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ORIGINAL ARTICLE

Diagnostic performance of predicted ionized calcium in dogs with total hypercalcemia and total hypocalcemia

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Abstract

Background: Abnormal total calcium (tCa) values do not necessarily imply dysregulated ionized calcium.

Objectives: We aimed to evaluate the diagnostic performance of predicted ionized calcium (piCa) regarding true calcium status in dogs with abnormal tCa.

Methods: This was a cross-sectional multicenter study. piCa and its prediction interval (PI) were calculated in 114 dogs, from three different hospitals, with either increased (62) or decreased tCa (52), All dogs also had ionized calcium and a biochemical profile available. The sensitivity, specificity, predictive values, and diagnostic discordance of piCa to confirm ionized hypercalcemia (iHyperCa) and ionized hypocalcemia (iHypoCa) were calculated using logistic regression analysis.

Results: iHyperCa was found in 28% and 66% of hyperphosphatemic and non-hyperphosphatemic dogs with tCa above the reference interval upper limit, respectively. The piCa correctly classified dogs with iHyperCa in 72.2% of those with hyperphosphatemia and 93.2% of those without hyperphosphatemia. Comparatively, elevating the tCa threshold to 12 mg/dL properly classified dogs 50% and 75% of the time in hyperphosphatemic and non-hyperphosphatemic dogs, respectively. iHypoCa was found in only 31/52 (60%) dogs with decreased tCa. The piCa correctly classified 55.2 to 100% of dogs with iHypoCa depending on the hospital. The PI demonstrated high sensitivity for iHyperCa (100%) and high specificity for both iHyperCa (100%) and iHypoCa (100%).

Conclusions: Evaluating tCa alone does not reliably determine ionized calcium status. Even with hyperphosphatemia, piCa and its PI represent a reliable alternative to interpret abnormal tCa values when ionized calcium measurements are not available. However, if the tCa reference interval is notably different from 7.6 to 11.4 mg/dL, piCa values might be under/overestimated.

KEYWORDS
abnormal total calcium, canine, hyperphosphatemia, piCa, sensitivity, specificity

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1 | INTRODUCTION

Although calcium exists in three forms in body fluids (ionized, protein-bound, and complexed with anions), only ionized calcium is metabolically active and tightly regulated.[1-3] The ionized calcium measurement is, therefore, considered mandatory for accurate calcium status evaluations.[4]

Calcium disturbances can be encountered in serious and life-threatening conditions. Regardless of the underlying cause, ionized hypocalcemia (iHypoCa) can lead to severe neurologic signs, due to increased neuronal excitability or cardiovascular disturbances.[5] Ionized hypercalcemia (iHyperCa) also might lead to serious complications if left untreated, and is a marker for life-threatening conditions, such as malignancies and hypoadrenocorticism.[6,7]

The ionized calcium measurement requires specific analyzers, which are not always available, especially in general practice.[8] Consequently, clinicians often rely on the interpretation of total calcium (tCa), which includes all three fractions of serum calcium. However, tCa does not always reflect true ionized calcium status, and its diagnostic performance has varied widely among previous studies. Positive predictive values (PPVs) of increased tCa to predict iHyperCa have varied between 19% and 93% in previous reports, with an average value around 67%.[9-12] Analogously, the PPV of decreased tCa to predict iHypoCa has varied between 45% and 87%, with an average value around 60%.[9-11] Based on these data, approximately one third of dogs with decreased or increased tCa values are improperly classified as hypocalcemic or hypercalcemic, respectively.

The diagnostic performance of tCa has varied widely among studies, likely because of differences in iHyperCa and iHypoCa prevalence, tCa threshold for hyper- and hypocalcemia, underlying conditions (eg, renal diseases), and concurrent biochemical abnormalities (eg, hypoalbuminemia and hyperphosphatemia). tCa concentration corrections for albumin and total protein concentrations have failed to improve a diagnostic discordance between tCa and ionized calcium.[7,9,13,14] A tCa threshold >12 mg/dL has been suggested to improve the accuracy in predicting iHyperCa in normophosphatemic dogs.[12] However, the accuracy of this threshold was assessed at only one institution, and its diagnostic performance was not validated in a different canine population, using different analyzers. Furthermore, the best tCa thresholds to diagnose iHyperCa in dogs with hyperphosphatemia and iHypoCa remain unknown.

A predictive multivariate adaptive regression splines (MARS) model was recently developed to predict measured ionized calcium (miCa) from routinely available canine biochemistry variables and is accessible online (https://pica-ice.shinyapps.io/appr/).[10] The performance of predicted ionized calcium (piCa) has been internally and externally validated in three different veterinary hospitals, using different biochemistry analyzers.[10,11] Overall, piCa had a higher PPV and positive diagnostic likelihood ratio (PDLR) than tCa for both iHyperCa (PPV: 69%-90%; PDLR: 20-157) and iHypoCa (PPV: 71%-89%; PDLR: 14-20).[10,11] The MARS model not only provides a piCa value but also a prediction interval (PI). When the PI was taken into account, PPV increased to 100% when the lower limit was >1.37 mmol/L and the upper limit was <1.11 mmol/L to predict iHyperCa and iHypoCa, respectively.[10,11] However, these performances were assessed in a general population of dogs with a prevalence of iHyperCa and iHypoCa around 5% (0% to 21%) and 20% (15% to 28%), respectively.[10,11] Thus, the performance of piCa and its PI in a population of dogs with abnormal tCa, which implies a higher prevalence of iHyperCa and iHypoCa, is still unknown.

Therefore, the primary objective of this study was to evaluate the diagnostic performance of piCa in regard to ionized calcium status in dogs with either increased or decreased tCa concentrations. A secondary objective was to assess the impact of hyperphosphatemia on the diagnostic performance of piCa in regard to ionized hypercalcemia.

2 | MATERIALS AND METHODS

2.1 | Study population and data collection

Medical records were retrospectively reviewed via computerized database searches at three referral veterinary hospitals located in three different countries: (1) University of Illinois Veterinary Teaching Hospital (UOI), USA, (2) the Ontario Veterinary College (OVC), Canada, and (3) the Animal Health Trust (AHT), United Kingdom. Because of data search restrictions specific to each hospital, computerized searches were performed between February 2010 and February 2016 at UOI, between January 2007 and September 2017 at OVC, and from February 2017 to November 2017 at AHT. All dogs that had both an ionized calcium measurement and serum biochemistry panel (creatinine, blood urea nitrogen, total protein, albumin, globulin, tCa, phosphorus, sodium, potassium, chloride, glucose, alkaline phosphatase, corticosteroid-induced alkaline phosphatase, alanine transferase, gamma-glutamyl transferase, total bilirubin, cholesterol, triglycerides, and bicarbonate) were recruited. Dogs were included in the study only if tCa was abnormal (ie, outside the specific institutions reference intervals), and if the ionized calcium measurement and biochemistry panel were performed within the same 24-hour time interval. Dogs were excluded if there were any missing laboratory values among those necessary to calculate piCa (ie, creatinine, albumin, tCa, phosphorus, sodium, potassium, chloride, alkaline phosphatase, and triglycerides), if their data were previously used to develop the predictive model,[10] or if the tCa concentrations were normal.

To guarantee independence of observations, an individual dog could not be included more than once; if the dog was evaluated multiple times, only the first evaluation was included.

Blood sampling and ionized calcium measurements were performed under conditions that guaranteed the accuracy of ionized calcium concentrations; blood was drawn using an anticoagulant-free syringe or a calcium-balanced heparinized syringe filled to full capacity. If an anticoagulant-free syringe was used, blood was immediately placed into a lithium heparin-coated plastic screw-top tube containing <15 USP units of heparin/mL and filled to full capacity, and the tube was immediately inverted 8-10 times to ensure

adequate anticoagulation. The tube remained closed until the time of sample analysis. For both calcium-balanced heparinized syringes and heparinized tubes, analysis was performed within 15 minutes of collection. The tube was opened only once, when the sample was drawn into a syringe for ionized calcium concentration measurements. Ionized calcium measurements were performed using an ion-selective electrode analyzer: NovaStat CCX (NOVA Biomedical), ABL800 Flex (Radiometer), and ABL90 Flex (Radiometer) at UOI, OVC, and AHT, respectively. The miCa value was uncorrected from the pH. The pH and pCO2 measurements were obtained at the same time as miCa using the same analyzers and were evaluated to ensure the absence of significant air exposure. The serum biochemistry variables were measured using six chemistry analyzers: Roche Hitachi 917 (Roche Diagnostics) from February to August 2010 and Olympus AU680 (Beckman Coulter) from August 2010 to February 2016 at the UOI, Cobas 6000 (Roche Ltd.) during opening hours and VetScan V2 (Abaxis) for after-hour samples at OVC, and an Indiko Plus wet chemistry analyzer (Thermo Fisher Scientific) during open hours and Olympus AU480 (Beckman Coulter) for after-hour samples at AHT. For each analyzer, tCa reference interval (RI) limits were: Roche Hitachi 917: 7.6-11.4 mg/L; Olympus AU680: 7.6-11.4 mg/L; Cobas 6000: 10.0-12.0 mg/dL; VetScan: 10.0-12.0 mg/dL; Indiko Plus: 8.0-12.4 mg/dL; and Olympus AU480: 8.4-11.2 mg/dL.

Data extracted from medical records included creatinine, albumin, tCa, phosphorus, sodium, potassium, chloride, alkaline phosphatase, and triglycerides. Additionally, miCa, pH, and pCO2 values were recorded for every dog. Additional data obtained from the medical records included age, breed, sex, body weight, and sampling method (ie, heparinized syringe or heparinized tube). Lastly, the final diagnosis made at the time of inclusion, particularly in regard to the underlying calcium disorders, was recorded for each case.

BOX 1 How to calculate and interpret the predicted ionized calcium (piCa) and its 95% prediction interval

- Predicted ionized calcium (piCa) is a statistical prediction of the measured ionized calcium concentration of a dog obtained from its age and biochemical variables by a nonlinear, nonparametric, multivariable model (Multivariate Adaptive Regression Splines or MARS model).[10]
- The model formula to calculate piCa is presented in Table S1. As with any prediction, piCa is associated with some degree of uncertainty expressed with a 95% prediction interval (PI), loosely interpreted as: *a clinician can be 95% confident that the measured ionized calcium of a dog is between the lower limit and the upper limit of the PI*.
- If both the lower and upper limits of the PI are above 1.37 mmol/L (ie, the upper limit of piCa reference interval), the clinician can be 95% confident that the dog has ionized hypercalcemia.
- If both the lower and upper limits of the PI are below 1.11 mmol/L (ie, the lower limit of piCa reference interval), the clinician can be 95% confident that the dog has ionized hypocalcemia.
- In other instances, the likelihood of ionized hypo/normo/hypercalcemia must be balanced by clinical and paraclinical variables.
- The piCa and 95% PI can be easily calculated online (https://pica-ice.shinyapps.io/appr/).

2.2 | Predicted ionized calcium calculation

The piCa was calculated for every dog using the previously published and validated MARS model (Table S1).[10,15] The 95% PI was obtained as described previously.[10,16] A brief explanation of piCa and the scientific basis of the 95% PI is presented in Box 1.

2.3 | Assessment of predicted ionized calcium diagnostic performance

Based on miCa RI limits, samples were classified as iHyperCa if miCa concentrations were >1.37 mmol/L if heparinized tubes were used and >1.45 mmol/L if heparinized syringes were used. Analogously, samples were classified as iHypoCa if miCa values were <1.11 mmol/L if heparinized tubes were used and <1.27 mmol/L if heparinized syringes were used. The RI for miCa using heparinized tubes was determined at UOI using 30 healthy dogs and was validated at AHT using 20 healthy dogs via the transference method. The RI for miCa using heparinized syringes was determined at OVC using 15 healthy dogs and was validated at AHT using 20 healthy dogs via the transference method. Samples were also classified according to their piCa status as hypercalcemic if piCa was >1.37 mmol/L and hypocalcemic if piCa was <1.11 mmol/L, based on piCa RI limits.[10]

Diagnostic performance of piCa and its PI was assessed via sensitivity (Sen), specificity (Spe), negative (NPV) and positive (PPV) predictive values, and negative (NDLR) and positive (PDLR) diagnostic likelihood ratios for the diagnosis of iHypoCa and iHyperCa.

PDLR corresponds to the proportion of true positives divided by the proportion of false positives. This aids a clinician running the diagnostic test to answer how more or less likely the disease being tested for is present and is independent of prevalence. It can be calculated by the formula:

$$\text{PDLR} = \frac{\text{probability animal with disease having a positive test}}{\text{probability animal without disease having a positive test}}.$$

When the PDLR is 2, animals testing positive are twice as likely to have the disease as not have the disease. The higher the PDLR, the more likely it is to rule the disease in if the diagnostic test result is positive.

NDLR corresponds to the proportion of false negatives divided by the proportion of true negatives, and is independent of prevalence. This aids a clinician running the diagnostic test to answer how more or less likely the disease being tested for is absent. It can be calculated by the formula:

$$\text{NDLLR} = \frac{\text{probability animal with disease having a negative test}}{\text{probability animal without disease having a negative test}}.$$

When the NDLR is 0.2 animals that tested negative are 0.2 times as likely to have the disease as not to have the disease. The lower the NDLR, the more likely a disease out can be ruled out if the diagnostic test result is negative.

The Sen, Spe, NPV, and PPV of piCa and their 95% confidence intervals (CIs) were calculated by logistic regression. The variables "Center" and "Hyperphosphatemia" were used as independent variables to assess differences in piCa diagnostic performances among the 3 hospitals, and depending on the presence or absence of hyperphosphatemia, based on the RI upper limit for serum phosphorus concentrations from each analyzer. The same method was applied to determine the diagnostic performance of piCa PI lower and upper limits. The PDLR and NDLR were directly calculated from contingency tables. Finally, the diagnostic discordance and its 95% CI were estimated for piCa and tCa by logistic regression, which also integrated "Center" and "Hyperphosphatemia" as independent variables using. If the tCa value was above the upper RI limit of the biochemistry analyzer and >12 mg/dL, regardless of the upper RI limit of the biochemistry analyzer, two iCa thresholds were used to estimate the diagnostic discordance using miCa to diagnose iHyperCa.

All statistical analyses were performed using STATA software version 14.2 software (StataCorp LLC). Significance was set at a value of $P < 0.05$ for all comparisons.

3 | RESULTS

3.1 | Study population

One hundred and fourteen dogs with abnormal tCa concentrations were included in the study. Sixty-nine dogs (61%) were recruited from UOI, 29 (25%) from OVC, and 16 (14%) dogs from AHT. Sixty-two dogs (54%) with tCa >upper RI limit and 52 (46%) dogs with tCa <lower RI limit were identified. In the group of dogs with increased tCa, 53/62 (85%) were recruited from UOI, none from OVC, and 9/62 (15%) from AHT. In the group of dogs with decreased tCa, 16/52 (31%) were recruited from UOI, 29/52 (56%) from OVC, and 7/52 (13%) from AHT.

Breeds of dogs with increased tCa values were mainly composed of mixed breed dogs (12/62 [19%]), and lesser numbers of Dachshunds, Labrador Retrievers, Miniature Schnauzers, Siberian Huskies (4/62 [6%] for each breed), and <5% of each remaining breed. Breeds of dogs with decreased tCa values were mainly composed of mixed breed dogs (5/52 [10%]), Miniature Schnauzers (5/52 [10%]), Yorkshire Terriers (5/52 [10%]), Cocker Spaniels (4/52 [8%]), Labrador Retrievers (3/52 [6%]), Maltese (3/52 [6%]), and ≤5% of each remaining breed. Demographic characteristics of both groups are outlined in Table 1. The most common diseases diagnosed in dogs with total hypercalcemia were: 22/62 (35%) with neoplasia, among which 14/22 (64%) were lymphomas, 9/62 (14%) with primary hyperparathyroidism, and 6/62 (10%) with endocrine diseases, among which 3/6 (50%) had hypoadrenocorticism. Other diseases included: 2/62 (3%) with renal diseases, 5/62 (8%) with liver and gastrointestinal diseases, 2/62 (3%) with fungal diseases, and 7/62 (11%) with miscellaneous diagnoses. No diagnosis could be retrieved from medical records in 9/62 (15%) dogs.

TABLE 1 Demographic data for dogs with increased and decreased total calcium concentrations

Variable	Dogs with tCa >upper RI limit (n = 62)	Dogs with tCa <lower RI limit (n = 52)
Age (y)	9 (0.3-14.7)	6.3 (0.9-13.5)
Weight (kg)	22.7 (2.7-64.0)	12.5 (1.4-43.4)
Sex		
Neutered males	31 (50%)	25 (48%)
Intact males	3 (5%)	3 (6%)
Spayed females	25 (40%)	17 (33%)
Intact females	3 (5%)	7 (13%)
Calcium status		
Hypercalcemic	34 (55%)	0 (0%)
Normocalcemic	27 (44%)	21 (40%)
Hypocalcemic	1 (2%)	31 (60%)

Note: Table entries represent median values (minimum-maximum) for continuous variables (age and body weight) and number of dogs (percent of dogs) for categorical variables (sex and calcium status).
Abbreviations: RI, reference interval (different for each analyzer); tCa, total calcium.

The diseases diagnosed in dogs with total hypocalcemia included 11/52 (21%) with protein losing enteropathies, 5/52 (10%) with pancreatitis, 5/52 (10%) with sepsis, 3/52 (6%) with renal diseases, 3/52 (6%) with trauma, 1/52 (2%) with hypoparathyroidism, and 20/52 (38%) with miscellaneous diagnoses, including 7/20 (35%) with chylothorax. No diagnosis could be retrieved in 4/52 (8%) dogs.

In dogs with total hypercalcemia, 34/62 (55%) were confirmed to have iHyperCa, and 27/62 (44%) and 1/62 (2%) had normocalcemia and hypocalcemia, respectively, based on miCa concentrations (Figure 1). The final diagnosis of dogs with iHyperCa included 18/34 (53%) with neoplasia (of which 14/18 [78%] had lymphoma), 9/34 (26%) with primary hyperparathyroidism, 2/34 (6%) with renal diseases, 1/34 (3%) with fungal disease, and 1/34 (3%) with a miscellaneous diagnosis. No diagnosis could be retrieved in 4/34 (12%) dogs. For all the hypercalcemic dogs, the median value for miCa was 1.37 mmol/L [minimum: 1.03; maximum: 2.01] using heparinized tubes, and was 1.87 mmol/L [1.43; 2.02] using heparinized syringes. In truly hypercalcemic (miCa >upper RI limit) dogs, the median value was 1.54 mmol/L [1.37; 2.01] using heparinized tubes, and 1.88 mmol/L [1.49; 2.02] using heparinized syringes. In dogs without iHyperCa (miCa <upper RI limit), the median value was 1.26 mmol/L

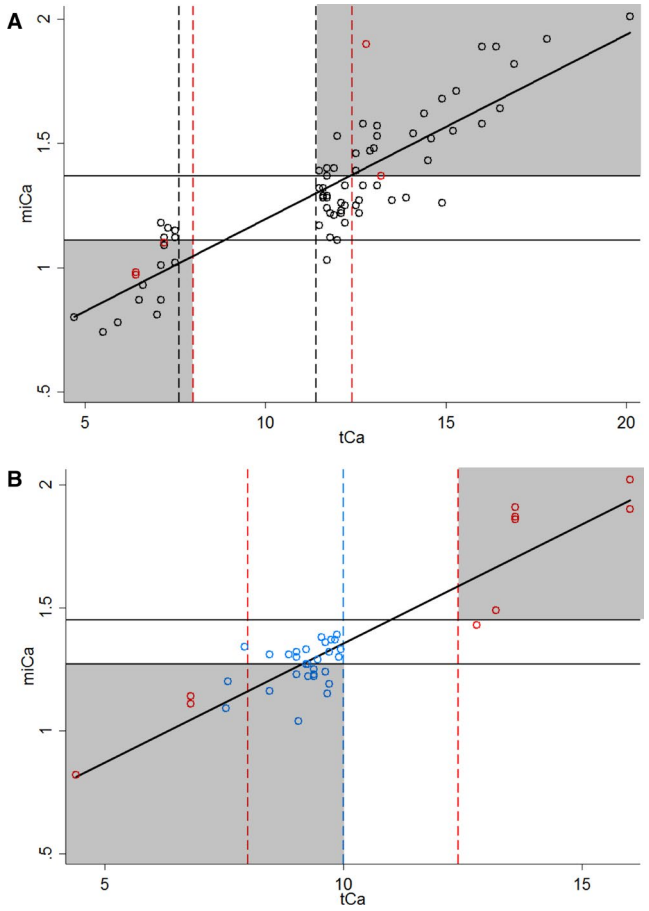

FIGURE 1 Scatter plots show the relationship between measured ionized calcium (miCa) and total calcium (tCa), stratified by the sampling methods: (A) lithium heparin-coated plastic screw-top tubes and (B) calcium-balanced heparinized syringes. The upper and lower limits of the measured ionized calcium reference interval (A: 1.11-1.37 mmol/L; B: 1.27-1.45 mmol/L) are represented by the horizontal solid lines. The upper and lower limits of the total calcium reference interval are represented by the vertical dashed lines (UOI: 7.6-11.4 mg/dL [Black]; AHT: 8.0-12.4 mg/dL [red]; OVC: lower limit = 10 mg/dL [blue]; upper limit not represented as no dogs had total hypercalcemia at OVC). All the dots above the upper solid line are ionized hypercalcemic dogs, and those below the bottom solid line are ionized hypocalcemic dogs. All the dots outside the gray squares are improperly classified by total calcium regarding the ionized calcium status

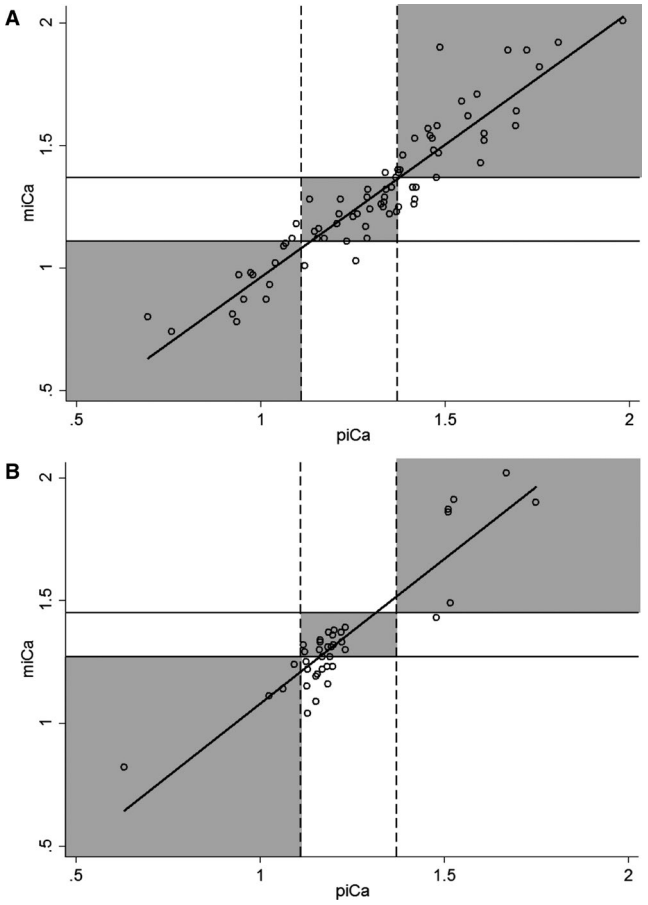

FIGURE 2 Scatter plots showing the relationship between measured ionized calcium (miCa) and predicted ionized calcium (piCa), stratified by the sampling methods: (A) lithium heparin-coated plastic screw-top tubes and (B) calcium-balanced heparinized syringes. The upper and lower limits miCa reference interval (A: 1.11-1.37 mmol/L; B: 1.27-1.45 mmol/L) are represented by the horizontal solid lines. The upper and lower limits of piCa reference interval are represented by the vertical dashed lines (1.11-1.37 mmol/L). All the dots above the upper solid line are ionized hypercalcemic dogs, and those below the bottom solid line are ionized hypocalcemic dogs. All the dots outside the gray squares are improperly classified by piCa regarding the ionized calcium status

[1.03; 1.33] using heparinized tubes, and miCa value was 1.43 mmol/L in the only dog for which a heparinized syringe was used.

In dogs with total hypocalcemia, 31/52 (60%) were confirmed with iHypoCa, and 21/52 (40%) had normocalcemia based on miCa (Figure 1). The final diagnosis of dogs with iHypoCa included 10/31 (32%) protein losing enteropathies, 3/31 (10%) renal diseases, 2/31 (6%) pancreatitis, 2/31 (6%) sepsis, 1/31 (3%) primary hypoparathyroidism, and 9/31 (29%) miscellaneous diagnoses. No diagnosis could be retrieved in 4/31 (13%) dogs. In total hypocalcemic dogs, the median value of miCa was 1.0 mmol/L [0.74; 1.18] using heparinized tubes, and 1.27 mmol/L [0.82; 1.39] using heparinized syringes. In truly hypocalcemic (miCa <lower RI limit) dogs, the median value was 0.95 mmol/L [0.74; 1.1] using heparinized tubes, and 1.2 mmol/L [0.82; 1.27] using heparinized syringes. In dogs without iHypoCa (miCa <lower RI limit), the median value was 1.13 mmol/L [1.12; 1.18] using heparinized tubes, and 1.33 mmol/L [1.29; 1.39] using heparinized syringes.

3.2 | Assessment of predicted ionized calcium diagnostic performance in dogs with increased tCa values

The observed-versus-predicted plot showing the relationship between miCa and piCa is presented in Figure 2. The Sen, Spe, NPV, PPV, NDLR, and PDLR of piCa and its PI for iHyperCa in dogs with

TABLE 2 Sensitivity, specificity, and negative and positive predictive values of predicted ionized calcium and its prediction interval for the diagnosis of ionized hypercalcemia in dogs with increased total calcium concentrations (prevalence of hypercalcemia = 54%)

	Hyperphosphatemia (n = 18)					
	Sen	Spe	PPV	NPV	PDLR	NDLR
piCa >1.37 mmol/L	99.2% (89.9-99.9)	61.2% (34.2-82.7)	49.6% (22.3-77.1)	99.5% (93.2-99.9)	2.6 (1.3-5.2)	0 (NC)
Lower end of PI >1.37 mmol/L	80% (28.4-99.5)	100% (75.3-100)	100% (39.8-100)	92.9% (66.1-99.8)	∞ (NC)	0.2 (0.03-1.15)
Upper end of PI >1.37 mmol/L	100% (47.8-100)	30.8% (9.1-61.4)	35.7% (12.8-64.9)	100% (39.8-100)	1.4 (1.0-2.1)	0 (NC)
tCa >12 mg/dL	100% (47.8-100)	30.8% (9.1-61.4)	35.7% (12.8-64.9)	100% (39.8-100)	1.4 (1.0-2.1)	0 (NC)

Note: Table entries represent diagnostic performance index values (95% confidence interval).

Abbreviations: NC, not calculable; NDLR, negative diagnostic likelihood ratio; NPV, negative predictive value; PDLR, positive diagnostic likelihood ratio; PI, prediction interval; piCa, predicted ionized calcium; PPV, positive predictive value; Sen, sensitivity; Spe, specificity; tCa, total calcium.

total hypercalcemia are presented in Table 2. No significant differences among hospitals were detected for Sen and Spe ($P = 0.40$), PPV and NPV ($P = 0.58$), and diagnostic discordance ($P = 0.36$). However, the Sen and Spe ($P = 0.04$), PPV and NPV ($P = 0.006$), and diagnostic discordance ($P = 0.04$) were significantly influenced by the presence/absence of hyperphosphatemia (Figure 3).

In dogs with total hypercalcemia and hyperphosphatemia, the Sen of piCa to detect iHyperCa was 99.2% (95% CI: 89.9-99.9), and the Spe to confirm iHyperCa was 61.2% (95% CI: 34.2-82.7). Sensitivity and Spe increased to 100% if the upper limit of the PI was >1.37 mmol/L and the lower limit of the PI was >1.37 mmol/L, respectively.

In dogs with total hypercalcemia without hyperphosphatemia, Sen and Spe of piCa were high: 93.2% (95% CI: 76.6-98.3) and 93.6% (95% CI: 65.8-99.1), respectively. Sensitivity and Spe increased to 100% if the upper limit of the PI was >1.37 mmol/L and the lower limit of the PI was >1.37 mmol/L, respectively.

Finally, diagnostic discordance between piCa and miCa was 27.8% (95% CI: 12.1-51.9) in dogs with total hypercalcemia and hyperphosphatemia and 6.8% (95% CI: 2.2-19.1) in dogs with total hypercalcemia without hyperphosphatemia.

For comparison purposes, the diagnostic discordance between tCa >upper RI limit and miCa was 72.2% (47.8-89.1) in dogs with hyperphosphatemia and 34.1% (21.0-54.1) in dogs without hyperphosphatemia ($P = 0.02$). With the threshold for hypercalcemia set at 12 mg/dL, the diagnostic discordance between tCa and miCa was 50% (28.0-73.2) in dogs with hyperphosphatemia and 25.0% (13.5-45.2) in dogs without hyperphosphatemia ($P = 0.01$) (Figure 4).

3.3 | Assessment of predicted ionized calcium diagnostic performance in dogs with decreased tCa values

Diagnostic performance indices of piCa and its PI for iHypoCa in dogs with total hypocalcemia are presented in Table 3. The Sen and Spe of piCa were significantly different among the centers ($P = 0.001$), unlike the PPV and NPV ($P = 0.28$) and diagnostic discordance ($P = 0.87$). None of these indices were significantly influenced by hyperphosphatemia ($P = 0.79$, $P = 0.22$, and $P = 0.82$ for Sen/Spe, PPV/NPV, and diagnostic discordance, respectively).

In dogs with total hypocalcemia, the Sen and Spe of piCa varied from 6.8% to 90.5% and from 67.6% to 99.6%, respectively, depending on the institution. Dogs whose the upper limit of piCa PI <1.11 mmol/L were all truly hypocalcemic (Spe = 100%). At AHT, 7/7 (100%) dogs with total hypocalcemia had iHypoCa and were classified as hypocalcemic by piCa.

Finally, the diagnostic discordance between piCa and miCa was 35.6% (95% CI: 17.5-60.9) in dogs with total hypocalcemia. Although no significant differences were detected among the centers, it could have been due to the low power of the analysis, as diagnostic discordance was 18.7% (95% CI: 5.2-49.1) and 0% (95% CI: 0-0) at UOI and AHT, respectively, while it was 44.8% (95% CI: 24.3-67.4) at OVC.

For comparison purposes, the diagnostic discordance between tCa <lower RI limit and miCa was 37.5% (15.5-66.3), 51.7% (30.7-72.8), and 0% (0-0) at UOI, OVC, and AHT, respectively (Figure 4).

4 | DISCUSSION

According to our results, about 45% of dogs were misclassified as hypercalcemic or hypocalcemic by tCa after restricting the studied population to dogs with abnormal tCa values. A lack of correlation between tCa and miCa is of great concern in human and veterinary medicine.[17,18] In a general canine population, tCa appears poorly sensitive but quite specific for assessing calcium status, suggesting a high rate of false-negative results, but a few false-positive results based upon previous studies.[9,10] This contrasts with the present study, as only 55% of dogs with total hypercalcemia and 60% of dogs with total hypocalcemia had iHyperCa and iHypoCa, respectively. This frequency of false-positive hypercalcemia and hypocalcemia results highlights that an abnormal tCa value should always be verified. Adjusting tCa for albumin or total protein concentrations is inefficient for this purpose.[9,10,17] A recent study evaluated a tCa threshold of 12 mg/dL in normophosphatemic dogs and found a high Spe, PPV, and NPV (100%, 93%, and 97%, respectively) for iHyperCa.

Normophosphatemia (n = 44)					
Sen	**Spe**	**PPV**	**NPV**	**PDLR**	**NDLR**
93.2% (76.6-98.3)	93.6% (65.8-99.1)	96.6% (75.6-99.6)	87.7% (62.0-96.9)	14.0 (2.1-93.0)	0.07 (0.02-0.28)
50% (31.3-68.7)	100% (79.4-100)	100% (78.2-100)	51.6% (33.1-69.8)	∞ (NC)	0.5 (0.35-0.72)
100% (88.4-100)	0% (0-20.6)	65.2% (49.8-78.7)	NC	1.0 (1.0-1.0)	NC
82.8% (64.2-94.2)	60% (32.3-83.7)	80% (61.4-92.3)	64.3% (35.1-87.2)	2.1 (1.1-3.9)	0.29 (0.12-0.71)

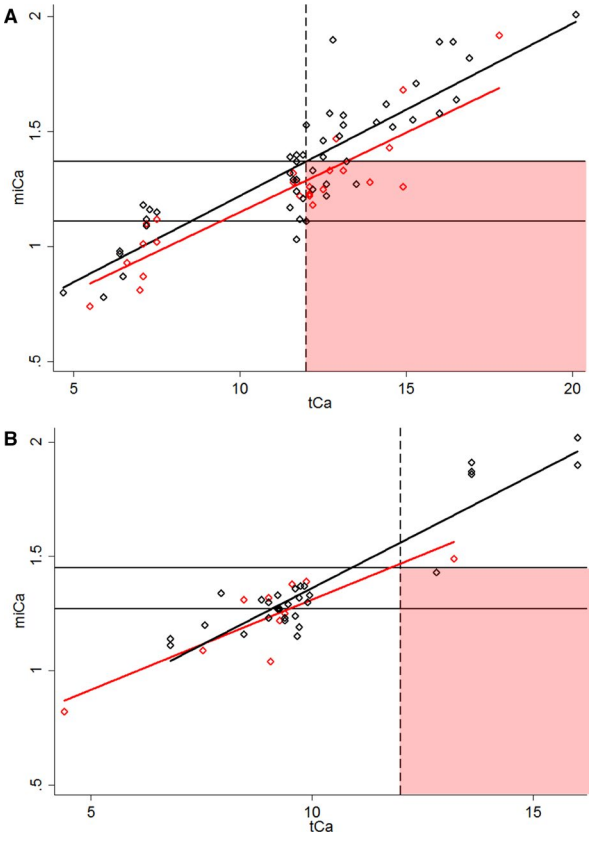

FIGURE 3 Scatter plots showing the relationship between measured ionized calcium (miCa) and total calcium (tCa), stratified by the sampling methods: (A) lithium heparin-coated plastic screw-top tubes and (B) calcium-balanced heparinized syringes. The upper and lower limits of measured ionized calcium reference interval (A: 1.11-1.37 mmol/L; B: 1.27-1.45 mmol/L) are represented by the horizontal solid lines. The vertical dashed line represents the tCa threshold of 12 mg/dL. Hyperphosphatemic dogs are represented by red diamonds and non-hyperphosphatemic dogs, by black diamonds, with their respective regression lines. All the dots above the upper solid line are ionized hypercalcemic dogs. All the dots inside the red square are improperly classified by tCa as hypercalcemic. A tCa >12 mg/dL was found in 44/62 (71%) dogs with total hypercalcemia and 15/44 (34%) did not have ionized hypercalcemia. Among these 15 dogs, 6 (40%) were normophosphatemic (black diamonds within the red square)

This threshold, however, was inaccurate for confirming iHyperCa in hyperphosphatemic dogs owing to the low PPV (19%).[12] The authors suggested that complexed calcium could be a major reason for the inaccuracy of tCa to predict miCa; thus, tCa >12 mg/dL could be very efficient in predicting iHyperCa in dogs without hyperphosphatemia, reducing the need for miCa or piCa in these dogs. This suggestion was not corroborated by the present study. Although the diagnostic discordance between tCa >12 mg/dL and miCa was lower for normophosphatemic dogs (25%) than for hyperphosphatemic dogs (50%), it was notably higher than the diagnostic discordance between piCa and miCa (6.8% and 27.8% for normophosphatemic and hyperphosphatemic dogs, respectively). A tCa >12 mg/dL was found in 44/62 (71%) dogs with total hypercalcemia, and 15/44 (34%) did not have iHyperCa. Among these 15 dogs, 6 (40%) were normophosphatemic. Therefore, according to the present study, tCa does not seem reliable enough to diagnose iHyperCa, even using a threshold of 12 mg/dL. The discrepancy between the two studies is likely explained by the difference in canine populations and laboratories.

Predicted ionized calcium has been developed as a solution to the poor reliability of tCa, when clinicians do not have easy access to miCa and is readily accessible online. Ionized calcium measurements require special analyzers, and sending a sample to a laboratory for delayed processing increases the risk of pre-analytical errors.[19] In a general canine population, piCa has shown good diagnostic performance for determining ionized calcium status compared with tCa or corrected tCa and has recently been externally validated using three different laboratories.[10,11] However, as for tCa,the diagnostic performance of piCa might change when assessed on a subset of dogs with abnormal tCa values and higher iHyperCa and iHypoCa prevalence. In practice, this population of dogs is the one for which piCa will be most commonly applied, as clinicians would likely use piCa to verify an abnormal tCa value, rather than calculate piCa on every presenting dog. In this setting and the setting of the present study, Spe of piCa is of greater importance than Sen. Indeed, when confronted with an abnormal tCa value, a clinician will want to know whether hyper/hypocalcemia is a true finding or a false-positive result. Like tCa, piCa performances

TABLE 3 Sensitivity, specificity, and negative and positive predictive values of predicted ionized calcium and its prediction interval for the diagnosis of ionized hypocalcemia in dogs with decreased total calcium concentrations (prevalence of hypocalcemia = 45%)

	UOI (n = 16)					
Hospital	**Sen**	**Spe**	**PPV**	**NPV**	**PDLR**	**NDLR**
piCa <1.11 mmol/L	90.5% (55.0-98.7)	67.6% (28.0-91.8)	82.3% (50.3-95.5)	81.1% (32.9-97.4)	2.7 (0.9-8.5)	0.15 (0.02-1)
Lower end of PI <1.11 mmol/L	100% (69.2-100)	0% (0-45.9)	62.5% (62.5-62.5)	NC	1.0 (1.0-1.0)	NC
Upper end of PI <1.11 mmol/L	50% (18.7-81.3)	100% (54.1-100)	100% (47.8-100)	54.5% (23.4-83.3)	∞ (NC)	0.50 (0.27-0.93)

Note: Table entries represent diagnostic performance index values (95% confidence interval). Data from AHT are not shown in the table, as only seven dogs with decreased total calcium values were identified and all seven dogs were classified as hypocalcemic by both measured ionized calcium and piCa.

Abbreviations: NC, not calculable; NDLR, negative diagnostic likelihood ratio; NPV, negative predictive value; PDLR, positive diagnostic likelihood ratio; PI, prediction interval; piCa, predicted ionized calcium; PPV, positive predictive value; Sen, sensitivity; Spe, specificity.

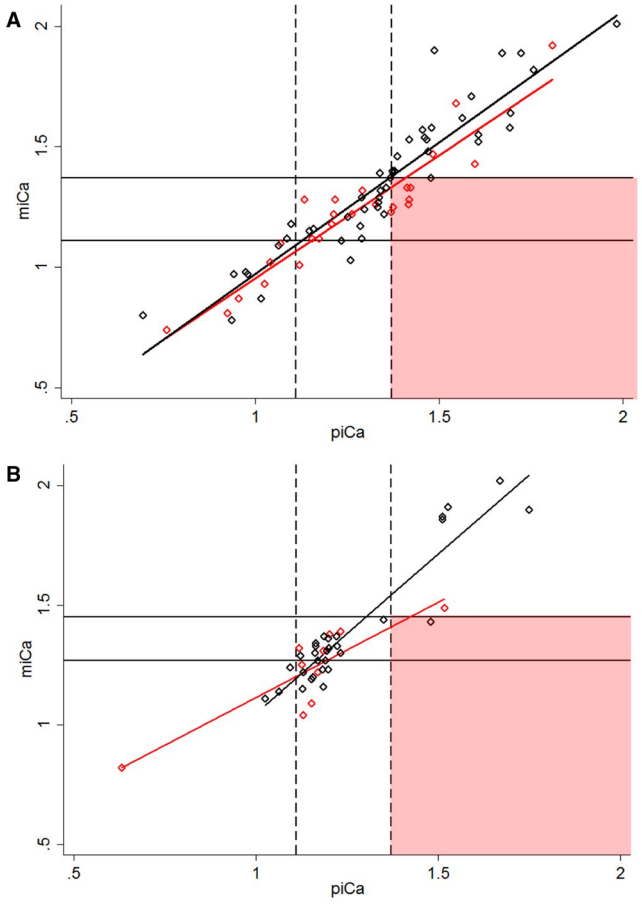

FIGURE 4 Scatter plots showing the relationship between measured ionized calcium (miCa) and predicted ionized calcium (piCa), stratified by the sampling methods: (A) lithium heparin-coated plastic screw-top tubes and (B) calcium-balanced heparinized syringes. The upper and lower limits of miCa reference interval (A: 1.11-1.37 mmol/L; B: 1.27-1.45 mmol/L) are represented by the horizontal solid lines. The upper and lower limits of piCa reference interval are represented by the vertical dashed lines (1.11-1.37 mmol/L). Hyperphosphatemic dogs are represented by red diamonds and non-hyperphosphatemic dogs by black diamonds with their respective regression lines. All the dots above the upper solid line are ionized hypercalcemic dogs. All the dots inside the red square are improperly classified by piCa as hypercalcemic

were significantly impacted by hyperphosphatemia in regard to iHyperCa. In normophosphatemic dogs with total hypercalcemia, piCa was efficient to verify tCa values, owing to the high Spe (94%), PPV (97%), PDLR (14), and NPV (88%), and the low NDLR (0.07). This can be appraised from Figure 3, where very few normophosphatemic dogs were misclassified regarding the hypercalcemia. piCa was less efficient in verifying tCa values in hyperphosphatemic dogs, as shown by the lower Spe (61%), PPV (50%), and PDLR (2.6). Nevertheless, these poorer performances are not obvious when looking at Figure 3. Five dogs out of the 18 hyperphosphatemic dogs with total hypercalcemia were misclassified as hypercalcemic. However, piCa values of these five dogs were all close to the RI upper limit (ie, 1.37 mmol/L) and the PI always included values <1.37 mmol/L, which meant that ionized normocalcemia was possible in these dogs. On the other hand, some of these dogs had very high tCa values (up to 14.9 mg/dL), with no possibility for the clinicians to relativize the results. PiCa PI provided a tremendous benefit compared to tCa. In dogs with total hypercalcemia, where both the lower and upper limits of the PI were >1.37 mmol/L, the Spe for detecting iHyperCa was 100% regardless of the presence/absence of hyperphosphatemia, meaning that no false-positive hypercalcemia results were found. Analogously, if both the lower and upper limits of the PI were <1.37 mmol/L, the Sen for detecting iHyperCa was 100% regardless of the presence/absence of hyperphosphatemia, meaning that no false-negative hypercalcemia results were found. If the PI included 1.37 mmol/L (ie, PI lower limit <1.37 mmol/L and upper limit >1.37 mmol/L), both iHyperCa and ionized normocalcemia were possible, and the measurement of ionized calcium would be recommended together with the use of clinical and paraclinical data to appraise piCa results.

The benefits of piCa PI were also true in dogs with total hypocalcemia: all the dogs with both the lower and upper limits of the PI <1.11 mmol/L had iHypoCa (Spe of the PI: 100%). Because no dogs with total hypocalcemia had both the lower and upper limits of the PI >1.11 mmol/L, the Sen of the PI could not be assessed. When comparing iHypoCa with iHyperCa, the high Spe of the PI counterbalanced the lower diagnostic performance of piCa, which had a higher overall discordance with miCa

OVC (n = 29)					
Sen	**Spe**	**PPV**	**NPV**	**PDLR**	**NDLR**
6.8% (1.0-35.6)	99.6% (92.0-100)	94.6% (54.4-99.6)	53.4% (35.3-70.6)	∞ (NC)	0.93 (0.80-1)
100% (76.8-100)	0% (0-21.8)	48.3% (48.3-48.3)	NC	1.0 (1.0-1.0)	NC
0% (0-23.2)	100% (78.2-100)	NC	51.7% (51.7-51.7)	NC	1.0 (1.0-1.0)

(36%). This was similar to previous studies.[10,11] The diagnostic performances of piCa significantly varied among hospitals. This was likely explained by the difference in the lower tCa RI limits between the analyzers at the different institutions. As tCa is the main contributor to the predictive MARS model that allows piCa calculation, the notably higher tCa RI limit likely made both tCa and piCa values higher at OVC compared with those at UOI. Thus, a piCa value >1.11 mmol/L calculated at OVC might have been <1.11 mmol/L calculated at UOI, explaining the lower Sen (ie, increased risk of false ionized normocalcemia prediction) of piCa to detect iHypoCa at OVC compared with that at UOI. On the other hand, a piCa value <1.11 mmol/L calculated at OVC would have been even lower if calculated at UOI, increasing the likelihood of correctly predicting iHypoCa and explaining the higher Spe of piCa at OVC than at UOI. It is noteworthy that differences in Sen between piCa obtained at UOI and OVC were already noted during the external validation process.[11] These differences were less marked when the Sen was calculated from the overall canine population than when the population was restricted to dogs with total hypocalcemia in the present study. The difference in tCa RI among hospitals did not alter the interpretation of piCa PI in this study; at both hospitals, all dogs that had both lower and upper limits of the PI <1.11 mmol/L had true iHypoCa and no dogs with iHypoCa had both the lower and upper limits of the PI >1.11 mmol/L. Nevertheless, it would be advisable for clinicians to compare the tCa RI limits of their biochemistry analyzer with those initially used to develop piCa (ie, 7.6-11.4 mg/dL) before interpreting piCa, as reported in previous study.[10] If the tCa RI limits are notably higher than 7.6-11.4 mg/dL (like at OVC), piCa values will likely be overestimated, and this will need to be considered when interpreting the results; a clinician should, therefore, consider that there is a higher risk of missing iHypoCa (lower Sen) when the piCa value is just above 1.11 mmol/L. Despite a likely overestimation of piCa at OVC, all the dogs with total hypocalcemia at this hospital had the lower limit of piCa PI <1.11 mmol/L, meaning that a low miCa value was possible. Therefore, iHypoCa would not have been overlooked in practice. Finally, only profound iHypoCa would prompt calcium or calcitriol supplementation, and all dogs with profound iHypoCa would be expected to be accurately diagnosed by piCa and its PI based on the present results.[3]

The present study had some limitations. First, due to its retrospective nature, some records were incomplete and some data were inconsistently reported, especially pertaining to the complete history, medication list, and final diagnosis. Second, proper sample collection and handling cannot be guaranteed due to the retrospective aspect of case recruitment. However, in the three hospitals, it is standard of care to analyze ionized calcium within 15 minutes of collection. Third, there were a relatively low number of dogs included in this study, likely because calcium abnormalities are relatively uncommon. This could have impacted the accuracy of the piCa diagnostic performance index estimation.[20] Fourth, the classification of the dogs into iHyperCa and iHypoCa was based on a single miCa value outside the RI. A dog with marginally increased or decreased miCa was thus classified as iHyperCa or iHypoCa, while it could have been normocalcemic. This could explain some discrepancies between piCa and miCa. Finally, RIs for miCa at each hospital were established based on a small number of healthy dogs, which could have negatively impacted the precision of the RI limit estimation.[21]

In conclusion, the present results showed that an abnormal tCa value should always be verified as false hypercalcemia/hypocalcemia diagnoses are common. Using a tCa threshold >12 mg/dL to diagnose iHyperCa in normophosphatemic dogs is not accurate enough to be recommended for use in practice. When ionized calcium cannot be measured under proper conditions, piCa represents a reliable alternative to verify an abnormal tCa value. When interpreting piCa values, clinicians should take into consideration the tCa RI limits of their biochemistry analyzers, the possible presence of hyperphosphatemia, and the piCa PI limits. Should the tCa RI limits of the biochemistry analyzer be notably different from 7.6 to 11.4 mg/dL, piCa values will likely be under- or overestimated. The risk of overdiagnosing (if tCa RI limits are notably lower than 7.6-11.4 mg/dL) or underdiagnosing (if tCa RI limits are notably higher than 7.6-11.4 mg/dL) iHypoCa should be considered when interpreting piCa values, especially if both the lower and upper limits of the PI are notabove or below 1.11 mmol/L. Further studies are needed to confirm this hypothesis. Values of piCa might be slightly overestimated in dogs with total hypercalcemia and hyperphosphatemia, although the clinical significance of this

is unknown. Finally, iHyperCa and iHypoCa should be considered highly probable when both the lower and upper limits of piCa PI are >1.37 mmol/L and <1.11 mmol/L, respectively, although a larger prospective study is warranted to strengthen these conclusions.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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