Comparative Analysis of Whole Blood Ionised Calcium, Lactate and Chloride Concentrations by Gem Premier 3000 and Edan I15 Vet and the Effect of Parity and Postpartum Blood Collection Time on Blood Calcium Status in Holstein With and Without Subclinical Hypocalcemia - Part 1

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Research Article

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Abstract

Lactating Holstein (n=125) were enrolled randomly for the coccygeal whole blood analysis by blood gas devices GEM Premier 3000 (GEM) and Edan i15 Vet (EDAN) between calving to postpartum day 3 (G1) and postpartum day 4 to 27 (G2). Blood pH, ionised calcium ($ICA_{7.4}$) and lactate analysis were significantly correlated between GEM and EDAN (r=0.86, 0.94, 0.87 respectively). The bias for $ICA_{7.4}$, lactate and pH analysis was -0.054, -0.344 mmol/L and +0.009 respectively. $ICA_{7.4}$ was correlated negatively with parity and chloride, but positively with lactate. The averages of $ICA_{7.4}$ and serum total calcium (TC) was significantly lower in G1 than G2. Chloride and lactate were significantly higher in G1 than G2. Subclinical hypocalcemia prevalence (SCH) (serum TC<2.15 mmol/L, as reference) was 52.9% in G1 and 21.1% in G2. Cows with SCH had frequently over 50% $ICA_{7.4}$/TC ratio. Sensitivity analysis provided a sensitivity of 57.4% for $ICA_{7.4}$ cut-points of 1.02 (GEM) and 1.05 (EDAN) mmol/L to detect SCH based on reference serum TC. Primiparous (PRP) with and without SCH in G1 had significantly higher $ICA_{7.4}$ than multiparous (MUL). Cows with SCH had significantly higher chloride in G1 than G2. MUL had significantly higher lactate and chloride in G1 than G2. Conclusively, $ICA_{7.4}$ and pH analysis between GEM and EDAN were correlated well with acceptable biases, but high differences occurred in lactate analysis. MUL was at risk in G1 due to lower $ICA_{7.4}$ and TC over PRP. Higher $ICA_{7.4}$ of PRP can reduce the risk and frequency of clinical hypocalcemia. SCH correlated negatively with Cl concentration in G1, but not lactate.

Introduction

Point-of-care testing in human medicine referred to near-patient or bedside biochemical testing by blood gas devices (Beneteau-Burnat et al. 2004; Steinfelder-Visscher et al. 2006) can also generate a rapid result for dairy cows or calves (Constable et al. 2019) so that quick appropriate treatment can be initiated during very early postpartum or acute care process. Especially, in dairy cows, it might be useful for the rapid determination of blood ionised calcium (ICA) for monitoring of physiologically active calcium levels (Ballantine and Herbein 1991, Mahen et al. 2018), as in the calves (Constable et al. 2019), because hypocalcemia related milk fever or subclinical hypocalcemia (SCH) is one of the most important metabolic diseases in dairy cows in early postpartum (Goff 2008). Calcium exists in three forms in the blood, which are physiologically active ionised form (43% - 57% of total), bound to anion salts (complexed form) (approximately 8%) and protein-bound form (35% - 49% of total) (Ballantine and Herbein 1991; Constable et al. 2019). Holstein cows are more susceptible to milk fever related to hypocalcemia in the first days of postpartum (Goff 2008; Caxieta et al. 2017). Screening serum total calcium concentration (TC) for the detection of clinical hypocalcemia (CH) or subclinical hypocalcemia (SCH) (Caxieta et al. 2017; Wilkens et al. 2020) and blood ionised calcium (ICA) (Mahen et al. 2018) are major tests about available calcium levels for physiological functions in the organism. Screening of blood ICA provides valuable information about physiologically active, free and diffusible forms of calcium, unlike total calcium concentration (TC) (Mahen et al. 2018). Blood ICA is primarily dependent on TC, albumin, pH, globulin, chloride, lactate as well as temperature and sodium concentration (Rehfeld et al. 1984; Seifi et al. 2005; Constable et al. 2019). The highest effect on the blood ICA is accounted to blood pH, which was already reported in calves (Agnes et al.

1993, Constable et al. 2019) and in cows (Kvart and Larson 1978). Blood ICA is negatively and linearly associated with plasma pH (Constable et al. 2019), that requires a correction to blood pH 7.40 in cows to support the interpretation of measured results comparable to a reference range (Dauth et al. 1984; Lincoln and Lane 1990; Ballantine and Herbein 1991; Szenci et al. 1994). Similarly, blood TC concentration has been corrected to the normal albumin concentration too, since elevated blood albumin concentration can have an effect on TC concentration (Seifi et al. 2005). Potentiometric devices with Ca-specific electrodes provide a rapid analysis of ICA in whole blood, plasma or serum (Raman 1970; Watson and Anbar 1985; Ballantine and Herbein 1991). Lincoln and Lane (1990) and Ballantine and Herbein (1991) indicated the validity and usefulness of potentiometric methods to analyse ICA in the blood of dairy cows. A recent study revealed that pH-corrective equations for bovine plasma were similar to those used for human plasma (Constable et al. 2019). Testing of serum TC could not be practical on the farm due to the long-lasting workload for a cow-side diagnosis of hypocalcemia in dairy cows, especially in the critical time just after parturition. Easy, accurate and rapid testing of blood calcium profile in dairy cows is important for the rapid detection of hypocalcemia, rather than serum TC, which needs a quite longer lab work labour. Different cut-points for the detection of SCH was reported in the literature, however serum TC concentration <2.15 mmol/L for SCH diagnosis was recommended due to association with postpartum diseases (Caixeta et al. 2017; Martinez et al.2012; Martinez et al. 2014; Neves et al. 2018). Screening of blood ICA levels was found as a valuable indicator for blood calcium status (Ballantine and Herbein 1991), however, others preferred to analyse serum TC because of the dependency of blood ICA on other blood parameters (Caixeta et al. 2017) or variations in early postpartum (Leno et al. 2017). The prevalence of hypocalcemia was observed more frequently in multiparous than primiparous according to serum TC screening tests (Reinhardt et al. 2011; Wilhelm et al. 2017). But, the interaction of ICA and TC with other parameters such as blood chloride, lactate and albumin in both multiparous and primiparous Holstein with and without SCH was not investigated in detail. The first objective of the present study was to compare a blood gas device Edan i15 Vet (EDAN), which was launched as a cow-side, the small handheld device in the dairy farm, with another blood gas device Gem premier 3000 (GEM), which was already compared with other blood gas devices in human (Beneteau-Burnat et al. 2004; Vukelić et al. 2007) and on the market to use in the laboratory. The comparison was specially performed for the analysis of whole blood ICA to detect subclinical hypocalcemia and its correlation with blood pH, chloride and lactate concentrations, as well as with serum TC, albumin and total protein concentration in early lactation of Holstein. Secondly, the present study aimed also to analyse the effect of parity and postpartum blood collection times (PBCT) on serum TC, albumin and globulin concentrations and whole blood ICA, lactate and chloride concentrations analysed by two different blood gas analysers ‘EDAN and GEM’ in lactating Holstein with and without SCH.

Materials And Methods

Study animals and groups

This study was a randomized field trial. One hundred twenty-five Holstein cows were enrolled randomly from various dairy farms (located in the Aegean Region of Turkey) without any selection criteria between calving to postpartum day 27 (PPD27). The distribution of PBCT were n=29 at calving, n= 30 at PPD1,

n=12 at PPD2, n=16 at PPD3, n=14 at PPD4-6, n= 10 at PPD7-10 and n= 14 at PPD11-27. Cows were allocated to group 1 (G1, n=87, between calving to PPD3) and group 2 (G2, n=38, between PPD4-27) based on hypocalcemia risk. Thirty-five out of 125 cows were primiparous (PRP), and the rest (n=90) were multiparous (MUL). According to the information received from the farms, cows were fed based on the nutritional requirements for their lactation stages (dry, close-up, early lactation) and they were served water ad-libitum. None of the study cows received additional calcium boluses or vitamin D3 injection at or before calving preventively. Study cows were healthy from clinical point of view and did not show clinical signs of hypocalcemia (paresis) or other metabolic diseases such as ketosis at the time of blood collection.

Blood collection and analysis

Whole blood was collected from the coccygeal vein in a sterile blood collection tubes (BD Vacutainer, Becton, Dickinson and company, UK) consisting no anticoagulant by using a 20-gauge needle (0.9 × 38 mm). Two ml of whole blood was drawn from the tube in the 100 µl lithium heparin containing injectors (ARD blood gas injector, Group ADR, Umraniye-Istanbul, Turkey) for using in the blood gas analyser. Lithium-heparinized whole blood was used in GEM Premier 300 (Instrumentation Laboratory Inc. Lexington MA, USA, distributor: Seray Medikal, Yenimahalle/Ankara, Turkey) and EDAN i15 Vet (Edan Instruments, Inc. Shenzhen, China, distributor: Hasvet, Kepez/Antalya, Turkey) for the analysis of blood pH, ICA, lactate and chloride molar concentrations. Chloride (Cl) concentration can only be analysed by Edan i15 Vet (EDAN, size: 23.8 x 15.3 x 31.0 cm, 3.6 kg), because GEM Premier 3000 (GEM) does not analyse chloride concentration. Both blood gas devices deliver ICA results as original measured value as well as corrected automatically to blood pH 7.40 ($ICA_{7.4}$). The following calculation is automatically performed by both blood gas devices:

$$ICA_{7.4} = ICA_1 \times 10^{[0.178 \times (pH1 - 7.40)]} \text{ mmol/L}$$

ICA_1: real ICA value analysed by blood gas device.

pH_1: blood pH value analysed by blood gas device.

ICA7.4: ICA value corrected to blood pH 7.40 by blood gas device.

Both blood gas analysers measure pH, ICA (GEM, EDAN) and Cl molar concentration (EDAN) by potentiometric sensors, and they analyse lactate by amperometric sensors (GEM and EDAN). Approximately 1 h after collection of whole blood, the coccygeal whole blood samples without anticoagulant were cool-centrifuged at 4100 rpm for 15 min (NUVE NF 800R, Nüve San. Mal., Ankara, Turkey). Supernatant serum was withdrawn from the tubes and injected into eppendorf sample tubes, which were marked and stored at -20 ^{o}C for one month until they were used for the analysis of serum total calcium (TC), total protein (TP) and albumin (Alb) concentrations using a commercial test kits of the manufacturer in an automated wet biochemistry analyser (Mindray BS-120 Vet Automatic Blood Chemistry Analyser, Shenzhen Mindray Animal Medical Technology Co., LTD., distributor: Hasvet, Kepez/Antalya, Turkey). The arithmetic difference between TP and Alb concentrations was accepted as

globulin (GL) concentration. The precision, reproducibility and linearity of EDAN were tested with Rapidpoint 400, Radiometer ABL 800, QC823 Blood Gas analyser and QC900 Hematocrit Control according to the manufacturer instructions (technical manual) of EDAN. Evaluations for imprecision and accuracy of GEM were performed in humans with another blood gas analyser 'Ciba Corning 865' (Steinfelder-Visscher et al. 2006; Vukelić et al. 2007) and 'Radiometer ABL725' (Beneteau-Burnat et al. 2004). The calibration frequency of GEM is automatic at timely-scheduled intervals. GEM performs one-point calibrations in every 20 min and two-point calibrations every 2 h. It performs also a one-point calibration after every sample analysis. GEM needs no gas tanks, electrode membranes or extraneous solutions, it analyse automatically with electrochemical sensors using 135 µl whole blood samples at fixed electrode chamber temperature of 37 ^{0}C. The calibration of EDAN was carried out always in advance using the respective calibration kits (i15 Calibrant Fluid Pack) delivered by the manufacturer before the analysis of the samples started in each farm. The storage (4 – 30 ^{o}C) and use (10 – 30 ^{o}C) of cartilages with relative humidity of 25 – 80% was recommended by the manufacturer of EDAN, which was fully followed by the present study. EDAN aspirates the sample directly and requires a minimum sample volume of 140 µl for the analysis with electrochemical sensors at a temperature of 37 ^{0}C. In both blood gas devices, no patient temperature was entered in the device since they were set up for fixed 37 ^{o}C. The calibration of Mindray BS 120 Vet (BS120) was always performed in advance using the respective calibration kits delivered by the distributor company before the analysis of the samples started.

Definition of hypocalcemia by serum total calcium

Different reference ranges for serum or plasma TC concentration were reported in Holstein as 2.00 – 2.15 mmol/L (Wilkens et al. 2020) for first 2 days after calving, >2.00 mmol/L (DeGaris and Lean 2009), 2.10 - 2.80 mmol/L (Chapinal et al. 2011; Reinhardt et al. 2011) and 2.10 -2.50 mmol/L (Goff 2008). Neves et al. (2018) and Caixeta et al. (2017) found significant correlation between the cut-point of TC≤2.15 mmol/L and postpartum health disorders. Definition of SCH was based on a cut-point of serum TC<8.6 mg/dl (2.146 mmol/L) (Caixeta et al. 2017) for first 3 days postpartum that was also accepted for the present study because the majority of study animals (70%) were between calving and postpartum day 3. Serum TC<1.50 mmol/L, <1.40 mmol/L and 1.25 mmol/L were reported to be cut-points for severe or clinical hypocalcemia (paresis) by Seifi et al. (2005), DeGaris and Lean (2009) and Melendez et al. (2020) respectively. None of study cows showed clinical paresis of hypocalcemia throughout the present study period, thus cows having serum TC<2.15 mmol/L and <1.50 mmol/L were accepted as SCH or severe SCH in the present study respectively.

Statistical analysis

Statistical analyses were performed using the SPSS (version 22) software. The level of significance for all statistical tests was set at α = 0.05. Normality of the data was evaluated by Kolmogorov-Smirnov test. Wilcoxon test was used to compare the groups for nonparametric data and some small sample sizes. The average concentrations of the parameters between GEM and EDAN were compared with Wilcoxon test. The comparison of the data between G1 and G2 were performed using the independent samples t test. The comparison of the data within the groups such as parity groups (PRP and MUL), cows with and without

SCH, G1 and G2 were performed using the independent-samples t-test. Mean (x), standard error (se) (x±se) and percentage ratio were presented as descriptive statistics where it was required. Least square regression analysis (LSRA) was used for the calculation of correlation coefficients of blood gas parameters (with 95% confidence interval) analysed by GEM and EDAN, as well as for correlation coefficients of the ratio between TC and ICA7.4, TP, AL and GL. The correlation of ICA7.4 with lactate and Cl was calculated by LSRA. LSRA was also performed between blood gas, serum biochemical parameters and parity and PBCT. Bland Altman test was employed for the determination of biases and agreements between quantitative results by both devices (Giavarina 2015). Plots of agreement were generated respectively. Receiver Operating Characteristics (ROC) analyses were performed to determine optimized cut-points and sensitivities for the definition of SCH (serum TC<2.15 mmol/L) or severe SCH (serum TC<1.50 mmol/L) using three devices. The optimum accuracy of the tests was determined if the resulting graph of the ROC analysis was closer to the left upper angle of the coordinate system. Area under curve (AUC) was a measure of the powerful detection of a test to identify animals as hypocalcemic cows respectively. An AUC of 1 showed an excellent test; but if it was below 0.5, it meant a worthless test. ROC analyses were based on the Youden-Index (*J*), AUC calculated sensitivity and specificity-1 if significant ($p<0.05$). The *J* indicates a valuable diagnostic test, if it is close to 1, whereas a value close to 0 implies a worthless test. Furthermore, percentage detection rates of GEM and EDAN for SCH or severe SCH were compared at multiple cut-points of ICA7.4 to the reference method BS120.

Results

The average parity of all study cows was 2.68±1.46 (min: 1, max: 7), it was 2.71±1.50 and 2.61±1.37 in G1 and G2 cows respectively. No significant difference was found between G1 and 2 ($p>0.05$). There was a significantly positive linear correlation between GEM and EDAN in the analysis of blood $ICA_{7.4}$ ($r=0.94$, $p=0.000$), pH ($r=0.86$, $p=0.000$) and lactate concentrations ($r=0.87$, $p=0.000$) (Fig. 1). Bland-Altman plots of agreement demonstrated the positive (pH) and negative (lactate and $ICA_{7.4}$) biases in the analysis of parameters between EDAN and GEM (Fig. 1). The bias for $ICA_{7.4}$ was -0.054 mmol/L between EDAN and GEM, 6 samples were out of 95% confidence interval. That meant an average of 5.1% higher $ICA_{7.4}$ values delivered by EDAN. The mean difference in the analyses of blood pH was +0.009 between EDAN and GEM which meant an average of -0.13% lower blood pH delivered by EDAN. The bias in the analysis of lactate was -0.344 mmol/L between EDAN and GEM (Fig. 1) which meant in an average of 46% higher lactate values of EDAN. Significant positive, but moderate correlation was found between $ICA_{7.4}$ and serum TC confirmed by both GEM and EDAN (Table 1, Fig. 2). The correlation between $ICA_{7.4}$ and Cl concentrations (EDAN) was significantly negative (Fig. 2). A significant positive correlation was observed between $ICA_{7.4}$ and lactate concentrations tested by GEM only, but not EDAN (Table 1). This was not confirmed by EDAN. Correlation coefficients were not significant between $ICA_{7.4}$ and serum TP, Alb, GL concentrations and between ICA and blood pH analysed by both GEM and EDAN ($p>0.05$) (Table 1). Furthermore, moderately significant positive correlation was found between serum TC and Alb concentrations ($r=0.223$, $p<0.05$, data not shown in tables). Serum GL and Alb concentrations were correlated significantly with the serum TP concentration ($r=0.90$, $r=0.22$, $p<0.05$). There was not a significant correlation between TC and TP ($r=0.12$,

r^2= 0.016, p>0.05) and between TC and GL concentrations (r=0.027, p>0.05). The bias in the analysis of $ICA_{7.4}$, lactate and blood pH between EDAN and GEM that were calculated by Bland-Altman test (Fig. 1) was also confirmed with the significant differences between averages by Wilcoxon test (Table 2). Despite high significant correlations between GEM and EDAN, there was a significant difference between average concentrations of the parameters and their ratio analysed by GEM and EDAN (Table 2). The average concentrations of serum TC and blood $ICA_{7.4}$ analysed by BS 120, GEM and EDAN were 2.25±0.49, 1.04±0.18 and 1.09±0.17 mmol/L respectively (Table 2). Serum TC and $ICA_{7.4}$ concentrations were significantly lower in G1 than G2. Oppositely, blood lactate and Cl concentrations were significantly higher in G1 than G2 (Table 2). Serum TP, Alb and GL concentrations did not change significantly between G1 and G2. But, their proportional ratio to TC were changed significantly due to low TC and $ICA_{7.4}$ in G1 (Table 2). LSRA showed that there was a positive correlation between PBCT and serum TC (r = 0.34, p<0.01) and whole blood ICA7.4 (r = 0.38, p<0.01 for GEM and r = 0.36, p<0.01 for EDAN). However, a negative correlation was found between PBCT and Cl (r = -0.39, p<0.01 for EDAN), lactate concentrations (r = -0.20, p<0.01 for GEM and r = -0.34, p<0.01 for EDAN). No significant correlation was found between TP, Alb and GL concentration and PBCT. Table 3 presented the correlation coefficients between parity and serum and whole blood parameters and their ratio. Significant negative correlations were confirmed by both blood gas devices between blood ICA, $ICA_{7.4}$ and parity. Serum TC was also negatively correlated with the parity, however, the coefficients were not high but moderately (p=0.07) significant. Serum TP and GL had a positive significant correlation with parity. Serum Alb, blood lactate, pH, Cl, the ratio of $ICA_{7.4}$ /TC, TC/Alb did not correlate significantly with the parity (p>0.05). But, the parity correlated negatively with serum TC/GL and TC/TP ratio. Blood $ICA_{7.4}$ values of GEM and EDAN represented almost half of serum TC concentrations (Table 2), but there was also significant difference between the results of both devices as in the average values of $ICA_{7.4}$. Therefore, individual cut-point for the definition of SCH needed to be set for each of the device. A ROC analysis was conducted based on different reference cut-points for the definition of SCH (serum TC<2.15 mmol/L) or severe SCH (serum TC<1.50 mmol/L) (Table 4). ROC analysis was intended to calculate the optimal thresholds to differentiate between hypocalcemic and normocalcemic values by GEM and EDAN blood gas analysers. Cut-points of <1.05 and 1.02 mmol/L for SCH detection were calculated for EDAN and GEM by ROC analysis respectively. Specificity of EDAN and GEM were 81.7% and 90.3% respectively, and sensitivity were 57.4% for SCH detection by both devices. AUC and *J* were calculated as 73%, 76% and 38% and 39% for EDAN and GEM respectively (Fig. 3, Table 4) that looked similar for both devices. The identification of SCH cases in the population was not satisfactory with these thresholds values received by ROC analysis, as it might be seen in the sensitivity values (Fig. 4). Under the consideration that serum TC concentration is doubling blood ICA concentration and a moderate correlation between serum TC and ICA, and $ICA_{7.4}$ concentrations may probably be influenced by Cl and lactate concentrations in the present study; ROC analysis can only give an indication about the cut-point trends of $ICA_{7.4}$ for the detection of hypocalcemic cows. Thus, optimum thresholds of $ICA_{7.4}$ calculated by multiple cut-points analysis to match with the reference prevalence data (n=54 SCH cases, 43.2%, based on serum TC<2.15 mmol/L detected by BS120 reference method) were calculated as <1.09 and <1.04 mmol/L for EDAN (n=53 SCH cases, 41.6%) and GEM (n=50 SCH cases, 40%) respectively (Fig. 4). These values matched with the average concentrations of $ICA_{7.4}$ produced by EDAN and GEM in 125 Holstein (Table 2),

as well as they represented proportionally 50.70% and 48.37% of serum TC concentration for the cut-point of 2.15 mmol/L respectively. High AUC, *J*, sensitivity and specificity values were observed both for $ICA_{7.4}$ cut-point <0.86 mmol/L by EDAN and $ICA_{7.4}$ cut-point <0.78 mmol/L by GEM based on a reference cut-point of serum TC <1.50 mmol/L for the detection of severe SCH (n=7, 8%) cases in G1, but no case in G2 (Table 4). But, this did not result in an acceptable correct detection rate, even much higher false positive cases were observed for severe SCH cases with these cut-points of EDAN (13.8%) and GEM (12.6%). However, by multiple cut-points analysis (Fig. 5), blood $ICA_{7.4}$<0.81 (EDAN) and <0.75 mmol/L (GEM) detected all severe SCH cases (n=7, 8% in G1, no case in G2) with full sensitivity. These cut-point values were within the ratio of blood $ICA_{7.4}$ /TC analysed by GEM and EDAN. However, it needs to be admitted that the number of affected animals (n=7, in G1) by severe SCH could not be sufficient for this and further evaluations. The average concentrations of serum TC, TP, Alb and GL, blood $ICA_{7.4}$, lactate and CI and their ratio in PRP and MUL cows were presented in Table 5. Overall, MUL cows had significantly lower TC and $ICA_{7.4}$ than PRP cows. TC of MUL cows was significantly lower both in G1 and G2 than PRP cows. But, $ICA_{7.4}$ of MUL cows was significantly lower in G1 only than PRP cows. $ICA_{7.4}$ of PRP cows did not change significantly between G1 and G2. That was confirmed by two different blood gas devices. However, MUL cows had significantly lower TC and $ICA_{7.4}$ in G1 than in G2. The concentrations of TP, Alb and GL was not changed between G1 and G2. A slightly increased GL was observed in MUL cows than PRP, which was moderately significant (Table 5). Lactate (GEM) and CI concentrations (EDAN) of MUL cows were significantly higher in G1 than G2, but no difference between parities was observed. The ratio of $ICA_{7.4}$ /TC was slightly different between PRP and MUL cows as well as between G1 and G2, although an increased ratio was observed in PRP cows in G1 compared to G2 that was close to significant difference (p=0.056) in EDAN analysis (Table 5). Overall, the rate of SCH prevalence was 52.9% in G1 and 21.1% in G2 (Odds: 0.24, p<0.01) based on serum TC<2.15 mmol/L (Fig. 4). Fourteen out of 35 PRP cows had SCH (40%), while 40 out of 90 MUL showed SCH (44.4%). An average of 85% of PRP (n=12) and MUL cows (n=34) affected by SCH were in G1. Table 6 presented the average concentrations of all study parameters in cows with and without SCH. Cows with SCH had an average serum TC of 1.82±0.27 mmol/L. Blood $ICA_{7.4}$ concentrations were 1.02±0.18 (EDAN) and 0.95±0.19 mmol/L (GEM) in cows with SCH, that were significantly lower (p<0.01) than normocalcemic cows confirmed by both devices. The difference between EDAN and GEM was also significant (p<0.05). Seven cows (5.6%) had severe SCH (Fig. 5) with an average serum TC concentration of 1.30±0.17 mmol/L and an average $ICA_{7.4}$ of 0.76±0.07 and 0.66±0.10 mmol/L analysed by EDAN and GEM respectively. All of them were in G1. Cows with SCH had significantly lower concentrations of TP and Alb, and a lower ratio of serum TC/Alb and TC/GL. But, significantly higher CI concentration and $ICA_{7.4}$ /TC ratio were observed in cows with SCH compared to normocalcemic cows. The ratio of ICA to TP, Alb and GL levels was lower in SCH cows when tested by GEM only, but not by EDAN. The ratio of ICA/TC was higher than 50% in SCH-cows. Table 7 presents the results of cows with and without SCH in G1 and G2. Cows with SCH in G1 and G2 had significantly lower serum TC, TC/TP, TC/Alb and TC/GL ratios compared to normocalcemic cows. $ICA_{7.4}$ concentration of SCH cows was significantly lower than cows without SCH in G1, but not in G2. However, a clearly and significantly increased ratio of $ICA_{7.4}$/TC of SCH-cows both in G1 and G2 were observed. The trend was much lower in G1 than G2

confirmed by both blood gas devices. Cl concentration of SCH cows in G1 was significantly higher than normocalcemic cows, but not in G2. No relation of SCH with lactate was observed. Lactate concentrations of cows with or without SCH did not change significantly, confirmed by both devices. MUL cows with SCH had significantly lower $ICA_{7.4}$ concentration compared to PRP cows with SCH and to MUL cows without SCH (Fig. 6). Serum TC concentration of MUL cows with SCH was significantly lower than PRP cows (Fig. 6), this was more clearly observed in MUL cows with SCH in G1 (Fig. 7). MUL cows with SCH had also significantly lower serum TC in G1 than in G2 (Fig. 7). If not taking the grouping effect (PBCT) into account between PRP and MUL cows (Table 7), blood $ICA_{7.4}$ levels did not significantly change in PRP cows with or without SCH (Fig. 6). $ICA_{7.4}$/TC ratio analysed by EDAN in PRP and MUL cows with and without SCH was presented in Fig. 8. Similar results were also observed by GEM (data not presented). Overall, cows with SCH had frequently over 50% of the $ICA_{7.4}$/TC ratio, while SCH negative cows had below 50% ICA/TC ratio both in G1 and G2. PRP cows with SCH had a significantly higher $ICA_{7.4}$/TC ratio than MUL cows with SCH and PRP cows without SCH in G1. MUL cows with SCH in G2 had a significantly higher $ICA_{7.4}$/TC ratio than cows without SCH. Both PRP and MUL cows with SCH had significantly higher Cl concentrations compared to normocalcemic cows (Fig. 9), this higher trend was also observed in G1 than in G2 (Table 7).

Discussion

Blood gas devices became an important part of the intensive care stations in the hospital (Beneteau-Burnat et al. 2004; Faggiano et al. 2015) and in the animal health sector (Ballantine and Herbein 1991; Constable et al. 2019). Portable small-sized and accurate blood gas devices can be useful for rapid analysis of the blood gases including ICA for the early diagnosis of hypocalcemia (Ballantine and Herbein 1991) because of their easy handling sizes in dairy farms. However, the correlation and accuracy of these devices need to be tested with already validated blood gas devices or reference methods. Evaluations for imprecision and accuracy of GEM were performed in humans (Westgard et al. 2003; Beneteau-Burnat et al. 2004; Steinfelder-Visscher et al. 2006; Faggiano et al. 2015) and its performance was compared with another blood gas device Ciba Corning 865 (Steinfelder-Visscher et al. 2006; Vukelić et al. 2007) and Radiometer ABL725 (Beneteau-Burnat et al. 2004), the correlations were found high between blood gas devices, except deviations in lactate. Blood pH, ICA, $ICA_{7.4}$ and lactate concentrations analysed by EDAN were correlated significantly well with the values analysed by GEM in the present study. However, there have still been significant deviations between average concentrations of these blood gas parameters and their ratio analysed by both devices that have also been confirmed by Bland-Altman's plots of agreement. The average blood pH looked similar between GEM and EDAN with a bias of 0.01 units and with an acceptable percentage deviation (-0.13% for EDAN). Bajcsy et al. (1999) reported using ABL4 Radiometer blood gas device significantly different average values of blood pH between jugular, coccygeal and milk vein (*V. subcutenea abdominis*) of fresh Holstein (calving to postpartum day 3) like in G1 of the presents study. The average value pH (7.42) of the coccygeal vein was lower than in the present study. They have even found different values of blood pH between ICA2 Radiometer and ABL4 Radiometer (difference of 0.06 unit), as in the present study between GEM and EDAN (difference of 0.01 unit). Accuracy of ICA analysis of GEM was compared with two blood gas devices Ciba Corning 865 and Radiometer ABL 725 (Beneteau-

Burnat et al. 2004; Steinfelder-Visscher et al. 2006) and found to be highly correlated, as in the present study with EDAN. The bias of 0.054 mmol/L created an average +5.1% deviation for EDAN that looked slightly comparable to GEM. The best and most acceptable difference would be less than a 5% deviation. On the other side, the average ICA or $ICA_{7.4}$ values of GEM and EDAN were in line with the literature reported in dairy cows for different lactation stages between 1.02 – 1.20 mmol/L (Riond et al. 1995; Martinez et al. 2016; Mahen et al. 2018). GEM has been used in animal health, especially in calves with diarrhoea to test the blood parameters like ICA, lactate and electrolytes (Aydoğdu et al. 2019), but no further information was given about the accuracy in calves. A good correlation between two methods can lead to misinterpretation; it does not necessarily mean that two methods deliver fully identical results in the analysis. A huge difference was found in the average lactate concentrations (+46%) between EDAN and GEM tough high significant correlation was found between both devices. This was confirmed with high biases with Bland-Altman analysis and Wilcoxon tests. The normal reference range of lactate concentration in lactating healthy Holstein was given as 0.42–0.74 mmol/L and an average of 0.54 mmol/L (Figueiredo et al. 2006), which looked close to the values provided by GEM, much lower than EDAN's value. Vukelić et al. (2007) reported that the precision and accuracy of lactate analysis of GEM was not satisfactory high in comparison with the reference method in humans. This might require that both devices should be tested against a gold reference method in lactating Holstein. Furthermore, Bland Altman analysis delivers the intervals of agreement and bias, but it does also not say the limits are acceptable or not (Giavarina 2015). This is in line with the results between GEM and EDAN. In the present study, there was no significant positive or negative correlation between blood pH and ICA concentrations analysed by both devices. Even, blood pH did not change significantly in G1 with decreased ICA concentrations than G2 unlike blood lactate and Cl concentrations. This was probably due to buffering system of the blood in the organism. An increasing H+ concentration decreases calcium binding to albumin or serum proteins which indicate a competitive relationship between H^+ and Ca^{2+} binding (Rehfeld et al. 1984). As all other blood gas devices, ICA was displayed as blood pH 7.40 adjusted value ($ICA_{7.4}$) by both blood gas devices, which was already reported by others to eliminate the effect of small blood pH deviations (Dauth et al. 1984; Watson and Anbar 1985; Ballantine and Herbein 1991; Constable et al. 2019). A negative correlation between blood ICA concentration and pH was already reported (Rehfeld et al. 1984; Goff 2008; Constable et al. 2019), which is the basis of the concept for anionic feeding in the close-up of pregnant cows for the stimulation parathormone by decreasing pH and consequently increasing blood calcium concentration by mobilisation from bones. The effect was clinically tested in the urine pH after calving (Goff 2008). ICA and $ICA_{7.4}$ concentrations were correlated negatively with the blood Cl concentration analysed by EDAN. Constable et al. (2019) reported a positive correlation between blood Cl and ICA concentrations in calves. This is not in line with the results of the present study. The average low $ICA_{7.4}$ concentrations in G1, even lower than the threshold of SCH definition was accompanied by significantly higher Cl concentrations which have confirmed this negative correlation trend. The present study provided the first evidence that blood Cl levels behaved controversially in PRP and MUL cows with SCH and it was dependent on PBCT. No parity, but a negative PBCT correlation was observed in Cl concentration which resulted in much higher Cl levels in MUL cows in G1 compared to G2. Cows with SCH had much higher Cl levels than SCH negative cows. Besides, reduced $ICA_{7.4}$ in SCH cows was accompanied by increased Cl concentration in G1, but not

in G2. The normal average value of Cl in healthy lactating Holstein was given as 99±4.3 mmol/L without pointing to the stage of lactation (Cozzi et al. 2011). This was almost in line with the average value reported by the present study, especially matched with the values of cows without SCH. However, Cl values of MUL and PRP cows with SCH in the present study was much higher than this value reported by (Cozzi et al. (2011). On the other side, the majority of animals was in the very early stage of lactation in the presented study. Metabolic acidosis caused by the negative dietary cation-anion difference (DCAD) via Cl addition in the diet was likely to decrease ICA reabsorption in the kidney, thus more ICA was excreted into urine that reduces blood ICA (Van Mosel et al. 1994; Wang et al. 2018). This can probably explain the controversial behaviour between blood ICA and Cl concentrations of SCH cows in the present study. Constable et al. (2019) observed a negative correlation between blood ICA and lactate concentrations in calves. In contrast, the present study found a weak positive correlation between ICA and lactate concentrations (GEM) or no correlation (EDAN) in lactating Holstein. This might be because of the low test accuracy of EDAN in Holstein that created a huge difference in average values between GEM and EDAN as mentioned previously. Lactate concentrations in G1 with reduced $ICA_{7.4}$ was significantly higher than G2. No parity, but a negative PBCT correlation was found in lactate levels which resulted in much higher lactate concentration in MUL cows (significant by GEM) between G1 and G2. But, there were no lactate deviations between cows with and without SCH, neither in G1 nor 2. Besides, cows with SCH in G1 had higher lactate levels than cows with SCH in G2. This was also a PBCT effect. Whole blood lactate levels analysed by GEM was much higher in calves that passed away due to severe diarrhoea, but their ICA levels were unchanged (Aydoğdu et al. 2019). In addition, higher lactate concentration (3.23 - 5.88 mmol/L) was seen as an indicator for the illnesses (displaced abomasum) in Holstein (Figueiredo et al. 2006). Values of lactate by GEM looked similar, but EDAN values looked quite high in comparison to this average reported in the literature. The reason for higher lactate concentration (out of reference) in G1 cows can be probably the other peripartal diseases or calving stress. Positive, but not a high correlation between blood $ICA_{7.4}$ and serum TC was previously well documented (Kvart and Larsson 1978; Agnes et al. 1993; Leno et al. 2017; Constable et al. 2019) was confirmed in the present study. However, there was no significant correlation between albumin, TP and $ICA_{7.4}$ concentrations analysed by both devices in the present study. This is in contrast to other studies that reported a positive correlation (Kvart and Larsson 1978; Agnes et al. 1993; Constable et al. 2019). Cows had significantly reduced serum TC and whole blood $ICA_{7.4}$ concentrations and reduced ratio of TC/TP and TC/Alb in very early postpartum (G1) compared to G2. Serum Alb was correlated positively with TC, but neither TP and GL nor Alb changed significantly between G1 and G2. A moderate positive correlation between serum Alb and TC was well documented previously, even a correction of TC to normal Alb concentration was recommended (Seifi et al. 2005). There are several cut-points (<2.0 to <2.20 mmol/L) defined for serum or plasma TC concentration for the diagnosis of SCH at 24 and 96 h postpartum (Goff 2008; DeGaris and Lean 2009; Chapinal et al. 2011; Reinhardt et al. 2011; Martinez et al. 2012; Martinez et al. 2014; Caixeta et al. 2017; Wilhelm et al. 2017; Wilkens et al. 2020). Once is important that the defined cut-point for serum TC should correlate with postpartum health problems and milk yield, this was not studied in several studies (Neves et al. 2018). Serum TC concentration <2.15 mmol/L for SCH diagnosis was defined for the present study, which was also recommended by Caixeta et al. (2017), Martinez et al. (2012) and Martinez et al. (2014). This threshold of

serum TC was found to be correlated with postpartum diseases (Caixeta et al. 2017; Neves et al. 2018). The present study defined the most closely and sensitive cut-points for blood $ICA_{7.4}$ concentration to catch SCH cases by using average concentrations of $ICA_{7.4}$ of GEM and EDAN which were within the ratio of blood $ICA_{7.4}$ to serum TC concentrations. This has been performed due to a moderately high positive correlation between $ICA_{7.4}$ and serum TC, which is Alb dependent as in other studies also reported (Agnes et al. 1993; Seifi et al. 2005; Constable et al. 2019), and therefore the threshold values provided by ROC analysis were not enough sensitive to catch the SCH cases in the present study. Leno et al. (2017) considered that the relationships between blood ICA and serum TC is not reliable to identify the same population of cows with SCH. The result of the present study confirmed this statement. The proportional ratio of $ICA_{7.4}$ to serum TC in the present study was in agreement with the previous reports (43%-57% of plasma or serum TC) in lactating cows (Raman 1970; Ballantine and Herbein 1991) and in calves (Agnes et al. 1993; Constable et al. 2019). Martinez et al. (2014) recommended blood ICA concentration<1.0 mmol/L for the definition of SCH which frequently caused a reduction of immune function, dry matter intake and altered the energy metabolism in nonpregnant, nonlactating Holstein cows. This cut-point looked close to the cut-points defined for GEM (<1.04 mmol/L) and a little lower than EDAN (<1.09 mmol/L) in the present study. But, Leno et al. (2017) identified a much higher ICA threshold (≤1.17 mmol/L) for serum TC of ≤2.0 and 2.125 mmol/L to define SCH which looked much higher than the results of the present study. They concluded that ICA cannot be used interchangeably as indicators of calcium status due to variation in the relationship between ICA and reference method TC in the postpartum period. Correct testing both serum TC or blood ICA in fresh cows is highly important because the association of reduced blood calcium level with postpartum health disorders have been reported (Chapinal et al. 2011; Neves et al. 2018). SCH between calving and postpartum day 3 can cause reduced neutrophils counts and functions which can result in the increased risk for metritis (Martinez et al 2012). Caixeta et al. (2017) reported impaired reproductive functions (low ovarian function, decreased pregnancy rate at first service) in Holstein with SCH (TC≤2.15 mmol/L at first 3 days postpartum). The present study detected 7 cows with severe SCH (5.6%, serum TC<1.50 mmol/L) in G1, however without clinical signs (paresis). Similarly, the defined cut-points for $ICA_{7.4}$ by both devices detected also 6 cows with severe SCH. Cows with serum TC<1.50 mmol/L or <1.40 mmol/L were described as clinical hypocalcemia or milk fever (Seifi et al. 2005; DeGaris and Lean 2009), but that was not observed in the present study. ICA, the physiologically active form of calcium, plays an important role in muscle contraction, nerve impulse transmission and hearth function (Wilkens et al. 2020), and therefore rapid testing of blood $ICA_{7.4}$ by portable devices makes more sense than testing serum or plasma TC concentration for detecting hypocalcemia in the very early postpartum period of lactating cows. Results of other studies (Lincoln and Lane 1990; Ballantine and Herbein 1991) indicate also this usefulness while some others preferred to analyse serum TC due to the dependency of blood ICA on other blood parameters (Caixeta et al. 2017) or variations in early postpartum (Leno et al. 2017). A negative correlation between parity and blood calcium levels resulted in reduced whole blood ICA7.4 and TC concentrations of MUL compared to PRP cows. Higher parity was a risk factor for low TC or ICA during very early postpartum days (Reinhardt et al. 2011; Caixeta et al. 2017; Neves et al. 2018; Mahen et al. 2018; Venjakob et al. 2019). A positive correlation between blood calcium levels and PBCT resulted in increasing whole blood $ICA_{7.4}$ and TC levels of MUL cows in G2 compared to G1. Interestingly, $ICA_{7.4}$ levels of PRP

cows was not affected by PBCT, but TC levels were significantly reduced in the first 3 days postpartum compared to G2. Hypocalcemia is a common metabolic disorder of lactating Holstein observed within the first days after calving (Goff 2008; Reinhardt et al. 2011). The lowest concentration of TC and ICA was found in the first 1-3 days postpartum (Szenci et al. 1994; Riond et al. 1995; Mahen et al. 2018; Venjakob et al. 2019). The incidence of CH and SCH increases with the age of lactating cows, frequently it was observed in mature cows (in their second or greater lactations) (Reinhardt et al. 2011). The average TC concentration of MUL cows was below the cut-point for SCH definition in early postpartum (G1), but the difference between PRP and MUL cows was not highly significant, PRP cows had a little higher TC. There was a highly significant difference between $ICA_{7.4}$ concentrations of PRP and MUL cows confirmed by two blood gas devices, thus PRP cows had much higher ICA in G1 and G2. This can indicate that physiologically active, free and diffusible form $ICA_{7.4}$ can differentiate hypocalcemia status between PRP and MUL cows in the very early postpartum period. That difference might be due to higher colostrum and milk production of MUL cows than PRP cows in the fresh period, which requires a much higher calcium supply into the udder. ICA in very early postpartum was related clearly to the clinical manifestation of the diseases and functional status of cow's calcium metabolism (Lincoln and Lane 1990). Similar SCH rates were observed in MUL and PRP Holstein in the present study, but it was observed more frequently in G1 than in G2, while others (Reinhardt et al. 2011; Wilhelm et al. 2017) reported hypocalcemia incidence was observed often in MUL cows. Both time and parity effect on SCH incidence was in line with most of the previous reports (Goff 2008; Reinhardt et al. 2011). There might be always a cut-point effect of serum TC on the prevalence. Wilhelm et al. (2017), reported an incidence of 14.6% (2.5% for PRP and 30.8% for MUL) at calving based on a much lower cut-point of TC (<2.0 mmol/L), compared to the present study. The overall prevalence of SCH in the present study was in line with the literature (Goff 2008; Reinhardt et al. 2011; Martinez et al. 2012). Cows with SCH in G1 and G2 had different concentrations of TC and $ICA_{7.4}$ in the present study. The reason was the effect of PBCT that was even effective in SCH cows as well as the effect of unchanged $ICA_{7.4}$ between G1 and G2 in PRP cows. This can indicate $ICA_{7.4}$ can effectively show SCH in the very early period after calving, especially in MUL cows, but probably less effective in the later stage after postpartum day 4. Serum TC and ICA analysed by GEM and EDAN between calving and postpartum day 3 looked similar to the results reported by others (Kvart and Larsson 1978; Szenci et al. 1994; Riond et al. 1995; Martinez et al. 2016; Mahen et al. 2018; Melendez et al. 2020). Blood ICA <1.00 mmol/L was recommended for the definition of SCH that frequently caused a reduction of immune function and altered the energy metabolism in Holstein cows (Martinez et al. 2014). This looked in line with average values produced in the present study. However, a much higher ICA threshold (≤1.17 mmol/L) was identified by Leno et al. (2017) to define SCH. Using ICA was not recommended as an indicator of calcium status due to variation in the relationship between ICA and reference serum TC in the postpartum period. In contrast, Lincoln and Lane (1990) reported that the concentration of ICA was related clearly to the clinical manifestation of calcium metabolism diseases. The reason for the reduced TC/TP, and TC/Alb ratio of MUL Holstein, especially in G1 was probably the high calcium excretion into milk and colostrum at an early stage after calving, although values of TP and Alb in the groups were unchanged between parities despite positive correlation. The effect can be due to increasing GL concentrations in MUL cows. A positive correlation between serum Alb and TC (Agnes et al. 1993; Sheifi et al. 2005), but not between TP and TC

(Agnes et al. 1993) was confirmed in reduced concentrations of TP, Alb and ratio of TC/TP and TC/Alb in SCH-cows in the present study. However, TC does not reflect the true concentration of calcium due to hypoproteinemia cases (Seifi et al. 2005). The average ICA/TC ratio between GEM and EDAN was significant both in PRP and MUL cows, this was due to the effect of significantly different averages of ICA between both devices. Various studies reported ICA/TC ratio between 43 - 57% in Holstein (Kvart and Larsson 1978; Ballantine and Herbein 1991; Riond et al. 1995; Joyce et al. 1997; Constable et al. 2019). The present study has shown that PRP cows had a clearly higher trend for the ratio of $ICA_{7.4}$/TC in G1 than G2, but this was not the case in MUL cows. A high ICA/TC ratio (55-57%) in 2 days after calving was reported by others (Ballantine and Herbein 1991; Joyce et al.1997), however, they did not distinguish the parity effect on this ratio. This effect came most probably from unchanged ICA concentration between G1 and G2 in PRP cows in contrast to MUL cows. The effect of parity was much clearer observed in cows with SCH. SCH-cows in G1 had lower serum TC, blood $ICA_{7.4}$ and $ICA_{7.4}$/TC ratio than G2, the reason was MUL SCH-cows, because they suffered from much lower blood ICA levels than PRP SCH-cows. In addition to this, two blood gas devices have confirmed both MUL and PRP SCH cows in G1 and G2 had much higher than 50% $ICA_{7.4}$/TC ratio while normocalcemic cows had frequently below 50%. Because of higher Ca ionisation in PRP cows with SCH, the ratio ICA/TC was higher than MUL cows with SCH in G1.

Conclusion

GEM and EDAN showed a good linear correlation for whole blood pH and ICA analysis in lactating Holstein, but significant deviations occurred in the average concentrations of these parameters. Average ICA results by both devices looked similar to the previously reported data in the literature. The differences were within acceptable deviations (roughly 5%). However, this was not applicable for lactate analysis. Both devices should be tested with reference laboratory method for the analysis of whole blood lactate in lactating Holstein due to high biases. ROC analysis did not provide sensitive cut-points of $ICA_{7.4}$ for the correct SCH and severe SCH detection based on serum TC threshold in lactating Holstein. This was probably because of the moderate correlation between ICA and TC as well as the dependencies of these parameters on other parameters and PBCT. Therefore, different cut-points for GEM (<1.04 mmol/L) and EDAN (<1.09 mmol/L) were necessary to define by multiple cut-points for the most correct cases with SCH compared to the reference serum TC. SCH was observed more frequently between calving and postpartum day 3 (2.5 times) and MUL Holstein was at risk for hypocalcemia based on lower serum TC and high SCH incidence. This was also clearly confirmed by the lower blood ICA concentrations in MUL cows analysed by two different blood gas devices. The ionisation of calcium in PRP cows with or without SCH was much higher within 3 days postpartum than MUL cows and this can be a reason for the lower incidence of CH in PRP cows, though serum TC was also low in PRP cows. Both PRP and MUL cows with SCH had frequently higher than 50% $ICA_{7.4}$ /TC ratio compared to normocalcemic cows that had below 50%; the even higher ratio in PRP cows was supportive within postpartum 3 days over MUL cows. The present study revealed that higher Cl concentration in PRP and MUL cows with SCH was probably due to negative DCAD, resulted in lower $ICA_{7.4}$ levels within 3 days postpartum. No clear interference and correlation were found between blood

lactate levels and $ICA_{7.4}$ or SCH, except the PBCT effect in G1. Reduced serum Alb levels in SCH-cows both in G1 and G2 indicated strong relation of serum TC with Alb, rather than TP and GL.

Declarations

Supplementary Information

Whole or part of this manuscript was not presented at a symposium or congress before. The present study is first part of an approved project by the Animal Ethics committee of Muğla Sıtkı Koçman University entitled 'Comparison of point-of-care blood gas devices (Gem Premier 3000 and Edan i15 Vet) for the analysis of blood ionised calcium concentrations to detect hypocalcemia and subclinical hypocalcemia'.

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Conflict of Interests Statement: The authors declare that they do not have any financial or business relation or benefits from the Manufacturer or Distributor Company of the blood gas devices (GEM Premier 3000 and EDAN i15 Vet) or automated biochemistry analyser Mindray BS 120 Vet.

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Tables

Table 1 Correlation coefficients, intercept and slope by Least Square Regression analysis between blood pH 7.40 corrected ionised calcium ($ICA_{7.4}$ mmol/L) concentrations analysed by EDAN and GEM and other blood parameters in 125 Holstein cows

Parameter	Device for $ICA_{7.4}$ analysis	r	r^2	Intercept+Slope (y)	p
TC (mmol/L)	EDAN	0.515	0.265	y = 0.700+0.18x	0.000
	GEM	0.566	0.320	y = 0.623+1.555x	0.000
Lactate* (mmol/L)	EDAN	0.059	0.003	y = 0.810+0.238x	0.516
	GEM	0.270	0.073	y = -0.417+1.097x	0.002
pH*[1]	EDAN	0.094	0.009	y = 7.471-0.022x	0.299
	GEM	0.034	0.010	y = 7.448+0.008x	0.709
TP (g/L)	EDAN	0.117	0.014	y = 6.245+0.559x	0.194
	GEM	0.107	0.011	y = 6.353+0.484x	0.237
Alb (g/L)	EDAN	0.133	0.018	y = 2.869+0.279x	0.139
	GEM	0.138	0.019	y = 2.888+0.275x	0.124
GL** (g/L)	EDAN	0.059	0.003	y = 3.376+0.280x	0.515
	GEM	0.046	0.002	y = 3.465+0.209x	0.609

TC: serum total calcium. Alb: serum albumin. GL: serum globulin. TP: serum total protein. *: Lactate and pH values refer to each blood gas device in which they were analysed. **: GL was calculated by deduction of Alb from TP. [1]: Correlation between blood pH and real value of ionised calcium concentration. Serum TC, TP and Alb concentrations were analysed by BS120

Table 2 Average concentrations (mean and standard errors, x±se) of serum and whole blood gas parameters analysed by EDAN and GEM blood gas analysers and BS120 automated wet-biochemistry analyser in 125 Holstein between calving to postpartum day 3 (group 1) and postpartum day 4 to day 27 (group 2)

Parameter	Device	All animals (n=125)	Group 1 (n=87)	Group 2 (n=38)	p (1)
TC (mmol/L)	BS120	2.25±0.49	2.14±0.48	2.49±0.43	0.000
$ICA_{7.4}$ (mmol/L)	EDAN	1.09±0.17	1.06±0.17	1.19±0.14	0.000
	GEM	1.04±0.18*	1.01±0.18*	1.13±0.14*	0.000
ICA (mmol/L)	EDAN	1.08±0.17	1.04±0.17	1.17±0.14	0.000
	GEM	1.02±0.17*	0.98±0.18*	1.10±0.13*	0.000
pH	EDAN	7.45±0.04	7.45±0.04	7.44±0.04	0.713
	GEM	7.46±0.04*	7.46±0.04*	7.45±0.03*	0.452
Lactate (mmol/L)	EDAN	1.07±0.69	1.21±0.73	0.76±0.42	0.001
	GEM	0.73±0.72*	0.83±0.82*	0.50±0.28*	0.001
Cl (mmol/L)	EDAN	102.66±3.86	103.69±3.79	100.29±2.91	0.000
TP (g/L)	BS120	68.58±8.09	67.80±8.59	70.38±6.59	0.102
Alb (g/L)	BS120	31.75±3.54	31.33±2.99	32.72±4.46	0.084
GL (g/L)	BS120	36.83±8.06	36.47±8.70	37.66±6.39	0.452
TC/TP ratio (%)	BS120	0.13±0.03	0.13±0.03	0.14±0.03	0.011
TC/Alb ratio (%)	BS120	0.29±0.07	0.27±0.06	0.31±0.07	0.010
TC/GL ratio (%)	BS120	0.25±0.07	0.25±0.07	0.27±0.07	0.061
Alb/TP ratio (%)	BS120	46.77±6.26	46.81±6.35	46.67±6.13	0.913
GL/TP ratio (%)	BS120	53.23±6.26	53.19±6.35	53.33±6.13	0.913
$ICA_{7.4}$/TC ratio (%)	EDAN	50.28±9.21	50.82±8.86	49.03±9.98	0.321
	GEM	47.56±8.13*	47.98±7.82*	46.60±8.83*	0.386

*: $p<0.01$ compared to EDAN value in the same column for the same parameter. p (1): significance between group 1 and group 2. $ICA_{7.4:}$ blood pH 7.40 corrected ionised calcium concentration, Cl: chloride, TC: serum total calcium, TP: serum total protein, GL: serum globulin, Alb: serum albumin concentrations. **: TC values in mmol/L were converted to mg/dl by dividing with 0.02495 to calculate the respective ratio; TP, Alb and GL values in g/L were converted to mg/dl to calculate the ratio to serum TC

Table 3 Correlation coefficients, intercept and slope between parity (min: 1, max: 7, average: 2.68±1.46) and blood parameters in 125 lactating Holstein

Parameters	Units	r	r^2	Intercept+Slope (y)	p
$ICA_{7.4}$ by GEM	mmol/L	-0.175	0.031	y=1.100-0.021x	0.051
$ICA_{7.4}$ by EDAN	mmol/L	-0.233	0.054	y=1.169-0.027x	0.009
ICA by GEM	mmol/L	-0.178	0.032	y=1.076-0.021x	0.047
ICA by EDAN	mmol/L	-0.224	0.050	y=1.146-0.026x	0.012
Serum TC by BS120	mmol/L	-0.162	0.026	y= 2.391-0.055x	0.070
Serum TP by BS120	g/L	0.208	0.043	y=65.485+1.156x	0.020
Serum GL by BS120	g/L	0.219	0.048	y=33.585+1.212x	0.017
Serum Alb by BS120	g/L	-0.023	0.001	y=31.900-0.056x	0.797
Ratio of $ICA_{7.4}$ /TC by GEM	%	-0.016	0.000	y=47.800-0.089x	0.859
Ratio of $ICA_{7.4}$ /TC by EDAN	%	-0.021	0.000	y=50.628-0.131x	0.818
Ratio of TC/GL by BS120	%	-0.261	0.068	y=0.289-0.013x	0.003
Ratio of TC/TP by BS120	%	-0.243	0.059	y=0.146-0.005x	0.006
Ratio of TC/Alb by BS120	%	-0.136	0.018	y=0.302-0.006x	0.132
Cl by EDAN	mmol/L	0.145	0.021	y=101.625+0.385x	0.107
Blood pH by GEM	pH	-0.035	0.001	y=7.458-0.001x	0.700
Blood pH by EDAN	pH	-0.057	0.003	y=7.450-0.002x	0.531
Lactate by GEM	mmol/L	-0.046	0.002	y=0.789-0.023x	0.609
Lactate by EDAN	mmol/L	-0.037	0.001	y=1.118-0.018x	0.680

TC: serum total calcium, $ICA_{7.4}$: blood pH 7.40 corrected ionised calcium (ICA), TP: serum total protein, GL: serum globulin, Alb: serum albumin, Cl: chloride concentration. BS120: an automated serum wet-biochemistry analyser

Table 4 Cut-points and respective test characteristics of EDAN and GEM to detect hypocalcemia (number of cases and prevalence) in coccygeal blood based on Receiver Operating Characteristics (ROC) analysis using different cut-points of serum total calcium concentration of <2.15 mmol/L (subclinical hypocalcemia, SCH) and <1.50 mmol/L (severe SCH) analysed by BS120 as reference (automatic wet-biochemistry analyser)

ROC analysis (n=125)							Detection of hypocalcemia					
							All cows (n=125)		Group 1 (n=87)		Group 2 (n=38)	
Cut-point of Ca (mmol/L) for devices		Spec (%)	Sens (%)	AUC (%95)	*j*	p	n	%	n	%	n	%
BS120	<2.15	100	100	1.00	1.000	0.000	54	43.2	46	52.9	8	21.1
EDAN	1.05	81.7	57.4	0.73	0.391	0.000	44	35.2	38	43.7	6	15.8
GEM	1.02	90.3	57.4	0.76	0.377	0.000	45	36.0	37	42.5	8	21.1
BS120	<1.50	100	100	1.00	1.000	0.000	7	5.6	7	8.0	0	0
EDAN	0.86	95.8	100	0.98	0.958	0.000	12	9.6	12	13.8	0	0
GEM	0.78	96.6	100	0.99	0.966	0.000	11	8.8	11	12.6	0	0

Spec: specification, Sens: sensitivity, AUC: area under the curve, *j*: Youden-Index. Group 1 (cows between calving to postpartum day 3), group 2 (cows between postpartum day 4 to 27)

Table 5 Average concentrations (mean and standard errors, x±se) of serum total calcium (TC), total protein (TP), albumin (Alb) and globulin (GL) concentrations analysed by BS120 (wet-biochemistry analyser), whole blood pH 7.40 adjusted ionised calcium ($ICA_{7.4}$), lactate and chloride (Cl) concentrations and their ratio analysed by EDAN and GEM in primiparous (PRP, n=35) and multiparous (MUL, n=90) Holstein between calving and postpartum day 3 (group 1) and postpartum day 4 to 27 (group 2)

Parameter	Parity	All animals (n=125)	Group 1 (n=87)	Group 2 (n=38)	p(1)
TC (mmol/L)	PRP	2.41±0.49	2.28±0.46	2.70±0.46	0.017
	MUL	2.18±0.48*	2.09±0.48+	2.40±0.40*	0.002
EDAN $ICA_{7.4}$ (mmol/L)	PRP	1.15±0.11	1.14±0.09	1.17±0.15	0.463
	MUL	1.08±0.18*	1.03±0.18**	1.19±0.13	0.000
GEM $ICA_{7.4}$ (mmol/L)	PRP	1.08±0.12	1.07±0.12	1.08±0.14	0.944
	MUL	1.03±0.19+	0.98±0.19**	1.15±0.13	0.000
TP (g/L)	PRP	66.60±8.15	65.50±8.67	69.01±6.62	0.242
	MUL	69.35±7.98+	68.68±8.46	70.93±6.62	0.221
Alb (g/L)	PRP	31.87±3.77	31.16±2.67	33.42±5.30	0.100
	MUL	31.70±3.47	31.39±3.13	32.43±4.15	0.249
GL (g/L)	PRP	34.73±7.58	34.33±8.25	35.58±6.15	0.658
	MUL	37.65±8.13+	37.29±8.79	38.50±6.41	0.519
GEM lactate (mmol/L)	PRP	0.73±0.55	0.81±0.62	0.55±0.31	0.209
	MUL	0.73±0.78	0.83±0.90	0.48±0.28	0.006
EDAN lactate (mmol/L)	PRP	1.08±0.47	1.15±0.51	0.93±0.34	0.199
	MUL	1.07±0.76	1.23±0.81	0.68±0.43	0.357
EDAN Cl (mmol/L)	PRP	102.09±3.36	102.71±3.58	100.72±2.45	0.107
	MUL	102.88±4.04	104.06±3.82	100.11±3.11	0.000
EDAN $ICA_{7.4}$ /TC ratio (%)	PRP	49.38±10.90	51.48±10.19	44.79±11.48	0.092
	MUL	50.63±8.51	50.57±8.38	50.76±8.96	0.922
GEM $ICA_{7.4}$ /TC ratio (%)	PRP	46.58±10.44	48.85±9.64	41.61±10.84	0.056
	MUL	47.94±7.07	47.65±7.07	48.63±7.14+	0.547
TC/TP ratio (%)	PRP	0.15±0.03	0.14±0.03	0.16±0.03	0.101
	MUL	0.13±0.03**	0.12±0.03*	0.14±0.02*	0.047
TC/Alb ratio (%)	PRP	0.31±0.06	0.29±0.05	0.33±0.08	0.121
	MUL	0.28±0.06*	0.27±0.06	0.30±0.07	0.022
TC/GL ratio (%)	PRP	0.29±0.08	0.28±0.07	0.31±0.09	0.165

	MUL	0.24±0.07**	0.24±0.07*	0.26±0.05^{+}	0.191

PRP cows: group 1 consists n=24, group 2 consists n=11. MUL cows: group 1 consists n=63, group 2 consists n=27. *: p<0.05 versus PRP in the same group, **:p<0.001 versus PRP in the same group, $^{+}$: p<0.07 versus PRP in the same group, $p^{(1)}$: significance of the difference between group 1 and group 2

Table 6 Average concentrations (mean and standard errors, x±se) of serum total calcium (TC), total protein (TP), albumin (Alb) and globulin (GL) concentrations analysed by BS120 (wet-biochemistry analyser), whole blood pH 7.40 adjusted ionised calcium ($ICA_{7.4}$), lactate and chloride (Cl) concentrations and their ratio analysed by EDAN and GEM in Holstein with subclinical hypocalcemia (SCH) (TC<2.15 mmol/L) and without SCH (TC≥2.15 mmol/L)

Parameter	Cows with SCH (n=54)	Cows without SCH (n=71)	$p^{(1)}$
TC (mmol/L)	1.82±0.27	2.57±0.36	0.000
EDAN $ICA_{7.4}$ (mmol/L)	1.02±0.18	1.16±0.13	0.007
GEM $ICA_{7.4}$ (mmol/L)	0.95±0.19*	1.11±0.14*	0.003
EDAN $ICA_{7.4}$ /TC ratio (%)	56.10±8.07	45.85±7.41	0.000
GEM $ICA_{7.4}$ /TC ratio (%)	52.14±6.99*	44.08±7.19*	0.000
TP (g/L)	66.38±8.19	70.26±7.66	0.008
Alb (g/L)	30.31±2.93	32.84±3.60	0.000
GL (g/L)	36.06±8.34	37.42±7.85	0.353
TC/TP ratio (%)	0.11±0.02	0.15±0.03	0.000
TC/GL ratio (%)	0.21±0,06	0.29±0.07	0.000
TC/Alb ratio (%)	0.24±0.04	0.32±0.06	0.000
EDAN Cl (mmol/L)	103.93±3.75	101.69±3.69	0.001
EDAN lactate (mmol/L)	1.04±0.49	1.10±0.81	0.184
GEM lactate (mmol/L)	0.65±0.46*	0.79±0.87*	0.107

SCH: subclinical hypocalcemia, TC: serum total calcium, $ICA_{7.4}$: pH 7.4 adjusted ionised calcium, TP: serum total protein, Alb: serum albumin, GL: serum globulin, Cl: chloride. *: p<0.01 indicates the difference between GEM and EDAN for the same parameter in the same column. $p^{(1)}$: significance between cows with and without SCH

Table 7 Average concentrations (mean and standard errors, x±se) of serum total calcium (TC), total protein (TP), albumin (Alb) and globulin (GL) concentrations analysed by BS120 (wet-biochemistry analyser),

whole blood pH 7.40 corrected ionised calcium ($ICA_{7.4}$), lactate and chloride (Cl) concentrations and their ratio analysed by EDAN and GEM in lactating Holstein with or without subclinical hypocalcemia (with SCH = serum TC<2.15 mmol/L, without TC≥2.15 mmol/L) in group 1 (calving to postpartum day 3) and group 2 (postpartum day 4 to 27)

Parameters	Group 1		p*	Group 2		p*
	With SCH (n=46)	Without SCH (n=41)		With SCH (n=8)	Without SCH (n=30)	
TC (mmol/L)	1.81±0.28	2.51±0.37	0.000	1.92±0.15	2.64±0.35	0.000
GEM $ICA_{7.4}$ (mmol/L)	0.93±0.19	1.09±0.14	0.000	1.06±0.15	1.15±0.13	0.112
EDAN $ICA_{7.4}$ (mmol/L)	0.99±0.17	1.13±0.13	0.000	1.15±0.18	1.20±0.13	0.391
EDAN $ICA_{7.4}$ /TC ratio (%)	55.44±7.69	45.63±7.09	0.000	59.89±9.70	46.14±7.95	0.000
GEM $ICA_{7.4}$ /TC ratio (%)	51.59±6.76	43.93±6.96	0.000	55.32±7.90	44.28±7.61	0.001
EDAN Cl (mmol/L)	104.65±3.21	102.61±4.12	0.011	99.75±4.13	100.43±2.57	0.563
EDAN lactate (mmol/L)	1.08±0.48	1.35±0.93	0.089	0.79±0.48	0.75±0.41	0.816
GEM lactate (mmol/L)	0.69±0.48	0.98±1.08	0.118	0.40±0.21	0.53±0,30	0.268
TP (g/L)	66.37±8.47	69.40±8.54	0.100	66.41±6.85	71.43±6.21	0.054
Alb (g/L)	30.56±2.79	32.19±3.02	0.010	28.91±3.52	33.73±4.16	0.005
GL (g/L)	35.81±8.58	37.21±8.88	0.456	37.50±7.09	37.70±6,32	0.939
TC/TP (%)	0.11±0.02	0.15±0.03	0.000	0.12±0.02	0.15±0.03	0.003
TC/GL (%)	0.21±0.06	0.28±0.07	0.000	0.21±0.05	0.29±0.07	0.005
TC/Alb (%)	0.24±0.04	0.32±0.06	0.000	0.27±0.03	0.32±0.07	0.007

p*: between cows with and without SCH in the respective group.

Figures

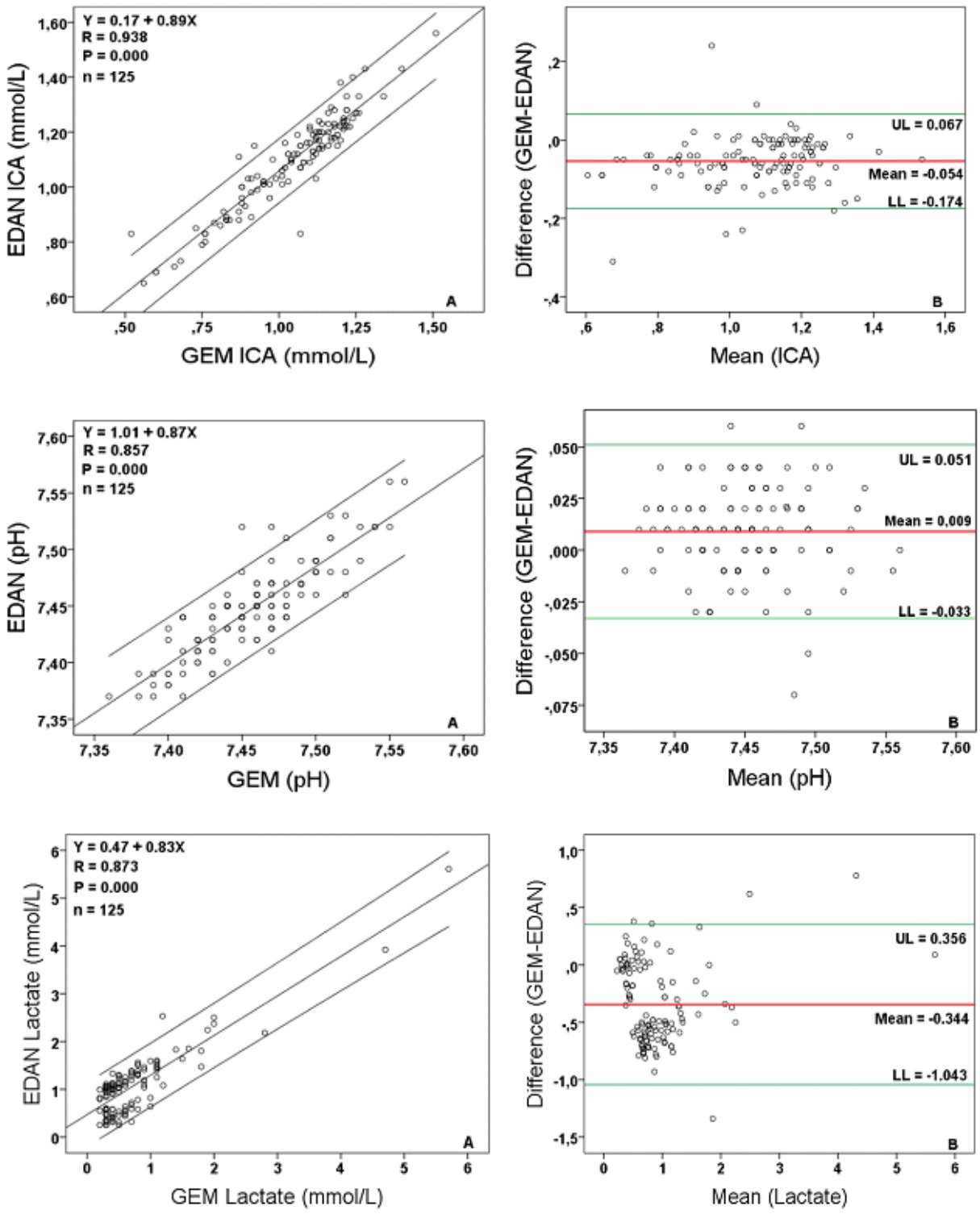

Figure 1

Correlation coefficients and confidence interval (95%) by Least Square Regression analysis (A) and plots of agreement by Bland-Altman (B) between blood pH 7.40-corrected ionised calcium concentrations (ICA, mmol/L), whole blood pH values and lactate (mmol/L) concentrations measured by GEM and EDAN blood gas devices. UL: upper limit, LL: lower limit

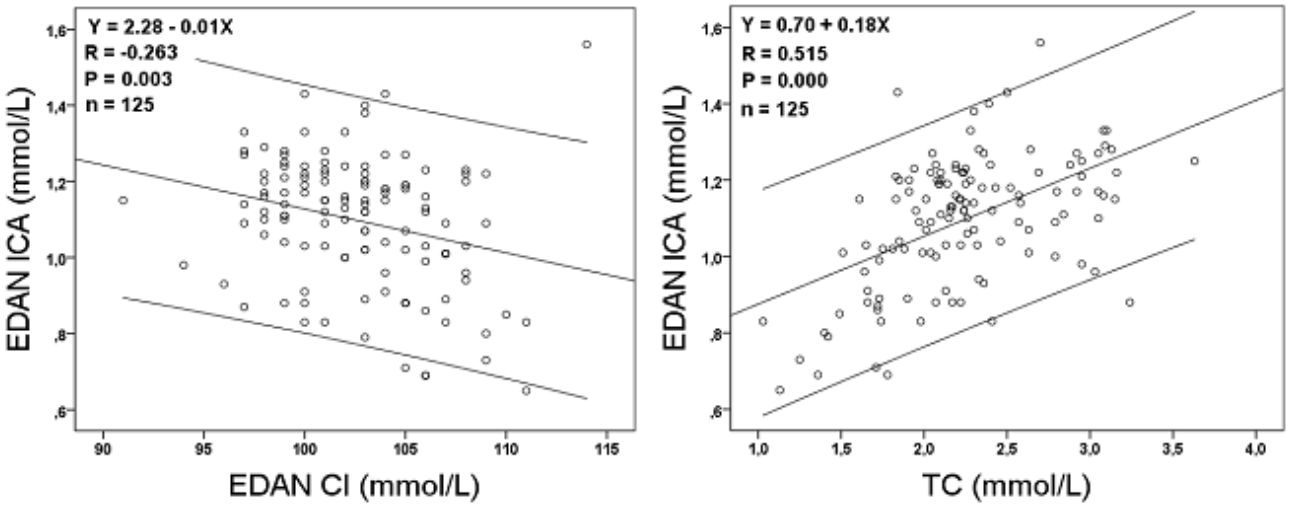

Figure 2

Correlation coefficients and confidence interval (95%) by Least Square Regression analysis between blood pH 7.40 corrected ionised calcium (ICA) and chloride (Cl, mmol/L, left figure) and serum total calcium (TC, mmol/L, right figure) concentrations analysed by EDAN blood gas device

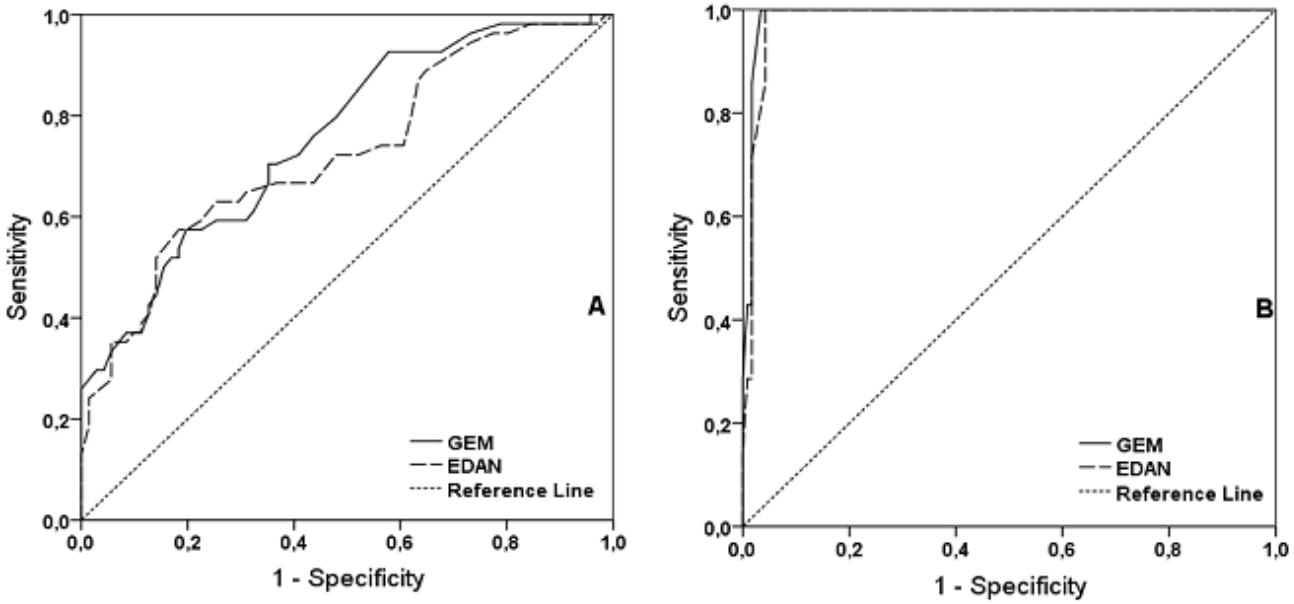

Figure 3

Receiver operating characteristics curves for the detection sensitivity of subclinical hypocalcemia (SCH) (A) and severe SCH (B) in the venous blood by EDAN and GEM based on a cut-points of serum total calcium concentration of <2.15 mmol/L for SCH and <1.50 mmol/L for severe SCH

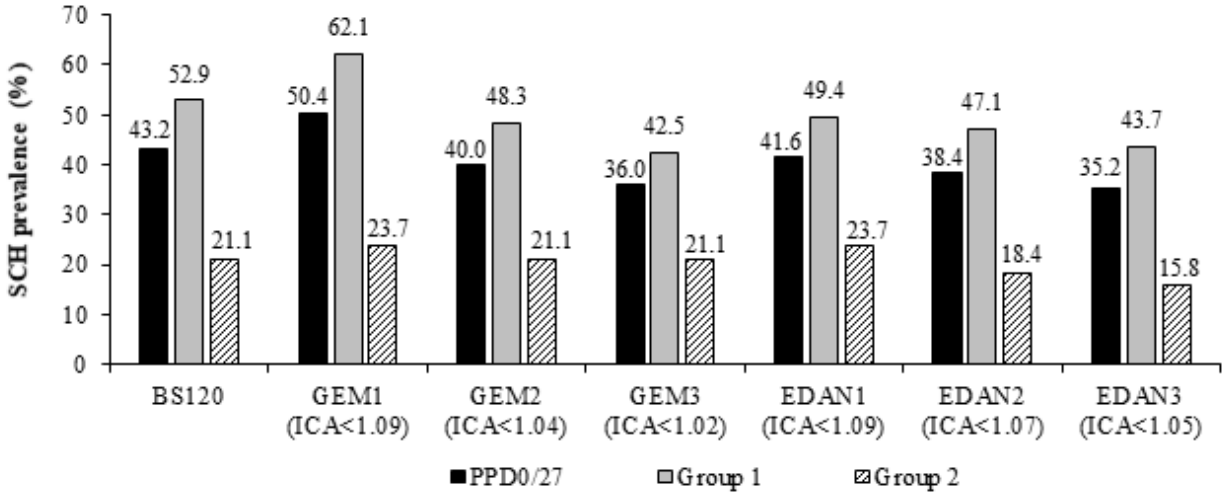

Figure 4

Prevalence of subclinical hypocalcemia (SCH) between calving to postpartum day 27 (PPD0/27, n=125), calving to postpartum day 3 (group 1, n=87) and postpartum day 4 to 27 (group 2, n=38) in Holstein. Cut-points for hypocalcemia detection were <2.15 mmol/L for serum total calcium concentration (TC) in BS120 (reference method) and <1.09 (EDAN1), <1.07 (EDAN2), <1.05 (EDAN3) and <1.09 (GEM1), <1.04 (GEM2) and <1.02 mmol/L (GEM3) for ICA7.4 (Blood pH 7.40 corrected ionised calcium concentration). Difference between group 1 and group 2 was significant in all devices for all cut-points (p<0.01). GEM3 and EDAN3 cut-points were calculated by receiver operating characteristics analysis based on reference method and cut-point (serum TC)

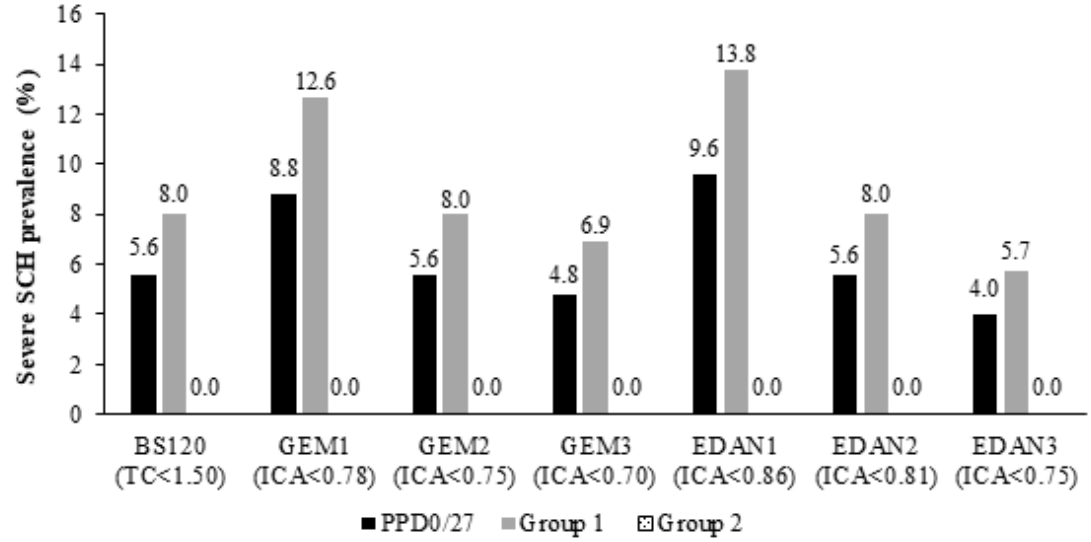

Figure 5

Prevalence of severe subclinical hypocalcemia (SCH) between calving to postpartum day 27 (PPD0/27, n=125), calving to postpartum day 3 (group 1, n=87) and postpartum day 4 to 27 (group 2, n=38) in Holstein. Cut-points for hypocalcemia detection were <1.50 mmol/L for serum total calcium concentration (TC) in BS120 (reference method) and <0.86 (EDAN1), <0.81 (EDAN2), <0.75 (EDAN3) and <0.78 (GEM1),

<0.75 (GEM2) and <0.70 (GEM3) mmol/L for ICA7.4 (Blood pH 7.40 adjusted ionised calcium concentration). GEM1 and EDAN1 cut-points were calculated by receiver operating characteristics analysis based on reference method and cut-point (serum TC)

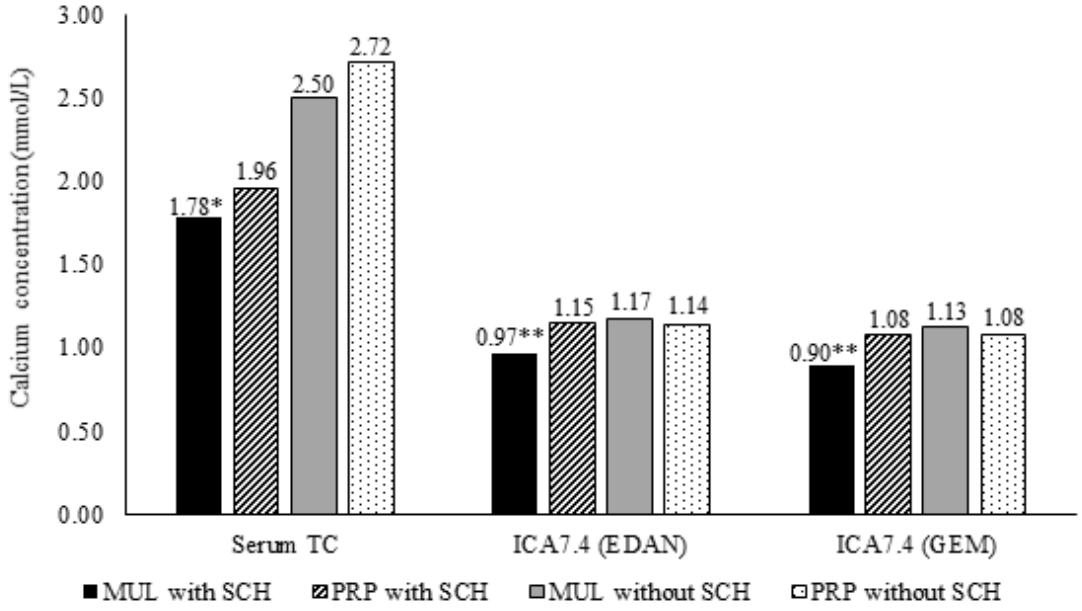

Figure 6

Average serum total calcium (TC) and blood pH 7.40 corrected ionised calcium concentration (ICA7.4) analysed by EDAN and GEM blood gas devices in primiparous (PRP) and multiparous (MUL) Holstein with subclinical hypocalcemia (SCH, serum TC<2.15 mmol/L) and without SCH (≥2.15 mmol/L). Number of PRP cows: n = 14 with SCH, n = 21 without SCH. Number of MUL cows: n= 40 with SCH, n = 50 without SCH. *: p=0.001, **p=0.016 compared to PRP cows with SCH

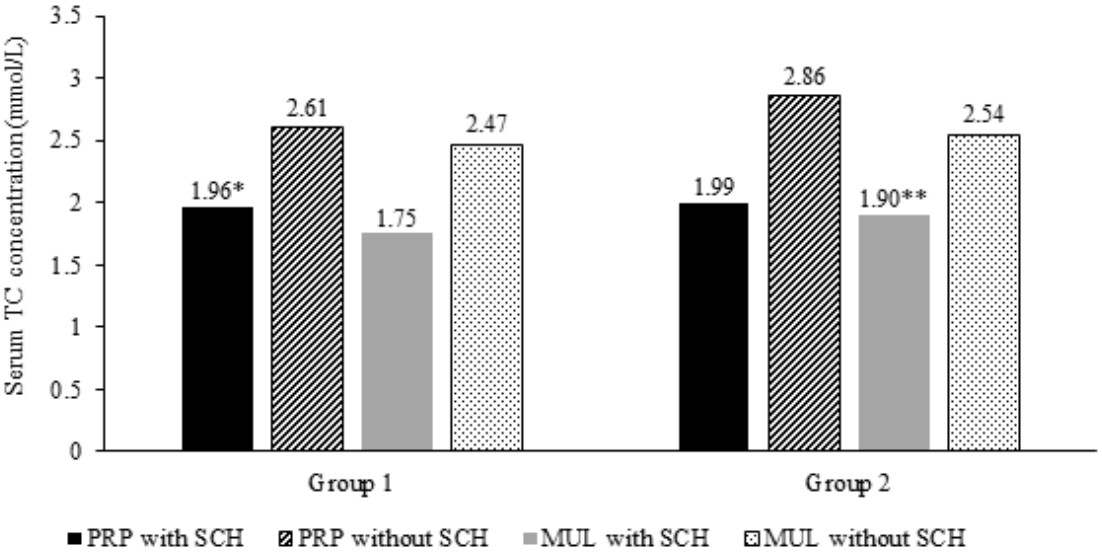

Figure 7

Average serum total calcium (TC) concentration in primiparous (PRP) and multiparous (MUL) Holstein with subclinical hypocalcemia (SCH: serum TC<2.15 mmol/L) and without SCH (TC≥2.15 mmol/L) between calving and postpartum day 3 (Group 1) and postpartum day 4 to 27 (Group 2). *:p=0.011 between MUL and PRP cows with SCH in the group 1. **: p=0.051 between group 2 and group 1 for MUL cows with SCH. Number of PRP cows with SCH was n=2 in group 2, no comparison was performed

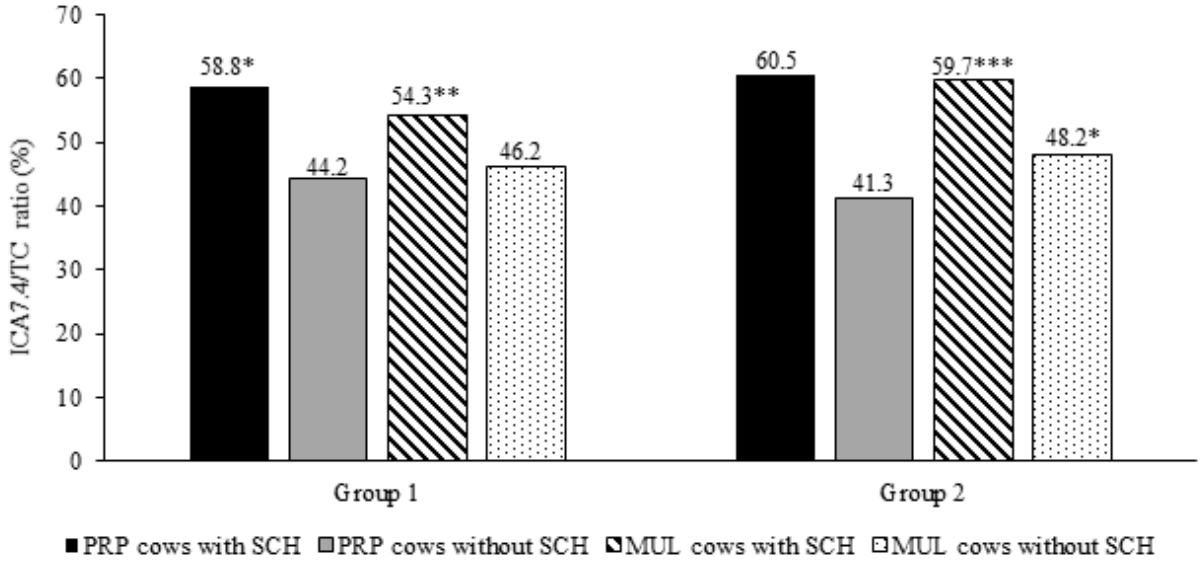

Figure 8

Average ratio of blood pH corrected ionised calcium (ICA7.4) tested by EDAN to serum total calcium (TC) between calving and postpartum day 3 (Group 1) and postpartum day 4 to 27 (Group 2) in primiparous (PRP) and multiparous (MUL) Holstein with subclinical hypocalcemia (SCH, serum TC<2.15 mmol/L) and without SCH (TC≥2.15 mmol/L). *:p=0.001 between cows with and without SCH in the group 1 (PRP) and group 2 (MUL). **p=0.041 between PRP and MUL cows with SCH in the group 1. ***p=0.080 between group 1 and group 2 of MUL cows with SCH. Number of PRP cows with SCH was n=2 in group 2, no comparison was performed

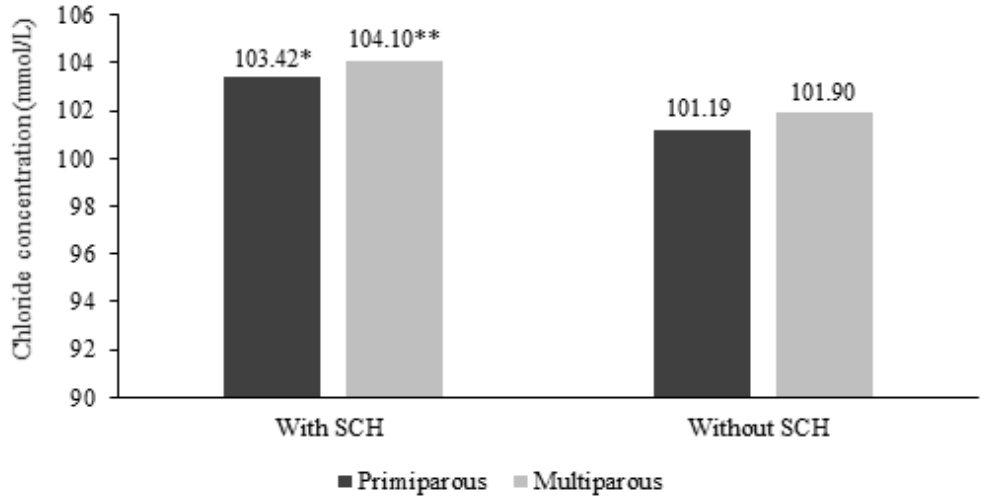

Figure 9

Chloride concentrations in in Holstein with subclinical hypocalcemia (SCH, serum total calcium <2.15 mmol/L, n=54) and without SCH (TC≥2.15 mmol/L, n=71) in group 1 (calving to postpartum day 3) and group 2 (postpartum day 4 to 27). *: p=0.014 compared PRP cows with SCH (103.42±0.86) to without SCH (101.19±0.70), **: p=0.009 compared MUL cow with SCH (104.10±0.62) to without SCH (101.90±0.54)