all conducted on a chemistry analyzer (Cobas Mira Plus CC, Hoffmann-La Roche AG, Basel, Switzerland)) as well as for the concentrations of total protein ([TP], colorimetric method with copper sulfate) and albumin ([Alb], bromocresol green method, all conducted on AU680, Beckman Coulter, Inc, Brea, CA). The lower detection limit for TCa was 0.01 mmol/L with intra- and interassay coefficients of variation of 0.91% and 1.14%, respectively.

The ratio of [iCa] : [TCa] was calculated for each cow at each sampling time.

2.4 | Statistical analysis

Results are expressed as LSM ± SEM or as median and interquartile range for variables not meeting the assumption of normality. The significance level was set at $P < .05$. Normality of residuals and homogeneity of variance were examined (Shapiro-Wilk test), variables violating the assumption of normal distribution of residuals were subject to log transformation; transformed variables were plasma [NEFA], [BHBA], [Gluc], and [Lact].

Mixed model repeated measures analyses of variance were first conducted on all blood parameters determined in this study, as well as on the ratio of [iCa] : [TCa] to identify the parameters affected by the varying dietary P supply in the experiment. The regression model included treatment (AP or LP) and time, as well as the interaction of treatment by time as fixed effects. This analysis was included with the purpose of identifying a potential bias resulting from to the experimental treatment of the primary experiment from which the data of this study were obtained. Animal ID was included as subject and time as repeated factor. The most appropriate covariance structure was chosen based on the lowest Akaike information criterion. Bonferroni-adjusted *P* values were used to assess differences between treatments at specific sampling times whenever the *F* test was significant. Values of both treatments (AP and LP) where pooled for those parameters for which a statistically significant treatment effect was not determined, whereas parameters with a significant treatment effect (only plasma [Pi]) are presented stratified by treatment.

Of the blood biochemical variables investigated for their association with the ratio of [iCa] : [TCa] only plasma [Pi] revealed a significant treatment effect ($P < 0.001$), with lower values in LP than AP cows from d −4 to d +1. For none of the other parameters a treatment- or treatment by time interaction effect was identified. The results of the plasma biochemical parameters and the values stratified by sampling time are provided as supplementary material (Table S1).

Spearman correlation and multivariate linear regression analyses using PROC CORR and PROC REG (SAS. 9.4, SAS, Inc, Cary, NC) were conducted for each time point separately to determine associations between the ratio of [iCa] : [TCa] as presumed dependent variable with [Pi], [TP], [Alb], pH, pCO_2, [HCO_3std], [Gluc], and the log transformations of [NEFA], [BHBA] and [Lact] as independent variables. Each regression model was checked for variance inflation by determining correlations between independent variables and screening tolerance. Cut-off values for variance of inflation and tolerance used were 10 and 0.1, respectively. Scatter plots were visually inspected for data points suspicious of being influential and Cook's distance (D_i) was calculated. In cases $D_i > 1$ the results of the regression analyses with and without the data point in question were compared.[18] All available data points were retained for the statistical analysis.

Pearson correlation analyses and Deming regression analyses were conducted to characterize the association of plasma [TCa] with blood [iCa] at the sampling times d 0 to d +7. receiver operating characteristics (ROC) analyses were conducted for each sampling time between d 0 and d +4 to assess the suitability of plasma [TCa] to predict the presence of hypocalcemia, defined as blood [iCa] < 1.10 mmol/L.[19] ROC curves were plotted, the area under the ROC curves (AUC) and associated *P* values were computed; the point on the ROC curve where the distance from the upper left corner of the graph (0, 1) is the smallest was determined for the time points where the AUC of the ROC curve was significantly different from 0.5. Deming regression and ROC

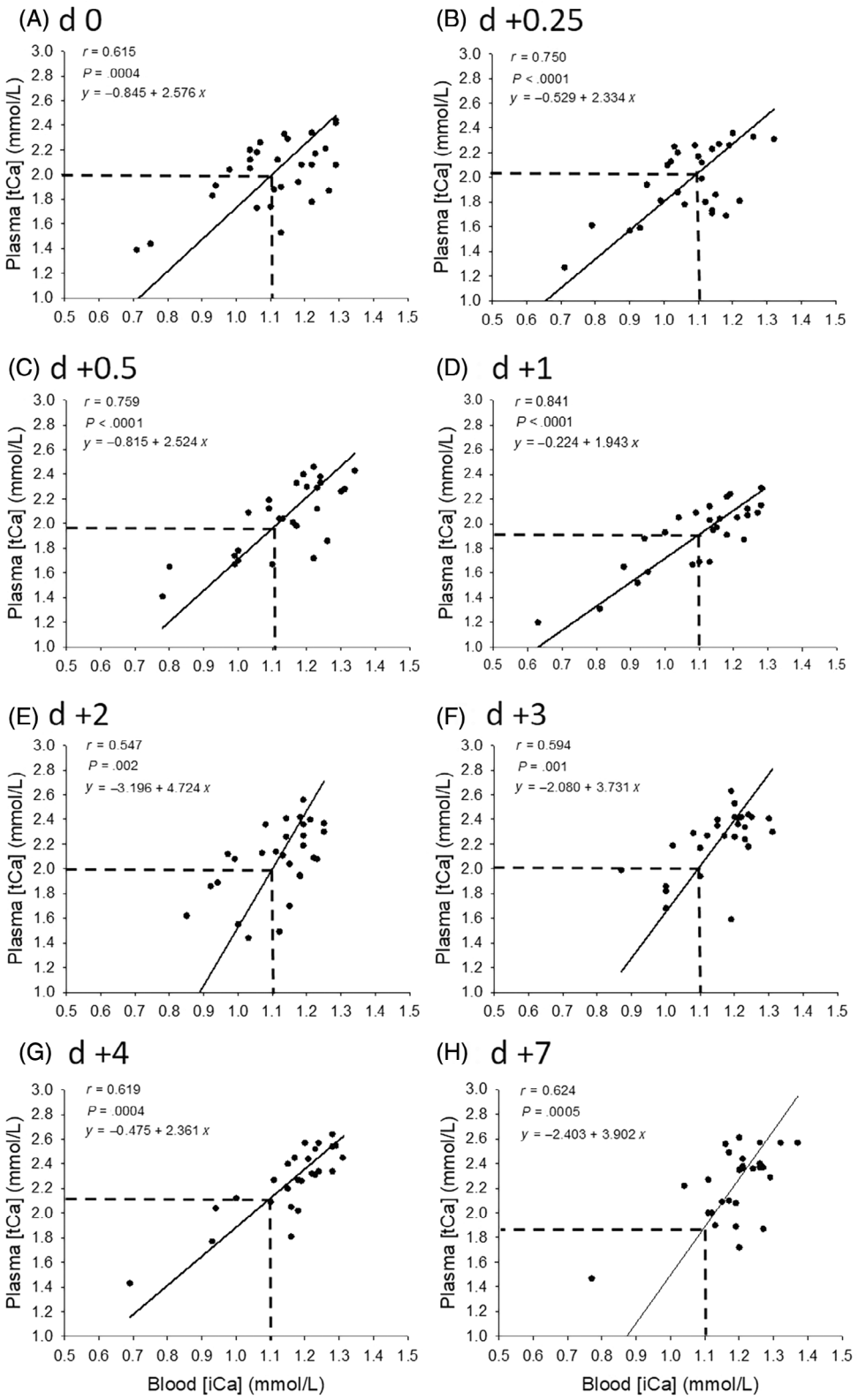

FIGURE 1 Relationship between the concentration of ionized Ca in blood ([iCa]) and the concentration of total Ca measured in plasma ([TCa]) at the times 0 hours (A, d 0, n = 29), 6 hours (B, d +0.25, n = 28), 12 hours (C, d +0.5, n = 27), 1, 2, 3, 4, and 7 d (D, d +1, n = 27; E, d +2, n = 26; F, d +3, n = 26; G, d +4, n = 26, and H, d +7, n = 26, respectively) relative to calving. The solid line represents the line of best fit from Deming regression. Pearson correlation coefficients (r^2) corresponding *P* values and the function of the Deming regression line are provided for each sampling time. Dashed lines regress the arbitrary cut-off value of 1.10 mmol/L for [iCa][19] to the corresponding [TCa] from the Deming regression line.

analyses were only conducted on samples obtained after calving, as subnormal values for [TCa] and [iCa] were not observed before calving. A scientific plotting software package was used to plot ROC and Deming regression curves (Sigma Plot 12.5, Systat Software, Inc, San Jose, CA). All remaining statistical analyses were conducted with a statistical software package (SAS 9.4, SAS, Inc, Cary, NC).

3 | RESULTS

The concentration-time curves for [TCa] and [iCa] as well as the incidence rates of clinical and subclinical hypocalcemia during this experiment are reported.[15] The range of [TCa] and [iCa] and the association between these variables at the postpartum sampling times are

visualized as scatter plots for the sampling times between d 0 and d +7 in Figure 1 together with results of the correlation and Deming regression analysis. Lowest values for both variables were determined at the sampling times within the first 24 hours of lactation. The correlation coefficients varied between 0.55 and 0.85 (Figure 1). The association between [iCa] and [TCa] varied considerably between sampling times, as did the slope of the Deming regression line (Figure 1). The cut-off value for blood [iCa] of 1.10 mmol/L arbitrarily used in this study was regressed to values of plasma [TCa] between 1.80 and 2.10 mmol/L (Figure 1).

Figure 2 presents the time curve of the ratio [iCa] : [TCa]. No treatment, time or treatment × time interaction effect was observed. The median ratio of [iCa] : [TCa] varied between 51 and 59% with a trend toward higher values at the postpartum sampling times. A considerable degree of variation between sampling times, with a maximum interquartile range from 47 to 64% was apparent.

Of the blood biochemical variables investigated for their association with the ratio of [iCa] : [TCa] only plasma [Pi] revealed a significant treatment effect ($P < .001$) with lower values in LP than AP cows from d −4 to d +1. For none of the other parameters a treatment- or treatment by time interaction effect was identified. Plasma [Pi] was also affected by a statistically significant time effect ($P < .001$) in the form of a transient decline from the moment of calving to d +2. A statistically significant time effect was also observed for [TP] ($P = .004$) and [Alb] ($P = .006$), which, in both cases, consisted in an increase from the time of calving compared to precalving values, that was transient for [Alb] but persisted until the end of the observation period for [TP]. Time effects were also identified for the log transforms of plasma [NEFA] and [BHBA] ($P < .0001$). Plasma [NEFA] started to increase at calving, and was followed by increased [BHBA] (Table S1). Both variables showed large inter- and intraindividual variation. The results of the plasma biochemical parameters and the values stratified by sampling time are provided as supplementary material (Table S1).

Values of variables characterizing the acid-base homeostasis as well as blood [Gluc] and [Lact] are presented as supplementary material in Table S2. Again, treatment effects related to the differing dietary P supply during the experiment were not observed for any of the variables. Significant time effects were found for blood pH ($P = .01$), [HCO_3std] ($P = .005$), blood [Gluc] ($P < .0001$), and blood [Lact] ($P < .0001$). The lowest pH was measured at d 0 with values increasing again at d +0.25. All acid-base related variables remained within or slightly above the reference range for cattle at all times.[20] Blood [Gluc] showed a transient peak at d 0 that was followed by a progressive decline throughout the study to values well below prepartum levels. For blood [Lact] highest values were measured at d 0 and d +25 (Table S2).

Results of the spearman correlation analysis for [iCa] : [TCa] with plasma [Pi], [TP], [Alb], [NEFA], parameters of the acid-base homeostasis, [Gluc] and [Lact] are presented in Table 1. Associations with [iCa] : [TCa] were most frequently identified for [Alb] (highest $r^2 = 0.58$, $P < .0001$) and [TP] (highest $r^2 = 0.45$, $P = .0001$) followed by positive associations with pCO_2 (highest $r^2 = 0.40$, $P = .0003$). Less frequently, negative associations were identified with pH (highest $r^2 = 0.22$, $P = .01$) and [HCO_3std] (highest $r^2 = 0.22$, $P = .01$). Strong associations of [Alb] with [TP] as well as of pCO_2 with pH are indicative of covariances requiring consideration. A negative association of the ratio [iCa] : [TCa] was identified with [Pi] at d +2 ($r^2 = 0.25$, $P = .009$), as well as with [Gluc] ($r^2 = 0.18$, $P = .028$), [Lact] ($r^2 = 0.16$, $P = .04$) and [NEFA] ($r^2 = 0.15$, $P = .05$). The multivariate regression analysis conducted for each sampling time yielded significant associations of the [iCa] : [TCa] with [Alb] at the sampling times d −1 (partial $r^2 = 0.37$, $P = .04$), d +0.5 (partial $r^2 = 0.34$, $P = .01$), d +3 (partial $r^2 = 0.26$, $P = .02$), as well as with log transform of [NEFA] at d +4 (partial $r^2 = 0.10$, $P = .02$).

The results of the ROC analysis assessing the suitability of plasma [TCa] to diagnose SCH, defined as blood [iCa] < 1.10 mmol/L, at the sampling times d 0 to d +4 are presented in Figure 3. A ROC analysis was not conducted for d +7 as only 1 animal with subnormal blood

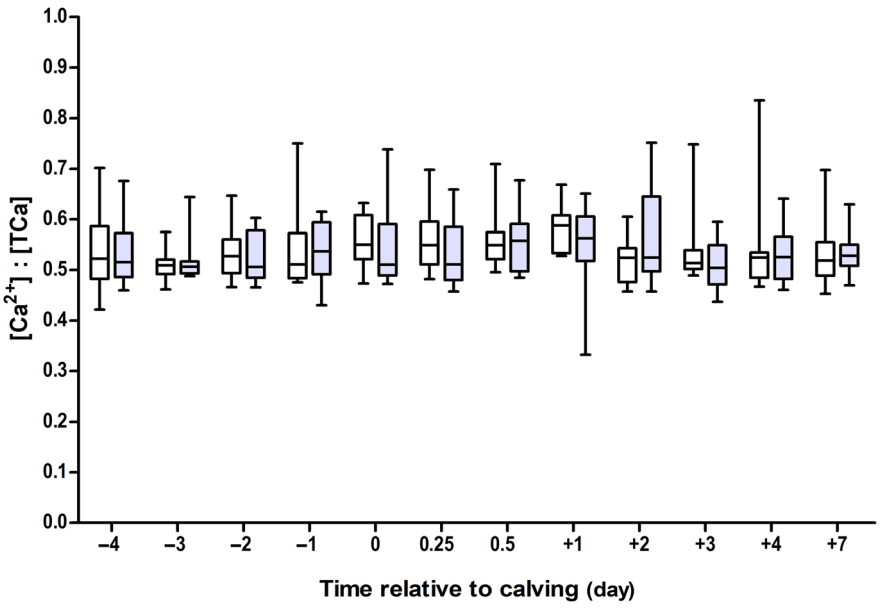

FIGURE 2 Ratio of blood ionized Ca to plasma total Ca ([iCa] : [TCa]) for adequate P (gray boxes) and low P (open boxes) treatment. Box and whisker plots represent median (horizontal line), lower and upper quartiles (bottom and top of box, respectively) and range (lower and upper end of whiskers, respectively). Each treatment comprised 15 multiparous dairy cows that were fed a dry cow ration with either adequate (AP, 0.30% P in DM) or low P content (LP, 0.16% P in DM) during the last 4 weeks of gestation. Sampling times were 0 hours (d 0) 6 hours (d +0.25), 12 hours (d +0.5), 1, 2, 3, 4 and 7 d (d +1, d +2, d +3, d +4, and d +7, respectively) relative to calving.

TABLE 1 Results of the spearman correlation analysis (Spearman rho and *P* values) for the ratio of blood concentration of ionized Ca to plasma concentration of total Ca ([iCa] : [TCa]) with plasma concentration of inorganic P ([Pi]), total protein ([TP]), albumin ([Alb]), nonesterified fatty acids ([NEFA]), blood pH, partial pressure of CO_2 (pCO_2), standard bicarbonate ($[HCO_3std]$), glucose ([Gluc]), and L-lactate ([Lact]).

Item	Day relative to calving	[Pi]	[TP]	[Alb]	NEFA	pH	pCO_2	$[HCO_3std]$	[Gluc]	[Lact]
[iCa] : [TCa]	d −4	NS	NS	NS	NS	NS	NS	NS	NS	NS
[iCa] : [TCa]	d −3	NS	−0.42 ($P = .03$)	−0.53 ($P = .006$)	−0.39 ($P = .05$)	−0.42 ($P = .04$)	0.51 ($P = .009$)	−0.47 ($P = .02$)	NS	NS
[iCa] : [TCa]	d −2	NS	−0.63 ($P = .0007$)	−0.61 ($P = .001$)	NS	NS	NS	NS	NS	−0.40 ($P = .04$)
[iCa] : [TCa]	d −1	NS	NS	−0.49 ($P = .007$)	NS	−0.42 ($P = .03$)	0.42 ($P = .03$)	−0.42 ($P = .02$)	NS	NS
[iCa] : [TCa]	d 0	NS	NS	−0.51 ($P = .005$)	NS	NS	NS	NS	NS	NS
[iCa] : [TCa]	d +0.25	NS	NS	NS	NS	NS	0.63 ($P = .0003$)	NS	NS	NS
[iCa] : [TCa]	d +0.5	NS	NS	−0.4 ($P = .039$)	NS	NS	NS	NS	NS	NS
[iCa] : [TCa]	d +1	NS	NS	−0.4 ($P = .04$)	NS	NS	0.47 ($P = .01$)	NS	NS	NS
[iCa] : [TCa]	d +2	−0.42 ($P = .03$)	−0.67 ($P = .0001$)	−0.58 ($P = .001$)	NS	NS	0.45 ($P = .02$)	NS	NS	NS
[iCa] : [TCa]	d +3	NS	−0.54 ($P = .004$)	−0.76 ($P < .0001$)	NS	NS	NS	−0.47 ($P = .01$)	−0.42 ($P = .03$)	NS
[iCa] : [TCa]	d +4	−0.5 ($P = .009$)	−0.41 ($P = .04$)	−0.46 ($P = .02$)	NS	NS	NS	NS	NS	NS
[iCa] : [TCa]	d +7	NS	−0.55 ($P = .006$)	−0.53 ($P = .008$)	NS	NS	NS	NS	NS	NS

Abbreviation: NS, no statistically significant effect.

Ca concentration was available at that time. The AUC of the ROC curves calculated for d 0 to d +4 increased from 0.64, that was not statistically significantly different from 0.5 at d 0 to 0.93 ($P = .007$). The AUC of the ROC curves remained below 0.85 from d 0 to d +2 (Figure 3). Most suitable cut-off values for [TCa] were 2.21 mmol/L (Se = 0.83, sp = 0.81) and 2.16 mmol/L (Se = 1.0, sp = 0.82) for d +3 and d +4, respectively.

4 | DISCUSSION

The objective of this study was to investigate changes in the relationship between [iCa] and [TCa] occurring during the periparturient period of dairy cows, and to identify variables having the potential of exerting a metabolically relevant effect on the ratio of [iCa] : [TCa] in this period. Our results indicate that the association between [iCa] and [TCa] during the first week of lactation is moderate and unstable, which corroborates the findings of earlier studies[6,12,14,21] while contrasting others.[22,23] Under the assumption that in clinical pathology [TCa] in plasma serves as convenient proxy for the metabolically relevant [iCa] in blood, this moderate and variable association between [iCa] and [TCa] in the first hours after calving casts doubt on the usefulness for the diagnosis of SCH of providing cut-off values for TCa in serum or plasma with a precision of 2 decimal points as is commonly done in the pertinent scientific literature.[24,25]

The mean ratio of [iCa] : [TCa] showed a trend toward higher values after- compared to before calving, and displayed a considerable degree of variation with a maximum interquartile range from 47% to 64%. The fraction of iCa is widely held to crudely account for approximately 50% of the TCa available in the extracellular space of healthy individuals and across species.[6] The periparturient period, however, like episodes of illness, has been identified as a phase where the equilibrium between iCa and the nonionized Ca fraction is prone to significant and metabolically relevant alteration.[4,6] Several authors reported increases of the iCa fraction relative to TCa around parturition from 50% to 56% and more,[7,19,26] while others found fairly constant ratios over time during the periparturient period.[12,22]

Several factors such as the plasma protein- and more specifically the albumin concentration, but also alterations of the acid-base equilibrium and the concentrations of Pi, NEFA or lactate in plasma have been discussed as factors affecting the ratio of [iCa] : [TCa] in cattle and other species.[4,6,7,22] Factors with strongest impact on the ratio determined in this study were plasma protein, and more precisely the albumin concentration, with increasing concentrations resulting in a decrease of the iCa fraction in blood, which is in agreement with earlier studies.[27,28] Sudden and short-lived changes of albumin concentration occur around calving, which presumably are the result of transient disturbances of the water balance in periparturient cows.[29-31] Water and feed intake in the hours before, and most of all shortly after calving are highly variable, and are likely to result in transient but metabolically relevant changes in extracellular volume. Such changes of the extracellular volume unavoidably alter the concentration of albumin in serum or plasma, and thereby the amount of albumin available to bind iCa.

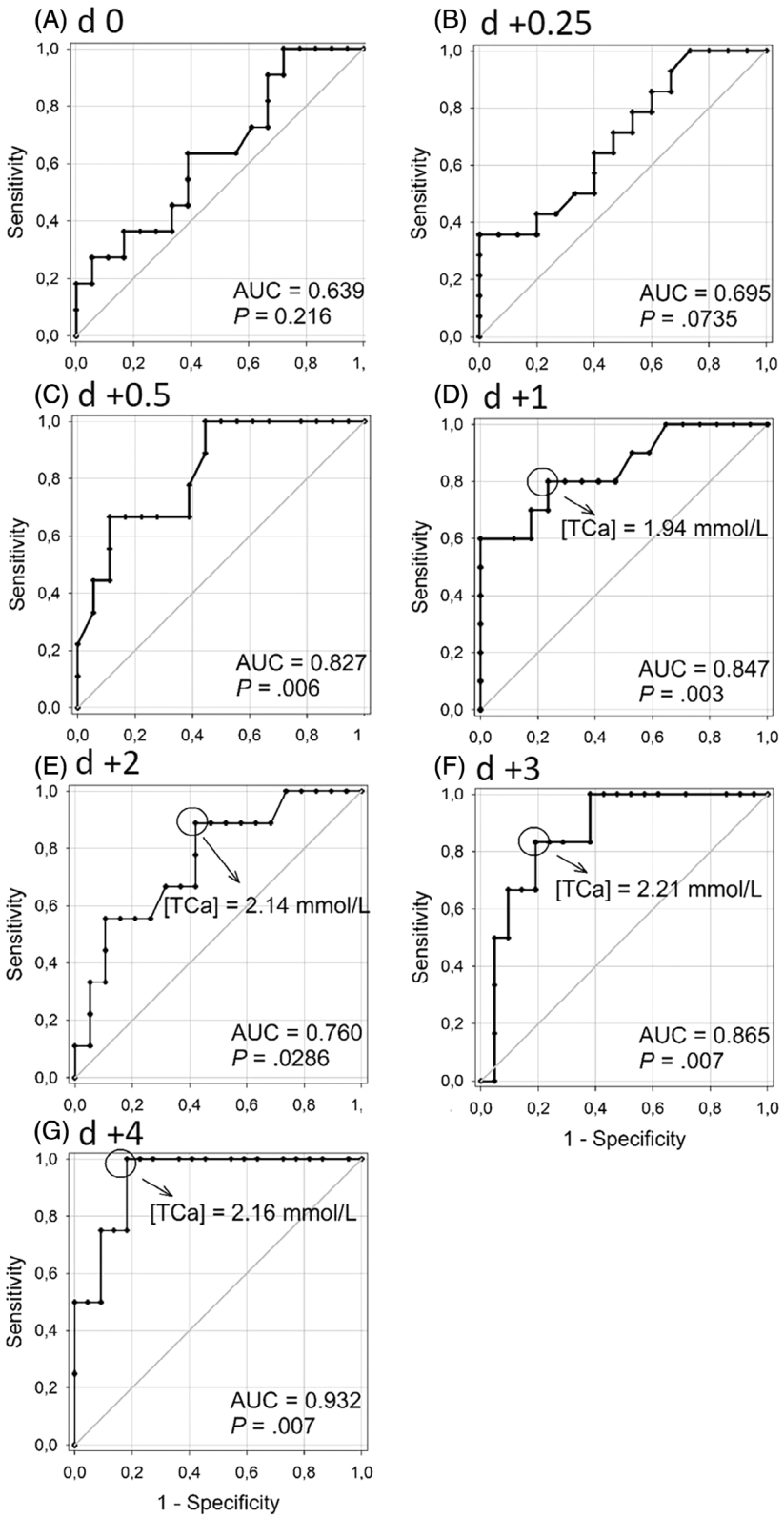

FIGURE 3 Receiver operator characteristic (ROC) analysis conducted for the sampling times 0 hours (A, d 0), 6 hours (B, d +0.25), 12 hours (C, d +0.5), as well as 1, 2, 3, and 4 d relative to calving (D, d +1; E, d +2; F, d +3, and G, d +4, respectively) for the concentration of total calcium in plasma ([TCa]) in periparturient cows to diagnose hypocalcemia in cattle, defined as concentrations of ionized Ca ([iCa]) in blood of below 1.10 mmol/L. The analysis at d +0 included 29 and 11 cows, at d +0.25 28 and 12 cows, at d +0.5 and d +1 27 and 9 cows, at d +2 26 and 9, at d +3 26 and 6 cows, and at d +4 26 and 4 cows in total and with an [iCa] < 1.1 mmol/L, respectively. The area under the ROC curve is provided in each panel, a *P* value <.05 indicates that the AUC is significantly different from the diagonal line (corresponding to an AUC of 0.5 corresponding to random chance of accurately predicting hypocalcemia). For the sampling times where the *P* value of AUC < 0.05 the value for [TCa] with the smallest distance from the upper-left corner of the panel is provided.

Another factor with considerable impact on the ratio of [iCa] : [TCa] in this study was the acid-base equilibrium with a slightly more prominent effect of the respiratory than the nonrespiratory component of the acid-base equilibrium.[7] A shift toward an acidotic state, either due to increased pCO_2 or decreased [HCO_3std] resulted in an elevation of the fraction of iCa, while the opposite occurred with decreasing pCO_2 or increasing [HCO_3std].[32,33] The standard HCO_3 rather than the actual HCO_3 was used in this study to determine the nonrespiratory effect of the acid-base equilibrium to avoid the bias resulting from an altered pCO_2 on HCO_3.[34] Earlier studies reported

that nonrespiratory alterations of the acid-base equilibrium have a stronger impact on the fraction of iCa than what is reported here.[7] These studies often included the use of low DCAD diets in late gestation, with the aim of inducing a compensated nonrespiratory acidosis which evidently would be associated with more pronounced changes in HCO_3 around calving.[7] In the study presented here cows neither were fed a diet with low DCAD, nor experienced pronounced acid–base disturbances around parturition.

The fraction of iCa in blood of periparturient cows was furthermore found to be inversely related to the plasma [Pi], an association that has been explained by the formation of complexes of iCa with Pi.[35] Remarkably this effect only became apparent from 2 days after calving, when the plasma [Pi] of LP cows had risen to approach values of AP cows. It is presumably the increase in variation of the plasma [Pi] occurring at this stage, resulting from the varying degree of hypophosphatemia around calving in combination with differing kinetics between individuals in restoring normal plasma [Pi] that drove this effect. The original experiment from which samples for this study were obtained used diets with either exceptionally low or with adequate P content. This resulted in a range of plasma [Pi] in this study that is unlikely to be encountered when feeding conventional dry cows diets. It is thus conceivable that the effect of plasma [Pi] on the ratio of [iCa] : [TCa] in the present study overestimates the effect this factor may have in practice. An association between [iCa] and the plasma [Pi] has, however, been reported previously in cattle and other species.[6,14,35]

Lactate, a negatively charged strong ion is also thought to form complexes with iCa in blood, which again reflects in a negative association of [Lact] with the fraction of iCa in this and earlier studies.[23,32] Again, this effect was most pronounced around calving, when cows displayed highest plasma [Lact], presumably due to physical stress associated with the calving process.

Another parameter with greatly increased degree of variation during the periparturient period with significant effect on the ratio of [iCa] : [TCa] is plasma [NEFA]. Elevated plasma [NEFA] in response to negative energy balance around calving as observed in this experiment is well established.[36,37] The decrease of the iCa fraction with increasing plasma [NEFA] without directly affecting the TCa is suggestive of the formation of NEFA-Ca complexes,[38] which is in line with earlier studies describing altered Ca binding due to conformational changes of the albumin molecule mediated by NEFA.[39,40] Another study, however, reported negative associations of plasma [NEFA] on the day of calving with [iCa] but not with [TCa], which was attributed to increased lipomobilization as blood [iCa] declines rather than to the binding of iCa by NEFAs.[41] This latter interpretation is corroborated by findings of a study where experimentally induced SCH in nonperiparturient cows resulted in decreased insulin secretion in association with elevated plasma [NEFA] and with lower dry matter intake.[42]

Hampered insulin secretion due to decreased availability of iCa in the extracellular space is also the presumed mechanism behind the negative association between the fraction of iCa and glucose identified in this and earlier experiments.[22,43] Pancreatic insulin secretion depends on the availability of iCa.[44] Following this sequence of events, lower blood glucose with increasing [iCa] should be interpreted as consequence rather than cause for elevated [iCa].

Taken together the results presented here indicate that a number of blood biochemical parameters, all characterized by a sudden and marked increase in variance around calving, and with a documented effect on the ratio of [iCa] : [TCa] are likely to have contributed to the weak association between blood iCa and plasma TCa in the first hours and days of lactation reported here.

The point was raised previously that defining SCH based on either blood iCa or TCa in serum or plasma does not reliably identify the same cohort of periparturient cows, and that clinical signs associated with clinical hypocalcemia do not always match serum [TCa].[6,12,14] This is of concern as for reasons of convenience, practicality and cost it remains routine practice in dairy production medicine to base the diagnosis of SCH on a subnormal [TCa] in serum or plasma.[24,45,46] A recent study found that occurrence of SCH in dairy cows determined based on the plasma [TCa] on the first day of lactation is predictive of an increased risk of developing disease or becoming a premature cull when diagnosed after the second day of lactation, but not when diagnosed in the first 48 hours after calving.[8,47] Consequently, the authors questioned the value of measuring the [TCa] in the first 24 hours of calving to diagnose SCH. Based on our results we propose that this lacking association of [TCa] in the first 48 hours after calving with increased risk of developing a fresh cows disease could also be attributable to the limited diagnostic value of [TCa] to identify cows with subnormal [iCa]. This hypothesis is corroborated by the observation that experimentally induced SCH, defined as subnormal plasma [iCa], was shown to have an impact for example on immune cell function within 24 hours of onset already.[42] Notwithstanding the association of the incidence of periparturient disease of dairy cows with the blood [iCa] in the first days after calving has not been studied. It thus remains to be determined if the [iCa] is indeed better suited than [TCa] to predict disease in recently calved cows, and if so what the best suited cut-off value and sampling time point would be.

The ROC analysis conducted for the different sampling times similarly underscores the importance of considering the sampling time relative to calving when assessing the diagnostic quality of [TCa] to predict subnormal [iCa]. The area under the ROC curve increased continuously throughout the study period from 0.64, that was not different from 0.50 and thus characterizing the diagnostic value of [TCa] at d 0 as limited, to a value above 0.90 at d +4, indicating good diagnostic quality of [TCa] at this time point.[48]

A number of preanalytical factors such as the type and amount of anticoagulant, anaerobic conditions, time to analysis and ambient temperature can affect the potentiometric blood gas and electrolyte analysis.[49] Care was taken in this experiment to standardize sample collection and handling, to minimize time intervals between sample collection and blood gas analysis, and to minimize exposure of the sample to ambient air. Blood was collected anaerobically in adequately filled lithium heparin tubes yielding blood samples with an activity of 17 IU of heparin/mL. At this concentration of heparin, Ca binding and thus an artifactual decrease of [iCa] in the range of up to 0.03 mmol/L must be expected.[49] We elected not to use syringes or capillaries coated with Ca balanced

heparin that are commonly used when measuring blood [iCa]. The Ca contained in electrolyte balanced heparin tends to artificially increase the [iCa] by on average 0.06 mmol/L with a blood [iCa] < 0.9 mmol/L.[50]

An important limitation of the present study is the small sample size resulting from the study design with intensive sampling schedule of the parent experiment on which the present study is based. The experimental treatment investigated in the parent study, that is feeding exceptionally low dietary P content was found to markedly affect the [tCa] and [iCa], but not the ratio of [iCa] : [tCa]. This provided a broader range of Ca values around parturition to be included in the analysis, but may at the same time have resulted in an unidentified bias. Although results reported here are in line with earlier studies investigating alterations of the association between iCa and tCa in early lactating cows a larger scale prospective study is certainly warranted.

Another limitation of this study resulted from the decision to treat study animals with clinical hypocalcemia in early stage, and thus before becoming recumbent. Since blood samples were only included until immediately before treatment, due to this approach more samples of hypocalcemic cows had to be excluded from the analysis, than if animals would only have been treated after becoming recumbent. This is likely to have resulted in an underestimation of the treatment effect on the Ca homeostasis in this experiment. This furthermore contributed to another weakness of the study that is the relatively small number of samples with subnormal [iCa] and [TCa] particularly toward the end of the observation period.

The increasingly narrowing range of values of TCa and iCa weakens the reliability of the comparison of the association between these parameters at the different sampling times primarily at the disadvantage of the later sampling times with narrower range of measured values. This, however, may have resulted in an underestimation of the association between [TCa] and [iCa] at the sampling times d +4 and later, but would not weaken the conclusions concerning the sampling times in the first 2 days after calving with considerable numbers samples with low Ca content.

In conclusion, the results reported here indicate that the [TCa] is a poor proxy for [iCa] in periparturient dairy cows in particular during the first 24 hours after calving. The association of the plasma [TCa] with blood [iCa], the presumed reference standard parameter to diagnose SCH, is affected by a number of parameters, all displaying a marked increase in the degree of variation around calving. The suitability of the [TCa] as proxy for the blood [iCa] improves considerably over the first 4 days of lactation. Based on these results, we discourage the practice of measuring the [TCa] in fresh cows in the first 24 hours to diagnose SCH.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

The study was conducted at the Educational and Research Centre for Animal Husbandry, Hofgut Neumühle, Münchweiler an der Alsenz, Germany and all procedures were approved by the Animal Welfare and Ethics Committee of the government of Coblenz, Rhineland Palatinate, Germany (permit no. 23-177-07/G 19-20-008).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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