# IMPACTS AND EVALUATION OF SUBCLINICAL HYPOCALCEMIA IN DAIRY CATTLE

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The initiation of lactation in dairy cattle requires a coordinated increase in supply of calcium to the mammary gland as part of the homeorhetic shift from supporting the demands of pregnancy to those of lactation (Bauman and Currie, 1980). The increase in release of calcium stored in bone, increase in absorption of calcium from the diet, and decrease in excretion of calcium at the kidney must provide at least a two-fold increase in supply of calcium for colostrum production compared to that required by the growing fetus within a very short time frame (House and Bell, 1993; Goff and Horst, 1997). A large proportion of dairy cows are unable to meet this challenge in the 48 hours after parturition and therefore have an acute drop in blood calcium (hypocalcemia). Whereas clinical hypocalcemia (blood calcium typically below 5.0 mg/dL) incidence can be reduced to 5% or less, the incidence of subclinical hypocalcemia (SCH; blood calcium <8.0 mg/dL) has been shown to be 47% in cows entering their second or greater lactation, and 25% in first lactation animals (Reinhardt et al., 2011).

## IMPACT OF SUBCLINICAL HYPOCALCEMIA

Clinical hypocalcemia is recognized as a risk factor for several other negative health events including dystocia, retained placenta, ketosis and mastitis (Curtis et al., 1983). Cows with clinical hypocalcemia have also been shown to produce less milk in early lactation (Rajala-Schultz et al., 1999). Recent research has shown similar negative associations between SCH and poor energy status, infectious disease, and productive and reproductive outcomes.

Cows with SCH have been shown to have higher plasma non-esterified fatty acids prepartum (NEFA), higher β-hydroxybutyrate (BHBA) postpartum and an increase in liver lipid accumulation at 7 and 35 days postpartum indicating that cows with low blood calcium have poorer energy status and excessive body fat mobilization (Martinez et al., 2012; Chamberlin et al., 2013). High prepartum NEFA and postpartum BHBA have been associated with losses in milk production (Ospina et al., 2010). Chapinal et al. (2012) showed that cows in herds with ≥35% incidence of SCH in the week postpartum had increased odds of displaced abomasum as well as a 3.8 kg/d reduction in milk production at the first test day. These data indicate that SCH exacerbates the negative energy balance experienced by the transition cow, resulting in increased susceptibility to other disorders as well as decreased productivity.

Calcium is an essential component for activation of many different cells throughout the body. A crucial role of calcium in the early lactation dairy cow is activation of the immune cells, primarily neutrophils, that are responsible for clearing the uterus of infectious agents after parturition. Kimura (2006) showed that immune cells of hypocalcemic cows had reduced calcium release from intracellular stores upon activation, causing a blunted response and furthering the immunosuppression experienced around the time of parturition (Kehrli et al., 1989). Martinez et al. (2012) also showed an association between hypocalcemia and reduced neutrophil function, as well as an increased risk for uterine disease in hypocalcemic cows compared to cows with normal blood calcium. Ultimately, hypocalcemic cows in this study tended to have delayed pregnancy. At the herd-level, a study conducted on commercial herds found that with higher incidence of SCH in the week postpartum, there was a 30% reduction in pregnancy at first artificial insemination, which occurred in 40% of herds sampled (Chapinal et al., 2012).

Subclinical hypocalcemia has serious implications in regards to the future health of the cow, as well as important economical consequences as a result of reduced milk production and poorer reproductive performance. Strategies for preventing milk fever, such as feeding a diet low or negative in dietary cation anion difference prepartum, are widely implemented, however there is a lack of evidence supporting these strategies as effective in reducing incidence of SCH. The ability to assess the calcium status of fresh cows on the farm will be an important tool to move forward in managing for lower incidence of SCH by providing a means for assessing prevention strategies as well as identification of cows in need of intervention.

## EVALUATION OF SUBCLINICAL HYPOCALCEMIA

Due to the lack of clinical symptoms associated with SCH, the disease can only by identified through the measurement of blood calcium. Blood calcium is present in three forms; ionized calcium and calcium bound to either proteins or anions (Rosol et al., 1995). It is hypothesized that ionized calcium should be a more accurate reflection of the functional calcium status of the cow because this is the form of calcium available to cells to perform intracellular signaling, necessary for such functions as contraction of muscles or activation of immune cells (Kimura et al., 2006).

Traditionally, the cutpoint used to identify SCH has been 8.0 mg/dL (2.0 mmol/L) total calcium, and 4.0 mg/dL (1.0 mmol/L) ionized calcium due to an assumption that total blood calcium is composed of 50% ionized calcium. This relationship is assumed to be fairly stable and therefore any variation caused by bound calcium when measuring total calcium is thought to be negligible. Some evidence suggests that the relationship between total and ionized calcium may be different around the time of parturition due to the rapid increase in mobilization of bone calcium and increased absorption from the diet (Ballantine and Herbein, 1991).

Ionized calcium is seldom used as a measure of calcium status due to challenges with accurate measurement. Exposure of samples to air as well as the use of

anticoagulants changes the amount of ionized calcium in the sample (Boinke et al., 1991). The iSTAT Portable Clinical Analyzer was identified as a tool for accurate measurement of ionized calcium due to the ability to analyze whole blood within minutes of sample collection and without the use of anticoagulants. The on-farm application of this tool is unlikely due to the cost of analysis but the accurate ionized calcium measurement allows for characterization of the ionized versus total calcium relationship.

Blood total calcium is typically measured to assess the calcium status of a cow due to ease of sample handling, storage and analysis. In research, samples are often sent to a diagnostic laboratory for analysis. The value of this method for on farm decision making is minimal due to the long turnaround time for results. Although potentially effective treatment options for SCH are available (Oetzel and Miller, 2012), methods for identifying cows affected with SCH within the time relevant for treatment are not widely implemented. A tool for measuring blood total calcium, the IDEXX VetTest Chemistry Analyzer, was identified as having potential as an on-farm tool because of its ease of use and relatively lower cost of analysis compared to the diagnostic lab.

A study was conducted in our laboratory to assess options for measuring calcium status of cows in the immediate postpartum period. The objectives of the study were to assess the interrelationships between blood minerals measured by the IDEXX VetTest and the reference method, as well as the relationship between ionized and total calcium measured in early postpartum dairy cows, and their relative values as predictors of neutrophil oxidative burst activity.

## EXPERIMENTAL APPROACH

Blood samples were taken from multiparous Holstein cows (n=33) twice in the 24 hours after calving and once daily through 5 days in milk. If cows became clinically hypocalcemia, only samples taken before treatment with intravenous calcium were included in the data set. All blood samples were analyzed for ionized calcium within 3 minutes of sample collection using the iSTAT Portable Clinical Analyzer (iSTAT; Abbot Point of Care, Inc., Princeton, NJ), which measures ionized calcium with an ion selective electrode. The sample was then centrifuged and the serum harvested for analysis of total calcium, magnesium and phosphorous on the IDEXX VetTest Chemistry Analyzer (VetTest; IDEXX Laboratories, Inc., Westbrooke, ME), the remaining serum was sent to the Michigan State Diagnostic Center for Population and Animal Health (DC) for a full mineral panel analysis, both of which use a colorimetric method for determining total calcium concentration. An additional blood sample was taken between 2 and 5 days in milk for flow cytometric determination of neutrophil oxidative burst activity using a commercial kit (Phagoburst, Glycotope GmbH, Berlin, Germany).

Data were analyzed using the statistical software SAS version 9.2 (Cary, NC). Correlations between mineral measurements were determined using PROC CORR. Cutpoints to diagnose SCH (defined as diagnostic lab total calcium ≤8.0 mg/dL) using ionized calcium and total calcium (measured by the VetTest) providing the highest

combined specificity and sensitivity were determined using the receiver operator characteristic. To determine the relative value of ionized versus total calcium as predictors of neutrophil oxidative burst activity, a multivariate model was created using PROC GLM in which the following variables were considered; parity ($2^{nd}$ lactation vs. $3^{rd}$ lactation and greater), calving ease (scale 1-5), block (summer 2013 vs. winter 2014), presence or absence of disease prior to neutrophil assessment (diseases included fever, retained placenta, displaced abomasum, ketosis, mastitis, and were diagnosed by the herd health staff), as well as biologically relevant 2-way interactions. Three models were created; a base model containing only variables significant at a p-value of less than 0.10, the base model plus the minimum total blood calcium, and the base model plus the minimum ionized blood calcium. Models containing calcium parameters were compared to the base model using a Chi-Square test and the ionized calcium model was compared to the total calcium model using the $R^2$ value.

## PERFORMANCE OF A POTENTIAL ON-FARM DIAGNOSTIC TOOL

Measurement of blood minerals by the VetTest were highly correlated with measurements by the DC. Magnesium ($r=0.92$, slope=0.94, $p<0.0001$) and phosphorous ($r=0.95$, slope=0.92, $p<0.0001$) measurements were highly correlated with minimal bias. Measurements of blood total calcium were highly correlated ($r=0.95$, $p<0.0001$), however, a slope of 0.82 indicated slight bias by the VetTest for higher measurements of blood calcium, with greater bias as the calcium content of the sample increased (Figure 1). Using a cutpoint of 8.9 mg/dL, SCH could be diagnosed with a sensitivity and specificity of 89% and 89%, respectively. This indicates that with an adjusted cutpoint, the VetTest has potential to be used on-farm as a tool for reliably diagnosing SCH.

## RELATIONSHIP BETWEEN IONIZED AND TOTAL CALCIUM POSTPARTUM

When all samples collected throughout the 5 day sampling period were considered, the correlation between ionized calcium, measured by the iSTAT, and total calcium, measured by the DC, was strong ($r=0.93$) and the slope indicated that ionized calcium constituted 53% of total calcium. However, this relationship varied throughout the first 5 DIM, with 57% of total calcium being ionized in samples taken in the 24 hours after calving (Figure 2). The cutpoint that diagnosed SCH with the highest combined sensitivity and specificity (95% and 79%, respectively) was 4.68 mg/dL, which differed from the assumed 4.0 mg/dL. This indicates that the dynamics between ionized calcium and total calcium in the day following calving may differ from a cow not experiencing such dramatic changes in calcium metabolism. Using cutpoints for ionized calcium that are based on assumptions of the relationship between ionized and total calcium may not be identifying the same cows as using total calcium for diagnosis of SCH.

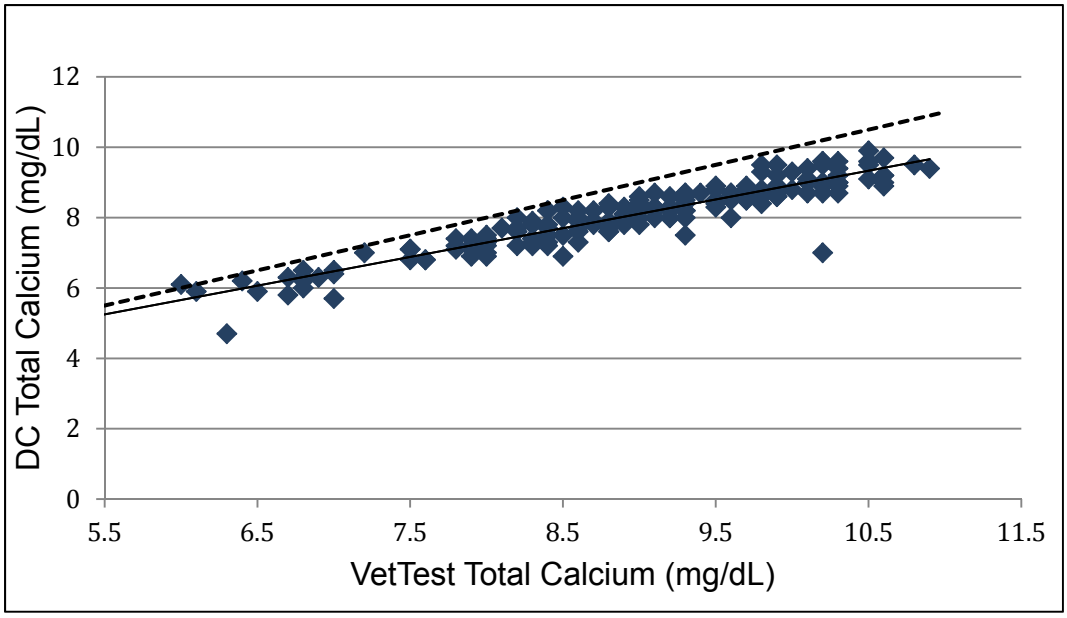


Figure 1. Relationship between total blood calcium measured by the IDEXX VetTest versus the Michigan State Diagnostic Center for Population and Animal Health. The dashed line represents unity.

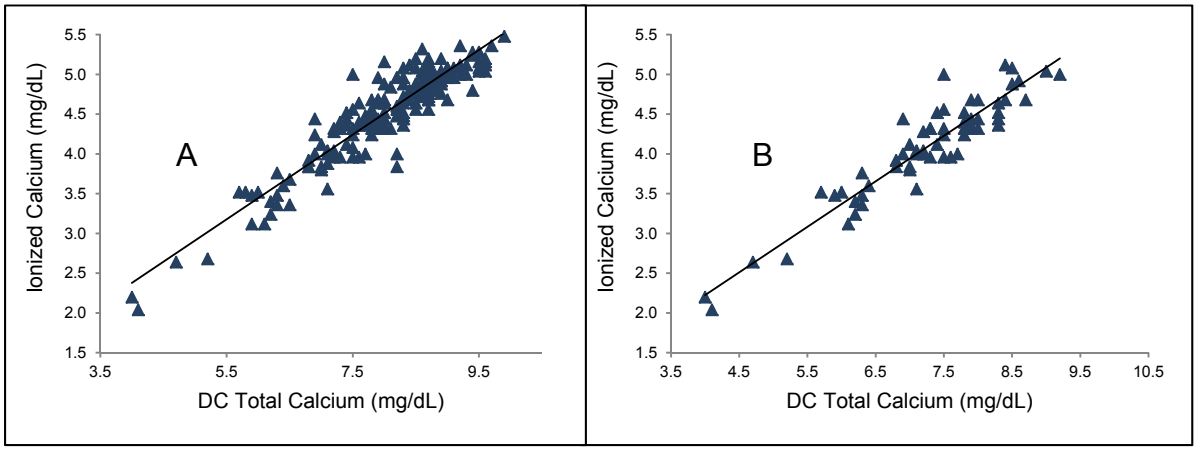


Figure 2. The relationship between ionized calcium and total calcium from samples taken throughout the 5 days after calving (A) and samples taken in the 24 hours after calving (B).

## IONIZED VS. TOTAL CALCIUM AS INDICATORS OF CALCIUM STATUS

The base model for explaining neutrophil oxidative burst activity contained parity, block and presence or absence of disease as explanatory variables. The model was significantly improved when either minimum total calcium ($p<0.05$) or minimum ionized calcium ($p<0.0025$) were added to the model, indicating that calcium status is an important factor in determining the ability of neutrophils to mount an oxidative burst response. The $R^2$ values for the models containing minimum ionized calcium and minimum total calcium were 0.61 and 0.52, respectively. In both models, the calcium

parameter had a significant interaction with diseases status such that cows which had one or more disease prior to measurement of neutrophil function had greater oxidative burst reaction as blood calcium content increased (Figure 3), whereas cows with no disease had no change in oxidative burst response as blood calcium increased. Considering that the model containing minimum total calcium provided the same information as the model containing ionized calcium, the added complication and cost of measuring ionized calcium does not seem to justify slight improvements in explaining this functional outcome related to calcium status.

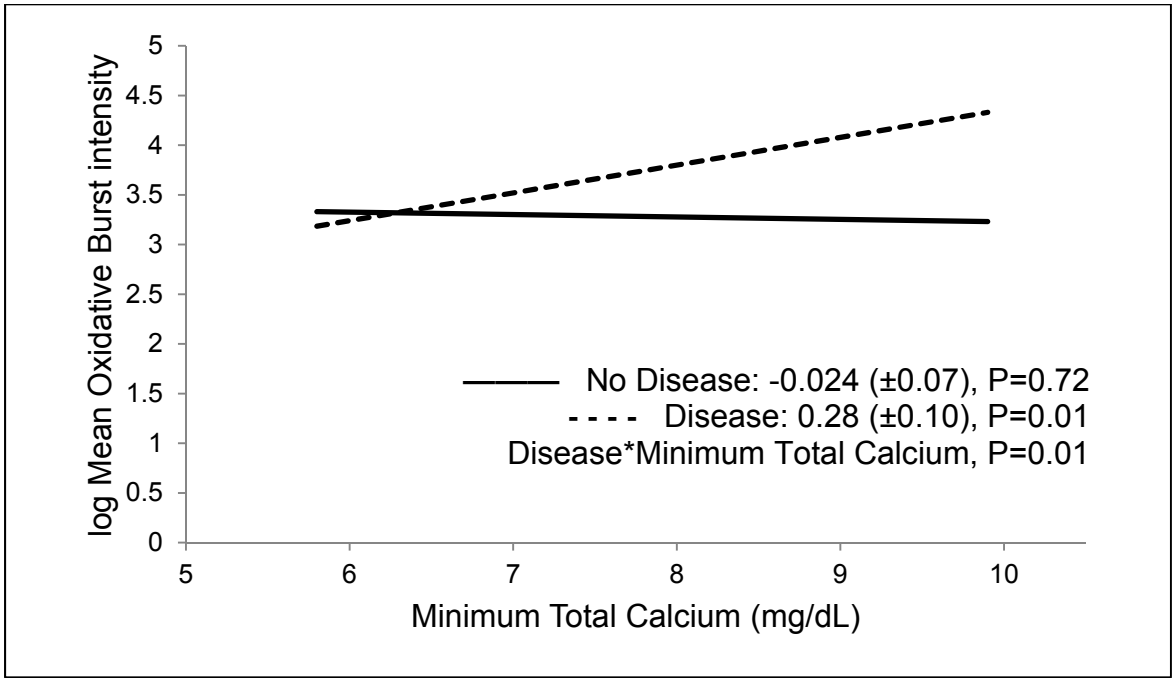


Figure 3. The interaction between disease status and minimum blood total calcium concentration in impacting the neutrophil oxidative burst response. Slope (±SE) is shown for each disease category.

## CONCLUSIONS

The relationship between ionized and total calcium in the immediate postpartum period may differ from the assumed relationship between the parameters, this may cause some discrepancies for diagnosis of SCH depending on the calcium parameter used. Ionized calcium does not show a strong advantage over total calcium as a predictor of neutrophil oxidative burst activity, suggesting that total calcium should be sufficient when measuring the calcium status of early postpartum cows. The IDEXX VetTest presents an opportunity for on farm determination of calcium status, which can allow for rapid diagnosis of SCH for treatment decisions as well as determination of SCH incidence on the farm. Measuring the calcium status of the fresh cow is the first step in making intervention or management decisions in order to decrease the long-term consequences of SCH on a herd.

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