PREDICTIVE MODEL TO ESTIMATE IONIZED CALCIUM FROM ROUTINE SERUM BIOCHEMICAL PROFILES IN DOGS

BY

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THESIS

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ABSTRACT

Ionized calcium is the gold standard to assess calcium status in dogs, but measurement is not readily available in private veterinary practices.

The objectives of this study were to (1) predict ionized calcium concentration from serum biochemical values and (2) compare the diagnostic performance of predicted ionized calcium (piCa) to those of total calcium (tCa) and two corrected tCa formulae; and (3) study the relationship between biochemical values and variation of measured ionized calcium (miCa).

This was a cross-sectional study. Records from 1,200 dogs who were patients at the University of Illinois Veterinary Teaching Hospital were randomly selected from a population of 1,719 dogs with mical and biochemical profile performed within 24 hours for the creation of a multivariate adaptive regression splines (MARS) model, with the final model being determined by backward elimination. Accuract and diagnostic performance of piCal and its prediction interval (PI) were tested on 519 dogs via Bland-Altman analysis, Pearson's R, and receiver operator characteristic (ROC) curves.

The final model included creatinine, albumin, tCa, phosphorus, sodium, potassium, chloride, alkaline phosphatase, triglycerides, and age, with tCa, albumin, and chloride having the highest impact on miCa variation. Predicted ionized calcium was better correlated to miCa than tCa and corrected tCa, and its overall diagnostic accuracy was significantly higher to diagnose hypocalcemia and improved for hypercalcemia. The average difference between the piCal and miCal was 0.002 +/- 0.080 mmol/L. The PI included miCal 94% of the time. For hypercalcemia, piCa was as sensitive (64%) but more specific (99.6%) than tCa and corrected tCa. For hypocalcemia, piCa was more sensitive (21.8%) than tCa, and more specific (98.4%) than

corrected tCa formulae. Positive predictive values of piCa were high for both hypercalcemia (90%) and hypocalcemia (70.8%).

Predicted ionized calcium can be obtained from readily available biochemical and patient variables, and seems more useful than tCa and corrected tCa to approach calcium disorders in dogs when miCa is not available. A webpage has been designed for piCa calculation (http://vetmed.illinois.edu/study/mars-model/VetMed.php)**.**

For my family,

Jon, Sue, Katie, and Brian Danner,

I love you guys!

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CHAPTER 1

INTRODUCTION

Calcium is a critical element in the body, as it promotes structural integrity of the skeletal system and plays an essential role as a messenger or a regulatory ion, both intracellularly and extracellularly (Rosol and Capen 1996; Schenck and Chew 2008b). In dog, the serum total calcium (tCa) is composed of three fractions: ionized, protein-bound, and complexed (to phosphate, bicarbonate, sulfate, citrate, lactate), with each comprising approximately 56%, 35%, and 10% of the serum tCa, respectively (Schenck, Chew, and Brooks 1996). The ionized calcium is the most biologically active fraction, which is responsible for the physiologic functions of calcium within the body and it is also a sensitive indicator of pathologic states when abnormal (Messinger, Windham, and Ward 2009). Ionized calcium measurement is therefore considered the gold-standard for evaluating calcium homeostasis in humans and animals (Schenck et al. 2012).

Ionized calcium in dogs can be assessed quickly and accurately using analyzers with ion-selective electrodes (Unterer et al. 2004) but, unfortunately, many veterinary practices do not maintain this equipment and clinicians often rely instead on the tCa measurement, present on most routine serum biochemistry profiles, to assess calcium status. However, tCa concentration may be poorly correlated with ionized calcium in a number of conditions and therefore inaccurate in reflecting the true calcium status of the animal (Schenck and Chew 2010; Sharp, Kerl, and Mann 2009; Messinger, Windham, and Ward 2009; Schenck and Chew 2005; Kogika et al. 2006). In an effort to improve the accuracy at predicting ionized calcium from tCa values, correction equations have been derived for canine patients (Meuten 1982). These equations

adjust the tCa for either albumin or total protein, on the assumption that a large fraction of tCa is protein-bound. These equations assume that serum tCa concentrations that are adjusted into the reference range are associated with a serum ionized calcium concentration within the reference range and that samples with values that fall outside the reference range after adjustment are associated with abnormal serum ionized calcium concentrations. While initially promising, subsequent studies have shown that adjusting tCa for albumin or total protein does not improve and may even worsen the correlation between tCa and ionized calcium (Schenck and Chew 2005; Sharp, Kerl, and Mann 2009; Messinger, Windham, and Ward 2009; Schenck and Chew 2008a), possibly because the relative proportion of the complexed form of calcium can vary widely amongst patients, particularly those with chronic renal disease, and that the complexed fraction of the tCa is not accounted for in the correction formulas (Kogika et al. 2006). A formula that takes into account this complexed fraction, as well as the protein-bound fraction, would therefore likely be more accurate at determining if the patient has normal calcium homeostasis.

Routine chemistry panels can be performed in the majority of veterinary clinics and laboratories. It is possible that calcium homeostasis could be predicted from tCa more accurately if a formula is created that takes other readily available factors besides albumin and total protein into account. The objectives of this study were to (1) create a model predictive of ionized calcium that integrates only routinely available biochemistry variables so that it could be readily usable to veterinarians, (2) describe the relationships between routinely available biochemistry variables and ionized calcium values, in order to better understand the factors that influence changes in ionized fraction of serum calcium and (3) assess the accuracy of the newly developed model for predicting ionized calcium levels (piCal) relative to three corrected total calcium (tCal) formulae for evaluating calcium disorders.

CHAPTER 2

LITERATURE REVIEW

Physiology of Calcium

Biological Roles of Calcium

Calcium has two distinct physiologic roles within the body: 1) providing structural support of the bones and teeth and 2) acting as a second messenger for a variety of intra- and extracellular processes including nerve conduction, neuromuscular transmission, muscle contraction, maintenance of vascular smooth muscle tone, hormone secretion, bone formation, hepatic glycogen metabolism, cell growth and division, blood coagulation, and membrane transport and stability (Rosol and Capen 1996; Schenck et al. 2012; Brown 1991). Many of these intracellular functions are accomplished through a "second messenger" system of interactions of calcium with intracellular binding proteins or receptors (largely calmodulin but also calbindin and troponin C), which then activate enzymes and other cellular effector systems (Means and Dedman 1980).

Part of the physiological effect of calcium is achieved as a result of the significant difference in the calcium concentration within the cell cytoplasm relative to the extracellular environment. Within the cytosol, there is approximately 100 nmol/L versus approximately 1.2 mmol/L within the extracellular fluid, generating a nearly 10,000 fold concentration gradient (Rosol and Capen 1996). This large electrochemical gradient is the driving force that favors calcium movement from the lumen of the endoplasmic reticulum into the cytosol, which then allows calcium to couple plasmalemmal and cytosolic events with intraluminal formation and transport of proteins, as well as with gene expression within the cell (Verkhratsky 2005). When a

cell is activated, the intracellular free calcium can rise by 10- to 100- fold due to the uptake of extracellular calcium and/or the release of calcium from the cellular stores, such as the endoplasmic reticulum or the mitochondria (Brown 1991).

The levels of activity of cells within the parathyroid glands, kidneys, and thyroid (the major organs responsible for hormonal regulation of calcium within the body via their secretion of parathyroid hormone, calcitriol, and calcitonin, respectively) are strongly influenced by variations in calcium concentrations within the extracellular fluid. This generally occurs via binding of extracellular calcium to membrane-bound calcium-sensing G-protein coupled receptors. These receptors are stimulated by excessive extracellular calcium ions to activate phospholipase C which will then transduce intracellular signals that alter cellular functions; for example, the parathyroid gland will decrease the secretion of the parathyroid hormone in response to excess extracellular calcium (Brown et al. 1995; Rosol and Capen 1996; Hall 2016). Any disruption of calcium metabolism resulting in variations in extracellular calcium levels will therefore alter the metabolic states of these organs.

Calcium Compartments

Body calcium is present both intracellular and extracellular compartments, in either insoluble or soluble forms. Most (approximately 99%) of total body calcium is combined with phosphorus and stored within the skeletal system as insoluable hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ (Schenck et al. 2012; Carafoli 1987). Overall, this form of calcium is necessary for protection of organs, mastication, locomotion, and mineral storage. While bone represents the largest reservoir of calcium, only about 1% can be accessed at any given time because its release is largely dependent on bone turnover. Normal bone turnover is directly related to osteoclast activity,

which is a delayed induction process that can take several weeks to increase (Hall 2016). Rather, the most readily available calcium in the skeletal system is either in the extracellular space between osteoblasts and osteocytes, or release through rapid activation of osteocytes

Extracellular fluid calcium, including serum or plasma, is in the soulable form, and only represents only approximately 0.1% of the body's complete calcium mass. Serum or plasma calcium exists in three subcategories or fractions: ionized calcium (Ca^{2+}), complexed calcium, and protein-bound calcium (Schenck et al. 2012). Ionized calcium represents approximately 56% of the total extracellular calcium, and since it is responsible for all the biologic effects of calcium within the body, it is the most significant form from a physiologic standpoint (Schenck 2007). Complexed calcium normally represents approximately 10% of serum calcium: calcium is generally complexed to and forming ionic salts with citrate, lactate, bicarbonate, or phosphate. However, it is natural that the amount of this fraction can vary widely with disease, depending on the levels of citrate, lactate, bicarbonate, and phosphate within the extracellular compartment. Combined, ionized calcium and complexed calcium can pass through the glomerular capsule and therefore make up the calcium present in the glomerular filtrate. Protein-bound calcium represents approximately 35% of total extracellular calcium and is principally bound to negatively-charged sites on albumin, with much less binding to other proteins such as globulins. There is no known physiologic role of protein-bound calcium, other than being used as a storage pool or buffering system for ionized calcium with alterations in blood pH (Schenck et al. 2012).

Intracellular calcium is stored within the cell cytosol, where it constitutes only approximately 0.00002% of total body calcium or it is associated with the cell membrane or sequestered in organelles, which represents approximately 0.9% of total body calcium. The main function of intracellular calcium is to act as a second messenger in response to signals transduced

from the cell surface. For calcium to operate efficiently and quickly as a second messenger, the amount of the calcium within the cytosol must remain at a very low level, approximately 10,000 fold less than the calcium present in the extracellular fluid. This marked concentration gradient allows for rapid flux of calcium from the extracellular fluid and intracellular organelles (mainly the endoplasmic reticulum or mitochondria) through voltage-gated, store-operated, ligand-operated, and non-selective channels that are located within the cell membrane or through several sets of intracellular calcium channels, including the ryanodine receptors and inositol trisphosphate receptors, in the endomembrane of the endoplasmic reticulum. Additionally mitochondria contain a calcium uniporter which is highly selective for calcium and a complex permeability transition pore which facilitate rapid flux of calcium. In general, in excitable cells such as neurons, the majority of the cytosolic calcium is acquired from activation of pumps within the plasmalemma. In non-excitable cells; cytosolic calcium is derived from intracellular organells, mainly the endoplasmic reticulum and mitochondria (Verkhrastsky 2005). After the necessary effects of the calcium are accomplished, several channels are utilized to decrease cytosolic calcium concentrations back to a resting level. A sustained increase in cytosolic calcium can cause cell toxicity and potentially cell death (Schenck et al. 2012). The cell membrane has calcium specific transporters including a Na^{+}/Ca^{2+} exchanger and a calcium pump (plasmalemmal Ca^{2+} ATP-ase), the endomembrane contains a sarcoendoplasmic reticular calcium ATPase, and several other channels are present on additional organelles; all work to decrease cytosolic calcium. In addition, calcium ions are rapidly buffered by intracellular calcium binding proteins within the cytosol (Verkhratsky 2007).

Calcium Balance and Hormonal Control

Regulation of calcium homeostasis within the body is accomplished by three different endocrine hormones acting on three different target organs. The primary hormones of control of calcium homeostasis are parathyroid hormone, calcitonin, and 1,25-dihydroxycholecalciferol (calcitriol) which act either directly or indirectly on the gastrointestinal tract, kidney, or bones (Peacock 2010). In addition to these three primary hormones, other hormones such as corticosteroids, prolactin, estrogens, thyroxine, and glucagon are important in calcium homeostasis during specific disease states, growth, or lactation (Schenck et al. 2012).

Overall, the function of hormones are to maintain a normal and steady concentration of ionized calcium within the body, mainly through regulation of intestinal and renal calcium absorption of excretion. Renal nephrons are directly stimulated by parathyroid hormone to reabsorb calcium, mainly in the late distal tubules, but also in the collecting tubules, early collecting ducts, and the ascending loop of Henle. The kidney has a large capacity to conserve calcium, as more than 98% of the filtered load of calcium is reabsorbed under the influence of parathyroid hormone. Approximately 50-60% of calcium is reabsorbed at the level of the proximal convoluted tubules via passive paracellular transport, 15% in the thick ascending limb by both passive paracellular and active mechanisms, and the remaining 10-15% is in the distal convoluted tubule by an active transcellular mechanism (Craven, Passman, and Assimos 2008). In addition, the kidneys are the site of synthesis of calcitriol from calcidiol (25 hydroxycholecalciferol) via activation of 1-alpha hydroxylase by parathyroid hormone. Calcitriol then acts on the gastrointestinal tract to increase absorption of both calcium and phosphorus. Parathyroid hormone also causes the kidney kidney to increase the excretion of phosphorus at the

proximal tubules. In contrast, calcitonin has a minimal effect on renal handling of calcium and phosphorus (Hall 2010).

The gastrointestinal tract typically absorbs approximately 35% of ingested calcium with the remaining calcium being lost in the feces (Hall 2010). Overall, the stomach has little impact on calcium absorption: the majority is absorbed in the ileum, with lesser amounts in the jejunum, duodenum, and colon (Cramer 1965). Intestinal calcium absorption occurs via three different mechanisms: active transcellular transport in the duodenum, nonsaturable paracellular transport, and vesicular transport primarily in the duodenum (Craven, Passman, and Assimos 2008). All three mechanisms are under the influence of calcitriol. In addition to dietary sources, intestinal calcium absorption can come from sloughed gastrointestinal mucosal cells and gastrointestinal secretions can also be absorbed. A variety of nonhormonal factors can also affect calcium absorption from the gastrointestinal tract, including the pH of the gastrointestinal contents, function of the villi, medications, and intestinal diseases. Calcitonin has only minor effects on the gastrointestinal tract (Schenck et al. 2012).

Typically, utilization of calcium stores within bone to increase serum calcium levels occurs only when there are significant total body deficits in calcium and when the kidneys and gastrointestinal tract under the influence of parathyroid hormone and calcitriol cannot meet the demands for calcium. For immediate response to hypocalcemia, extracellular calcium located between osteocytes within bone is available to be used emergently to help increase ionized calcium. This exchangeable calcium is elsewhere in the body, such as in the liver and gastrointestinal cells. This pool is small (0.4-1% of the total bone calcium) and depletion occurs rapidly. For longer-term calcium homeostasis, calcium can either be deposited into the bone via osteoblasts, or resorbed from the bones by osteoclasts. Calcitonin from the thyroid C cells

functions to trigger deposition of calcium into bone during periods of hypercalcemia (Schenck and Chew 2003a). Resorption of bone is due to the influence of parathyroid hormone and calcitriol via activation of osteoclasts. This is not through direct mechanisms as osteoclasts lack receptors for parathyroid hormone. Instead, parathyroid hormone binds to receptors on adjacent osteoblasts, which in turn signal to osteoclasts to mature and activate. When activated, osteoclasts form villus projections toward the bone matrix that secrete proteolytic enzymes from lysosomes as well as citric and lactic acid from the mitochondria and secretory vesicles of the osteoclasts. The enzymes digest the organic matrix of bone and the acids cause dissolution of bone salts to release calcium (Hall 2016).

Both calcitriol and parathyroid hormone can increase bone and gastrointestinal resorption of both calcium and phosphorus. That being said, the phosphaturic effects of parathyroid hormone on the kidney far exceed the amount of phosphorus that is absorbed from the gastrointestinal tract or released from the bones; therefore, the net result of parathyroid hormone action is to decrease phosphorus levels, thus preventing calcium from complexing with phosphorus which would prevent increases in ionized calcium. Once extracellular calcium concentration increases to a normal level, negative feedback reduces the secretion of parathyroid hormone, reduces production of calcitriol, and decreases osteoclastogenesis and osteoclastic bone resorption.

Calcium Regulating Hormones

Parathyroid Hormone

Parathyroid hormone (PTH) is synthesized, secreted, and degraded by the chief cells of the parathyroid gland, and the major stimulus for PTH secretion is a reduction in serum ionized

calcium. When needed, PTH is synthesized de novo from new messenger RNA with subsequent translation, as little PTH is actually stored within the parathyroid glands. After translation, PTH is present as preproPTH, which is 115 amino acids in length. A 25-amino acid sequence is cleaved from the amino terminus in the endoplasmic reticulum, creating proPTH, leaving a 90 amino acid peptide. Finally, proPTH is moved to the Golgi apparatus where a 6-residue sequence is removed, creating PTH with a length of 84 amino acids as a single chain polypeptide. Circulating PTH has a half-life of 3-5 minutes, so constant synthesis may be required.

The parathyroid glands respond controlled mainly to calcitriol and the extracellular ionized calcium concentration, but are also be influenced either directly or indirectly by extracellular magnesium and phosphorus concentrations (Silver, Kilav, and Naveh-Many 2002). Calcitriol binds to vitamin D receptors on parathyroid cell membranes, and ionized calcium binds to a plasmalemmal calcium receptor. Calcitriol is responsible for expression of the calcium receptor, so it is said to have complete control PTH secretion by the parathyroid glands. Parathyroid secretion is constant but can have pulsatile changes in response to fluctuations in the serum ionized calcium as PTH works efficiently to maintain a steady ionized calcium concentration. During normocalcemia, PTH is secreted but only at 25% of the maximal rate and, even during severe hypercalcemia, PTH continues to be secreted (Kronenberg, Bringhurst, and Segre 2017).

Overall, PTH is responsible for minute-to-minute control of calcium concentration via direct action on bone and kidney, and by indirectly influencing calcium absorption from the gastrointestinal tract (Schenck et al. 2012). The function of PTH is to increase the blood calcium concentration via three mechanisms: 1) increase renal tubular reabsorption of calcium thus decreasing urinary excretion, 2) increasing bone resorption, and 3) increasing formation of active

vitamin D (calcitriol) by activating 1 alpha-hydroxylase in the renal proximal tubular epithelial cells. After secretion, the N-terminal region of PTH binds to a seven-transmembrane domain receptor (PTH/PTHrp receptor) that is present on renal epithelial cells and osteoblasts. The PTH/PTHrp receptor is associated with hypercalcemia of malignancy rather than calcium homeostasis. It binds PTH and PTH-related peptide (PTHrp) with equal affinity and in neoplastic cells, such as colorectal carcinoma, prostate cancer, renal cell carcinoma, and osteosarcoma in people (Lupp et al. 2010), and most commonly lymphoma and apocrine gland anal sac adenocarcinoma in dogs. In addition to increasing blood calcium concentrations, PTH also decreases blood phosphorus concentrations by stimulating phosphaturia via removal, internalization, and degradation of Na/Phosphorus-IIa and IIc cotransporters in the renal proximal tubular cells (Michiko, Shimizu, and Fukumoto 2016, Hori et al. 2010).

Following secretion and circulation, the majority of intact PTH is cleared from the circulation principally by hepatic macrophages. In addition, the renal and skeletal systems participate in the degradation of intact PTH: PTH fragments are created and subsequently filtered through the glomerulus. The carboxyl-terminal fragments (C fragments) of PTH, can only be removed from the circulation via glomerular filtration; therefore, removal is directly related to the glomerular filtration rate, which results in them having a much longer half-life than intact PTH. The blood conentrations of C-terminal-fragments can therefore be increased in patients with renal disease, in which renal clearance is reduced, or in patients that have increased secretion of PTH, such as is the case in primary, secondary, and tertiary hyperparathyroidism (Donadio et al. 2007). While C-fragments have no biological role in calcium homeostasis, they can interfere with the correct interpretation of diagnostic PTH immunoassay results by cross-reacting with anti-PTH test antibodies (Endres et al. 1989).

Inhibition of PTH synthesis and secretion occurs in several ways. First, elevated ionized calcium will inhibit PTH secretion from the parathyroid gland within minutes, mediated by the calcium receptor on the parathyroid cell surface. During hypercalcemia, over 90% of PTH is degraded within the parathyroid gland (Silver, Kilav, and Naveh-Many 2002). Over a longer period of time, an elevated calcium will also decrease the synthesis of PTH mRNA and the subsequent translation to preproPTH by inhibiting the production of cyclic AMP within the chief cells, which is required to stimulate phosphorylation of transcription factors (Nagode, Chew, and Podell 1996). Calcitriol also plays a role in inhibiting PTH synthesis and secretion via two negative feedback loops (Carnevale et al. 2010). The shorter negative feedback loop is directly mediated by calcitriol binding to the vitamin D receptor (VDR) in the parathyroid gland. After the VDR and calcitriol complex together, it binds to vitamin D response elements to inhibit transcription of PTH. A longer negative feedback loop is a consequence of calcitriol increasing increasing intestinal calcium absorption, which results in the inhibition of PTH synthesis and secretion. One last inhibitor of PTH secretion is fibroblast growth factor-23 (FGF-23). FGF-23 is an amino acid protein that is synthesized and secreted by osteocytes and osteoblasts. The main effects are to decrease serum phosphorus by increasing renal excretion and decreasing gastrointestinal absorption via calcitriol inhibition (de Brito Galvao et al. 2013; Liu and Quarles 2007). In addition, FGF-23 directly affects PTH synthesis by decreasing gene expression and secretion via activation of the mitogen-activated protein kinase pathway in the chief cells (Galitzer et al. 2008).

Besides its role in regulating calcium homeostasis, PTH has been shown to have cause many deleterious effects when its secretion is chronically increased, as for example with chronic kidney disease (de Brito Galvao et al. 2013). These include acting as an uremic toxin, affecting

cardiac muscle contractility, promoting atherosclerosis and vascular calcification, and potentiating cardiac fibrosis and hypertension, ultimately leading to increased morbidity and mortality in both human and veterinary patients (Björkman, Sorva, and Tilvis 2008; Shalhoub et al. 2012).

Parathyroid Hormone-Related Peptide

Parathyroid hormone-related peptide (PTHrp) is a polyhormone that plays a pivotal role in the development of hypercalcemia of malignancy (HHM) in some neoplasias, but is also important in fetal development, lactation, and adult animals with no evidence of neoplasia. The N-terminal portions of PTHrp and PTH have 70% sequence homology over the first 13 amino acids which is responsible for both peptides having a similar binding affinity to PTH receptors in bone and kidney. In the fetus, PTHrp is synthesized by the placenta and acts as an endocrine and paracrine hormone to increase fetal parathyroid gland development and bone formation. PTHrp is also produced by mammary tissues during lactation and is likely present to facilitate calcium mobilization from bone to meet the high demands of calcium during the lactation period. In healthy adults, PTHrp has a paracrine function on the skin, muscle, lymphoid tissues, kidney, bone, and brain. In HHM, PTHrp acts as an abnormal hormone, mimicking the effects of PTH.

Calcitriol

Synthesis and secretion of calcitriol requires interaction of three different organ systems. While metabolism of calcitriol (1,25-dihydroxyvitamin D3) is similar between people and veterinary patients, the source of vitamin D is different. In people, vitamin D can either be absorbed from the gastrointestinal tract as vitamin D3 (cholecalciferol) or vitamin D2 (ergocalciferol), Vitamin D3 can also be synthesized in the skin from ultraviolet light exposure.

In dogs and cats, there is insufficient synthesis of vitamin D3 in the skin; therefore, the primary source of vitamin D3 in these species is absorption from the gastrointestinal tract. After absorption, vitamin D3 is transported to the liver via vitamin-D transport proteins, where it is subsequently hydroxylated by 25-hydroxylase to form calcidiol (25-hydroxyvitamin D3). This process occurs independent of any influence of calcium, phosphorus, or PTH. During times of excessive vitamin D3 ingestion, it can be stored in adipose tissue and released continuously to be converted to calcidiol (Heaney et al. 2008). However, calcidiol can be converted to calcitriol within the proximal tubules of the kidney via 1-alpha hydroxylase. Calcitriol is the most biologically active form of vitamin D and has the highest affinity for vitamin-D receptors in target cells. Also present in the kidney is 24-hydroxylase which transforms calcidiol to 24,25-dihydroxyvitamin D3, which is an inactive form of vitamin D and is eventually excreted in the feces.

Calcitriol synthesis is controlled by PTH, phosphorus, calcium, calcitonin, FGF-23, calcitriol itself, and other hormones such as testosterone, estrogens, growth hormone, and prolactin. Phosphorus and calcium can directly influence calcitriol synthesis independent of the presence of PTH but, in the face of severe hypocalcemia, will additionally increase calcitriol indirectly via the activation and secretion of PTH. Hypophosphatemia will increase calcitriol as will low levels of circulating calcitriol. Calcitriol synthesis is decreased when hyperphosphatemia, hypercalcemia, elevated calcitriol, or increased FGF-23 is present. Again, hypercalcemia does this both directly and indirectly due to the negative feedback hypercalcemia has on the parathyroid glands to decrease PTH secretion.

Calcitriol has effects on bone, the gastrointestinal tract, kidneys, and parathyroid glands. The largest effect of calcitriol is on the gastrointestinal tract, where it increases the absorption of

calcium and phosphorus by several mechanisms: increasing brush border permeability to calcium, removing calcium and phosphorus from enterocytes, and stimulating rapid calcium transport across the cell. The rapid and most efficient calcium absorption occurs in the duodenum, with a steady decrease in efficiency along the gastrointestinal tract to the level of the ileum. In bone, calcitriol is important for bone formation and mineralization, as it provides a source of calcium and phosphorus, but is also necessary for normal bone resorption. During states of hypocalcemia, calcitriol alone is a weak stimulus for bone resorption but acts synergistically with PTH. In the kidney, calcitriol has several effects including increasing calcium and phosphorus reabsorption and providing negative feedback to 1-alpha hydroxylase during times of increased calcitriol. Finally, calcitriol has many effects on the parathyroid gland by both directly and indirectly decreasing PTH secretion, and calcitriol is necessary for PTH synthesis and secretion as it is the primary controller of PTH transcription.

Calcitonin

Synthesis and secretion of calcitonin occurs in the C cells (parafollicular cells) of the thyroid gland, which are present in between the thyroid follicles. It is a peptide hormone consisting of 32 amino acids whose main function is decreasing serum calcium concentration within the body. The overall effects of calcitonin are minimal compared to that of calcitriol and PTH. The primary effects of calcitonin are on the bone metabolism, with minor effects on the renal and gastrointestinal systems unless administered at supraphysiologic doses. In bone, calcitonin decreases the absorptive capacity of osteoclasts, which is an immediate effect, and also decreases the overall formation of osteoclasts, which is a prolonged effect, to decrease serum calcium concentrations.

Disorders Causing Hypercalcemia

Hypercalcemia is a relatively uncommon electrolyte abnormality in dogs but is of great importance when it is present (Hall 2016). Unlike most electrolyte abnormalities, even mild elevations in serum calcium can cause marked clinical signs and hypercalcemia can also serve as a marker for certain disease processes. Common clinical signs of hypercalcemia include polyuria, polydipsia, anorexia, lethargy, and weakness. In some cases, hypercalcemia is identified on bloodwork but the patient is asymptomatic.

In general, the causes of hypercalcemia can be grouped into three categories: non-pathologic, transient/inconsequential, or pathologic/consequential. Non-pathologic causes of hypercalcemia include post-prandial sampling, sample handling error, a spurious result due to lipemia or detergent contamination, or a sample from a young patient. Transient causes of hypercalcemia include severe hypothermia, hyperproteinemia, hypoadrenocorticism, and hemoconcentration. These calcium abnormalities are typically mild with the patient exhibiting no clinical signs of hypercalcemia. Hypercalcemia is common dogs with hypoadrenocorticism, being present in up to 45% of cases, and is thought to be due to severe volume depletion and decreased glomerular filtration rate, but can also be caused by metabolic acidosis which can lead to hypercalcemia by reducing protein binding (Schenck et al. 2012). In hypoadrenocorticism, the hypercalcemia resolves with fluid administration and appropriate treatment for the disease.

There are several causes of persistent hypercalcemia. Primary hyperparathyroidism is one common cause of hypercalcemia, and can occur due to an adenoma, adenocarcinoma, or hyperplasia of one or more of the parathyroid glands. Malignancy-associated hypercalcemia is common is veterinary patients and is the most common cause of hypercalcemia in dogs,

accounting for up to 2/3 of cases (Messinger, Windham, and Ward, Holowaychuk et al. 2009; Holowaychuk and Martin 2007; Bergman 2012). Malignancy-associated hypercalcemia can be due to hypersecretion of PTHrp by neoplastic cells, such as lymphoma, anal sac apocrine gland adenocarcinoma, thymoma, and other carcinomas (squamous cell carcinoma, mammary carcinoma, thyroid carcinoma) or due to bone marrow osteolysis or local osteolysis secondary to hematologic malignancies, such as lymphoma, multiple myeloma, leukemia, or metastatic/primary bone neoplasia. In addition to neoplasia, other skeletal diseases, including osteomyelitis and hypertrophic osteodystrophy, can cause hypercalcemia. Causes of vitamin D-associated hypercalcemia include vitamin D intoxication (iatrogenic, rodenticide, plants) and fungal diseases (associated with calcitriol synthesis from activated macrophages during inflammation). Renal failure (acute or chronic) can cause hypercalcemia, which is typically due to secondary or tertiary hyperparathyroidism, but can also be due to excessive calcitriol or calcium-carbonate intestinal phosphate binder administration. Other less common causes of hypercalcemia include hypervitaminosis A, grape/raisin intoxication, retained fetuses, and excessive calcium intake.

Due to the wide variety of diseases that can cause hypercalcemia and the fact that neoplasia is a common cause, it is imperative to thoroughly evaluate a patient when persistent hypercalcemia is detected. A complete and thorough physical examination is required, and all lymph nodes should be palpated and a rectal examination performed. A serum chemistry panel, complete blood count, urinalysis, and a venous blood gas can all be good initial first steps in assessing hypercalcemia. Thoracic and abdominal radiographs should also be performed to evaluate for metastatic or mass lesions. If the cause of the hypercalcemia is not initially clear, further diagnostics, including measurement of PTH, PTHrp, and potentially vitamin D

metabolites (calcitriol and calcidiol), are indicated. Additional testing may include abdominal and cervical ultrasound to assess the parathyroid glands, fungal disease testing, aspiration/biopsy of any abnormal organs or bone lesions, and, potentially, bone marrow aspirate or core biopsy.

The prognosis for curing hypercalcemia is highly dependent on the underlying cause and whether it can be definitively treated, such as through removal of a functional parathyroid nodule in primary hyperparathyroidism. In addition, there is no specific level of calcium concentration that defines a need for supportive therapy and the need for emergency treatment of hypercalcemia is highly dependent on the patient and the clinical signs that are being observed. The goal of therapy is to decrease these symptoms of hypercalcemia and to prevent mineralization and organ dysfunction, particularly renal dysfunction, which can lead to death. Supportive therapy can include fluid therapy, diuretics, calcitonin, glucocorticoids, and bisphosphonates. The fluid of choice for treating hypercalcemia is 0.9% sodium chloride, which promotes calciuresis through increased competition for calcium reabsorption with sodium in the proximal renal tubules, as well as inducing increased glomerular filtration rate that augments the filtered load of calcium. If 0.9% sodium chloride is unavailable, lactated Ringer's solution is a valid option but it does contain and therefore contribute some calcium. That being said, pure volume expansion is likely the most important factor determining the benefit of fluid therapy; therefore, any balanced electrolyte solution can be considered in an emergency situation. Furosemide is the diuretic of choice for hypercalcemia and can be used in moderate to severe hypercalcemia after volume expansion with intravenous fluid therapy. Furosemide causes calciuresis by blocking the Na/K+/2Cl- in the thick ascending loop of Henle, which subsequently decreases calcium reabsorption. Furosemide is readily available to most practitioners. Thiazide diuretics should never be considered as a treatment for hypercalcemia, as they can enhance the

reabsorption of calcium in the distal tubules, thus worsening hypercalcemia. Glucocorticoids have a variety of effects, including inhibiting bone resorption, reducing intestinal calcium absorption, and increasing renal calcium excretion, that counter hyperglycemia but their use is usually restricted to treatment of hypercalcemia associated with neoplasia, Addison's disease, fungal disease, or hypervitaminosis D (Ettinger 1975; Lifton, King, and Zerbe 1996). Due to the high prevalence of lymphoma in patients with hypercalcemia, it is ideal to wait on utilizing glucocorticoids for treatment of hypercalcemia until a diagnosis is confirmed, as they can complicate diagnosis and treatment of lymphoma. Calcitonin and bisphosphonates are typically reserved for severe hypercalcemia, and calcitonin specifically is best utilized for treatment of hypervitaminosis D associated with rodenticide toxicity. Both medications decrease bone resorption by decreasing the activity of osteoclasts.

Disorders Causing Hypocalcemia

As opposed to hypercalcemia, hypocalcemia is a relatively common finding in veterinary patients, and ionized hypocalcemia has been found to be present in up to 31% of ill dogs (Schenck and Chew 2005). Causes of hypocalcemia include hypoalbuminemia, renal failure (acute and chronic), puerperal tetany, acute pancreatitis, hypoparathyroidism, ethylene glycol toxicity, intestinal malabsorptive conditions, nutritional deficits, tumor lysis syndrome, hypomagnesemia, laboratory error, sepsis (Holowaychuk and Martin 2007) and a variety of other critical illnesses. The clinical signs associated with hypocalcemia are generally related to an increased excitability of neuromuscular tissue, and the severity of clinical signs typically correlate well with the magnitude of hypocalcemia. Common clinical signs include seizures, muscle tremors/fasciculations, facial rubbing/pruritus, muscle cramping, behavior change, and

gastrointestinal signs. Less commonly, patients can have increased thirst/urination, panting, tachycardia, hypotension, respiratory arrest and death.

Hypoalbuminemia accounts for approximately 50% of cases of hypocalcemia, though the hypocalcemia is generally mild and clinical signs are typically not observed. If the underlying cause of the hypoalbuminemia is corrected, the hypocalcemia will resolve. Hypocalcemia with renal failure is more common than hypercalcemia and can occur from a variety of mechanisms. The kidneys are the site of calcitriol synthesis and, with reduced calcitriol synthesis due to renal dysfunction, calcitriol is unavailable to increase absorption of calcium and phosphorus from the gastrointestinal tract and the kidneys. Phosphorus is typically elevated in patients with renal disease and will complex with available calcium, decreasing the amount of ionized calcium. Subsequently, the combination of decreased calcitriol and decreased ionized calcium will increase PTH cause renal secondary hyperparathyroidism.

Puerperal tetany usually occurs 1-3 weeks post-partum and hypocalcemia is caused by increased calcium loss through lactation and fetal mineralization, and may be exacerbated by a calcium deficient diet. However, this condition is more commonly seen in bitches that have been fed high calcium foods prior to whelping, which through negative feedback decreases PTH secretion; thus, when demands for calcium during lactation rise exponentially, there is a lag between hypocalcemia and the response of increased PTH synthesis and secretion. Hypocalcemia is observed in some cases of acute pancreatitis and is hypothesized to be due to saponification of peripancreatic fat, but may also be due to increased calcitonin and increased PTH resistance or deficits. Gastrointestinal malabsorptive disorders may cause of hypocalcemia and can occur due to inability to absorb vitamin D. Intestinal malabsorption of magnesium may result in concurrent hypomagnesium, which, when severe, can cause decreased PTH secretion,

increased end-organ resistance to PTH, and decrease calcitriol synthesis (Schenck et al. 2012). Patients with severe gastrointestinal disease may develop nutritional secondary hyperparathyroidism, which can also be seen in healthy dogs fed diets high in phosphorus and low in calcium.

Hypoparathyroidism is an uncommon endocrine disorder in dogs, and can occur due to idiopathic chronic inflammation of the parathyroid tissue, sudden removal of a source of hypercalcemia, or iatrogenic surgical removal of the parathyroid glands. Diagnosis of primary hypoparathyroidism is based on the observation of a low blood ionized calcium and PTH concentration, generally in conjunction with an increased blood phosphorus and decreased calcitriol concentration.

Hypocalcemia has been identified in critically ill human and veterinary patients. Up to 88% of critically ill humans, specifically patients with sepsis, have been found to have significant hypocalcemia that can also be associated with severity of illness (Zivin et al. 2001). This association of hypocalcemia with critical illness has also been demonstrated in veterinary patients, and a lower blood ionized calcium for a prolonged period of time has been associated with a poorer outcome with decreased survival and longer hospitalization (Luschini, Fletcher, and Schoeffler 2010; Holowaychuk et al. 2009). In dogs, sepsis, renal failure, diabetic ketoacidosis, and pancreatitis were the severe illnesses most commonly associated with hypocalcemia, but hypocalcemia has also been detected in cases of trauma (Luschini, Fletcher, and Schoeffler 2010). The causes of hypocalcemia in these disease is unknown, but has been postulated to be due to a variety of mechanisms including intracellular calcium accumulation, altered function of the parathyroid gland, altered vitamin D concentration or activity, renal loss

of calcium, increased pro-inflammatory cytokines, and increased calcitonin (Wills, Bohn, and Martin 2005).

Determining the etiology of hypocalcemia can typically be determined from a thorough history including diet, serum chemistry panels, PTH, and vitamin D metabolite measurement. PTH is typically be increased in all diseases associated with hypocalcemia except primary hypoparathyroidism. If a patient is hypoalbuminemic, blood ionized calcium concentration offers the best diagnostic accuracy, and, in the case of hypoalbuminemia, the ionized calcium is often normal. In addition, evaluation of the serum phosphorus concentration can be useful in determining the etiology of hypocalcemia, as it is usually decreased in paraneroplastic syndromes and hypoalbuminemia, and is normal or increased in conditions such as hypoparathyroidism, ethylene glycol toxicosis, and renal disease.

Treatment of hypocalcemia is dependent on the degree to which the calcium concentration is of decreased, as well as the patient's clinical signs. Increasing the calcium is usually accomplished with a combination of parenteral calcium supplementation with calcium gluconate or calcium chloride, as well as administration of oral calcium (calcium carbonate, calcium lactate, calcium chloride, and calcium gluconate), calcitriol, ergocalciferol, and potentially exogenous PTH administration. Treatment of primary hyperparathyroidism in involves surgical removal of the parathyroid gland adenoma, which results in a post-surgical hypocalcemia in up to 50% of dogs 3-6 days after surgery. Post-surgical hypocalcemia is a sequela of parathyroid gland atrophy, which arises secondary to the chronic state of hypercalcemia imposed by excessive and prolonged blood PTH concentration from the functional adenoma. The condition is most likely to occur in patients with a serum total calcium concentration of >14 g/dL. Preoperative treatment with calcitriol shortly before surgery (1-2

days) can help prevent hypocalcemia, and, post-operatively, calcitriol can be gradually tapered over time.

Calcium Measurement

Total Calcium

Total blood calcium is routinely available on most chemistry analyzer machines used in veterinary clinical medicine, and therefore is usually the first calcium value observed as abnormal in veterinary patients. This measurement includes all three circulating fractions: protein bound, ionized, and complexed. Typically, total calcium is measured on a fasting sample of serum or heparinized plasma, and then subsequently evaluated clinically using colorimetric spectrophotometry or atomic absorption spectrometry methodologies. A more accurate method is isotope dilution with mass spectrometry, but this method is not routinely available, and the previously mentioned methods are typically adequate for measuring total calcium concentration.

As might be expected, colorimetric test methods are susceptible to interference from substances in the blood and plasma that absorb or reflect colors of a similar wavelength to the test substrates and products. Hemoglobin in a sample with hemoloysis can falsely increase the measured calcium concentration through formation of a hemoglobin-chromogen complex that interferes with the analysis. Increased bilirubin concentrations can falsely decrease the measured calcium concentration. Lipoproteins creating lipemia within a sample can cause significant abberations in the measured calcium concentrations. In addition, inappropriate handling or dilution of a sample can also create abnormalities in the calcium level. Oxalate, citrate, and ethylenediaminetetraacetic acid (EDTA) anticoagulation tubes should not be used for blood

collection, as calcium can become bound to such chemicals and therefore falsely lower the measurement (Bowen and Remaley 2014).

Serum total calcium has been shown to be inaccurate in correctly identifying ionized hypocalcemia, hypercalcemia, and normocalcemia in a variety of clinical settings, particularly in patients with renal disease, with up to 36% of dogs having improper calcium classification when using their total calcium (Schenck and Chew 2005; Kogika et al. 2006; Schenck and Chew 2010). For example, when using total blood calcium measurements as a means of assessing calcium homeostasis in a large population of dogs with chronic renal failure, and verified by the actual measurement of the ionized calcium concentration, the percent of dogs that were hypercalcemic was greatly overestimated, and the percent of dogs determined to be hypocalcemic or normocalcemic was found to be underestimated. This is similar to what has been described in people with chronic renal failure (Burritt, Pierides , and Offord 1980). Most diagnostic discordance is likely due to variations in patients protein-bound or complexed calcium fractions as demonstrated in dogs with chronic renal failure, in which complexed calcium as well as the protein-bound form were found to be highly variable and therefore likely impact the total calcium measurement (Schenck and Chew 2003a). In addition to these findings, a study evaluating canine patients that had received fresh frozen plasma transfusions that contained the anticoagulant, citrate, found an increase in the total calcium, but a decrease in the ionized calcium that was attributed to increased calcium complex formation (Mischke 1996). Overall, such findings support the abandonment of the use of blood total calcium to diagnose disorders of calcium homeostasis should be abandoned in favor of ionized calcium.

In addition to renal disease, alterations in albumin, age, diet, and the fasting state may also impact the apparent serum total calcium concentration. A positive linear correlation between

albumin and total calcium has been documented in humans, dogs, cats, horses, and cattle (Bienzle, Jacobs, and Lumsden 1993). The association between albumin and total calcium concentration is due to the need to maintain physiologic concentrations of blood ionized calcium in the face of increased albumin-calcium binding. With regard to age, young, growing dogs less than one year of age are most likely to have an increased serum total calcium due to the effects of increased growth hormone synthesis and active bone growth. Diets that are high in fat or a lack of fasting may also impact serum total calcium measurements by causing gross lipemia of the sample.

Corrected Calcium Equations

As alluded to above, the relationship between blood albumin and total protein to serum total calcium has been studied in both humans and dogs, and a positive linear correlation between total calcium and albumin, as well as total calcium and total protein, is well known to exist. Because calcium status is inaccurately assessed by total calcium measurements in patients with hypoalbuminemia or hyperalbuminemia due to the large amount of calcium that is bound to protein, correction formulae were subsequently created for dogs using linear regression (Meuten 1982). These correction formulae were created to help increase the diagnostic accuracy of total calcium in patients with abnormal serum protein levels: if the total calcium "corrected" into the normal range after taking into account the degree of hypoalbuminemia or hyperalbuminemia, the patient then was more likely to not have an issue maintaining proper calcium homeostasis. The commonly used corrected calcium formulae proposed for use in dogs are as follows:

1. Total Calcium (mg/dL) – albumin (g/dL) + 3.5
2. Total Calcium (mmol/L) – [0.025 x albumin (g/dl)] + 0.0875

3. Total Calcium (mg/dL) – [0.4 x Total Protein (g/dL)] + 3.3
4. Total Calcium (mmol/L) – [0.1 x Total Protein (g/L)] + 0.825

After application of the correction formulas to patients with hypoalbuminemia, 91% of dogs had a serum calcium concentration that was then within the normal range. These formulae were created before the development of ion-selective electrodes to measure ionized calcium and could they not be accurately validated. Since measuring serum ionized calcium has been validated, these formulae have been tested in a variety of settings and have globally been shown to be inaccurate and potentially even worse at predicting calcium homeostasis than if total calcium was assessed alone in both dogs and people (Crews et al. 2007; Holowaychuk 2013; Sharp, Kerl, and Mann 2009; Schenck and Chew 2005; Slomp et al. 2003; Bienzle, Jacobs, and Lumsden 1993; Clase et al. 2000; Messinger, Windham, and Ward 2009; Jafri, Khan, and Azeem 2014). This is likely due to the fact that the formulas only adjust for total protein or albumin, and ignore the possible influence of the complexed forms of calcium on the total calcium.

Ionized Calcium

Ionized calcium is the test of choice for assessing calcium homeostasis, as the ionized fraction of total calcium is responsible for all the biological effects of calcium, and is also the fraction responsible for providing feedback and autoregulation by binding directly to the parathyroid gland, thyroid C cells, and renal tubular cells to maintain serum ionized calcium. Ionized calcium measurement has been found to be more accurate at assessing calcium homeostasis compared to total calcium in many conditions including patients with both hyperalbuminemia and hypoalbuminemia, renal disease, hyperparathyroidism, acid-base

abnormalities, and critical illness (Holowaychuk and Martin 2007; Kogika et al. 2006; Messinger, Windham, and Ward 2009; Ong et al. 2012).

Prior to the advent of ion-selective electrodes, ionized calcium was measured using complicated, impractical methods including rachitic cartilage bioassays, metal ion indicators (murexide), and frog-heart experiments (Moore 1970). Calcium electrodes were first created using two difference methods: static and flow-through. With static-type electrodes, a glass tube was filled with an ion-exchanger solution and calcium salt, and then contact to the tube was made with a chloridized silver wire that was attached to an electrometer allowing a measurement to be recorded. Flow-through electrodes involve a sample that is continuously pushed through a sample chamber that is connected to an organic exchanger solution via a porous membrane. The sample that does not diffuse through the membrane continues through to be added to a solution of potassium chloride that is then measured via a colomel electrode connected to an electrometer. The measurement from the organic exchanger solution is also recorded using an Ag/AgCl wire connected to the same electrometer as the colomel electrode. The flow-through system has several advantages to the static-type electrode including more rapid measurement, increased stability, less sample volume needed, and the sample remains anaerobic (Moore 1970). Ion-selective electrodes employing membranes of various compositions that are permeable to specific ions were subsequently developed, and have made the routine measurement of ionized calcium clinically feasible. Indeed, ion-selective electroles have improved to the point where cage-side analyzers are nor regularly employed to provide fast and accurate assessments of ionized calcium in blood, plasma, and serum (Bowers, Brassard, and Sena 1986). Ion selective electrodes quantify the number of ions that diffuse across the semi-permeable membrane by measure the charge induced by their migration using either an electrometer or galvanometer. In

addition to ion-selective electrodes, portable clinical analyzers that utilize a disposable cartridge that contain impregnated biosensors for ionized calcium have been developed, which can be used on heparinized whole blood (Tappin et al. 2008). The accuracy of the ion-selective electrode and impregnated biosensors have found to be similar in dogs and horses (Looney et al. 1998), though a large human multi-institutional study evaluating the two methods found that the biosensor method resulted in a 2.6% lower ionized calcium value, which is likely clinically insignificant (Lindemans et al. 1999).

Appropriate handling of samples is imperative when measuring ionized calcium as multiple factors can interfere with accurate measurement. The largest interference arises from alterations in the pH of the sample. Ideally, all blood samples should be handled anaerobically, although most analytes can be accurately measured from both aerobic and anaerobic samples. The pH of the blood has been shown to have a significant effect, however, on the ionized calcium, with calcium changing by as much as 0.36 mmol/L per change in pH unit (Wang et al. 2002b). This is due to hydrogen competing for binding sites on albumin in states of acidosis, which increases the ionized calcium concentration. The opposite effect is observed in states of alkalosis. When blood is exposed to air, there is a loss of CO_2 and a subsequent increase in the pH of the sample, resulting in decreased measured ionized calcium (Schenck, Chew, and Brooks 1995). This has been studied using dog blood by filling blood collection tubes to either 25%, 50%, or 100% of blood capacity. Blood from the tubes filled to 50% or less had a significantly decreased ionized calcium concentration as compared to the the tubes filled to 100% (Unterer et al. 2004). In an attempt to negate the need for anaerobic handling of blood samples and the effects of pH, mathematical formulas have been created for use in human medicine to correct the ionized calcium after adjusting the pH to 7.4, but these formulas have not been validated for dogs

and have also been questioned in human medicine as well. (Krahn and Lou 2008; Lam, Dhaliwal, and Mamo 2013).

In addition to inaccuracies caused by exposure of blood samples to air, other factors in sample collection and handling must be considered to prevent artefactual changes to the ionized calcium concentration. The type of blood collection tube utilized, for example, is very important; silicone separator, lithium heparin, zinc heparin, and ethylenediaminetetraacetic acid (EDTA), and citrate tubes should all be avoided. Silicone separator tubes can falsely increase the ionized calcium due to release of calcium from the silicone gel (Larsson and Ohman 1985) and tubes containing lithium heparin can falsely decrease the measured ionized calcium (Lyon et al. 1995). A more recent study showed that tubes containing lithium heparin as well as tubes containing gel-barriers did not have significantly different measured ionized calcium compared to blood-gas syringes (Haverstick et al. 2009). Zinc heparin has been shown to falsely increase the ionized calcium, mainly by decreasing the pH of the sample (Lyon et al. 1995). Heparin-containing tubes of any kinds should not be used as heparin can increase the pH of the blood sample, therefore decreasing the measured ionized calcium. In contrast, syringes filled with blood to yield final heparin concentrations of either 150 IU/ml or 40 IU/ml and evaluated showed no significant difference in the measured ionized calcium concentration. However, blood samples with very high heparine concentrations of 5,000 IU/mL produced clinically significant underestimations of the ionized calcium concentration (Tappin et al. 2008). Hence, if whole blood is used to measure ionized calcium, the quantity of heparin should be standardized, with blood collection tubes that contain a premeasured amount of heparin are likely optimal to establish consistency. The anticoagulants EDTA, potassium oxalate, and citrate all chelate calcium, and therefore their use should always be avoided when measuring ionized calcium (Bowen and Remaley 2014).

To avoid interferences with its measurement, serum is the fluid of choice for evaluating ionized calcium (Schenck et al. 2012; Kang et al. 2014; Jafri, Khan, and Azeem 2014; Jonna et al. 1998). The use of serum eliminates the need for anticoagulants and has also been shown to stabilize the sample for a longer period of time (Schenck, Chew, and Brooks 1995), allowing for a more accurate measurement when shipping is necessary. Because severe abnormalities in calcium may warrant emergency therapy but most veterinarians have the inability to perform ionized calcium measurements in-house, alternatives to evaluating serum total calcium, serum ionized calcium, or using adjusted calcium formulas are needed. This would allow for more rapid and accurate assessment of calcium homeostasis, decrease client expense as ionized calcium typically has to be sent to a referral clinic for most veterinarians, and decrease potential patient morbidity and mortality. In addition, since total calcium has been shown to be inaccurate at predicting ionized calcium, a new formula could also allow for patients to be identified as having abnormal calcium levels when it would have otherwise been missed due to normal total calcium.

CHAPTER 3

MATERIALS AND METHODS

Study Population and Data Collection

Medical records from dogs presented to the University of Illinois Veterinary Teaching Hospital between February 2010 and February 2016 were identified by computerized database search. Dogs that had both an ionized calcium measurement and a serum biochemistry panel (creatinine, blood urea nitrogen, total protein, albumin, globulin, tCa, phosphorus, sodium, potassium, chloride, glucose, alkaline phosphatase, corticosteroid-induced alkaline phosphatase (cALP), alanine transferase, gamma-glutamyl transferase, total bilirubin, cholesterol, triglycerides, and bicarbonate) performed were selected and individually reviewed by one investigator (JD).

Ionized calcium measurement was routinely performed via a stat blood gas analyzer[a] which uses ion-selective electrode technology. The biochemistry panel was obtained from two chemistry analyzers (one[b] from February to August 2010, and another[c] from August 2010 to February 2016). Patient data was included in the study only if the ionized calcium measurement and the chemistry panel were performed within the same 24 hour time interval. Patients were excluded if there were any missing laboratory values. In order to guarantee independence of observations, an individual could not be included more than once.

All chemistry values and the measured ionized calcium (miCa) value were tabulated for every patient, with the exception of cALP, which is not routinely available on most chemistry analyzers. Additional data obtained from the medical records included age, breed, sex, body weight, and body condition score. When available, information regarding drug therapy that could

influence calcium levels (such as calcitriol, calcium supplementation, prednisone, non-steroidal anti-inflammatory drugs, phenobarbital, and diuretics) and final diagnosis (particularly with respect to the presence/absence of renal disease and/or calcium disorders) that was made at the time the bloodwork was performed, was also noted. A patient was assessed to have parenchymal renal disease if the combination of a creatinine value > 2.0 mg/dL and a urine specific gravity < 1.030 was identified.

Multivariate Adaptive Regression Splines Predictive Model Creation

Data from 2/3 of the included dogs was randomly selected using a random number generator and assigned to the training set, which was used to develop the model. The data from the remaining 1/3 of dogs was assigned to the test set, which was used to evaluate the performance of the newly created model. Comparisons of age, weight, body condition score, sex, and calcium status between the dogs of the training set and those of the test set were performed via the Mann-Whitney test and the chi square test respectively.

The predictive algorithm was created via a multivariate adaptive regression splines (MARS) model (Friedman 1991). Basically, a MARS model is a nonparametric regression method that estimates complex nonlinear relationship by a series of hinge functions of the predictors expressed using the following formula:

$$\hat{y} = a_0 + \sum_{k=1}^{p} a_k * h_k(x),$$

where $\hat{y}$ is the predicted value (i.e., predicted ionized calcium), a_0 is the intercept of the model, p is the total number of hinge functions, and a_k, the coefficient of the kth hinge function $h_k(x)$.

The hinge functions are pairs of two-sided truncated functions applied to all the predictors and described by the following equations:

$$h(x-t) = \begin{cases} (x-t) \; if \; x > t \\ 0 \qquad if \; x \leq t \end{cases}$$

$$h(t-x) = \begin{cases} (t-x) \; if \; x < t \\ 0 \qquad if \; x \geq t \end{cases}$$

where x is a predictor variable and t is the joining point of the polynomial called knot. The hinge functions break each predictor variable into two groups centered on a knot value and determine a linear relationship between the predictor and the outcome (i.e., miCa) in each group. The final relationship between the predictors and the outcome is therefore nonlinear.

The MARS model selection followed a two-step process involving an initial forward pass and then multiple backward passes as needed until the final model was obtained. Briefly, for the forward pass, hinge functions associated with the predictor variables (biochemical variables, age, breed, sex, body weight, body condition score, renal disease, drugs) were added in pairs into the model until any of the following conditions were met: (1) there were no more terms to add because all of them had been added into the model or (2) adding a term did not increase the predictive performance of the model. At the end of this process, the full model was obtained, including all the biochemical variables that were useful *a priori* to predict miCa, regardless of the complexity of the model. This full model is however over-fitted and therefore has poor predictive performance. Backward elimination was then used for final model selection and predictive performance optimization. Hinge functions were removed one by one from the model through all possible combinations. Each time a variable was removed, the accuracy and the

complexity of the new model was assessed by the generalized cross-validation (GCV) statistics. The smaller the GCV, the more accurate and less complex the model. The model with the lowest GCV value was selected as the final model. Finally, a prediction interval (PI) was defined as follows: predicted calcium ± 2 x regression standard error (Pardoe 2013). Because of the persisting complexity of the final model, a webpage that integrates the predictive algorithm has been developed. This webpage, called Canine Ionized Calcium Calculator, allows clinicians to obtain a prediction of the ionized calcium and its prediction interval after entering the mandatory biochemistry values. The Canine Ionized Calcium Calculator is available at: http://vetmed.illinois.edu/study/mars-model/VetMed.php

Assessment of Predictive Model Performance

The test set was used to evaluate the performance of the model in predicting miCa. The correlation between predicted ionized calcium (piCa) and miCa was assessed graphically via the observed-versus-predicted plot and mathematically via Pearson's R with its confidence interval (CI). Accuracy of the prediction was measured by calculating the root mean squared error (RMSE), which can be interpreted as the average distance between the observed values and the model prediction. The accuracy of the PI was evaluated by measuring how often the miCa values were included inside the PI.

To compare the diagnostic performance of piCa with other available calcium status estimators, tCa was adjusted for albumin and total protein in order to obtain 2 corrected tCa variables according to the following formulae:

1. tCa (mg/dL) – albumin (g/dL) + 3.5

2. tCa (mg/dL) – [0.4 x TP (g/dL)] + 3.3

Samples were further classified as hypercalcemic if miCa values were >1.37 mmol/L, and as hypocalcemic if miCa values were <1.11 mmol/L. Correlations with miCa were compared among piCa, tCa, and the 2 corrected tCa via Pearson's R. Ability of piCa, tCa, and the 2 corrected tCa formulae to properly classify dogs as hypercalcemic and hypocalcemic was assessed using receiver operator characteristic (ROC) curve analysis. The areas under the ROC curves (AUC) were used as a measure of diagnostic performance for these variables, and were compared via chi-square analysis.

Finally, sensitivity, specificity, and negative and positive predictive values were calculated for piCa and it's PI, tCa, and the 2 corrected tCa equations for diagnosis of hypocalcemia and hypercalcemia. Sensitivity and specificity of piCa were compared to those of tCa and both corrected tCa formulae using McNemar's test.

Description of the Relationship between Biochemistry Predictors and Ionized Calcium

Variable importance of particular biochemical parameters in miCa prediction was assessed using three criteria:

1) The number of model subsets (i.e., with the best set of terms for a given model size) that include the independent variable of interest during the backward elimination process. Variables that were included in more subsets were considered more important.

2) The residual sum of squares (RSS) criterion, which corresponds to the decrease in RSS (i.e., loss of prediction accuracy) when a variable is removed from the model. For ease of interpretation, the decreases in RSS were scaled so the largest decrease was 100. Variables that caused larger decrease in RSS were considered more important.

3) The GCV criterion, which corresponds to the increase in GCV when a variable is removed from the model. For ease of interpretation, the increases in GCV were scaled so the largest increase was 100. Variables that caused larger increase in GCV were considered more important.

The predicted relationship between ionized calcium and the biochemical predictors was depicted graphically for each biochemistry variable. Statistical analyses, including MARS model creation, were performed using public domain[d] software. Significance was set for all comparisons at a value of $p < 0.05$.

CHAPTER 4

RESULTS

Study Population

Two thousand dogs met the search criteria and 281 were excluded, leaving 1,719 dogs which met criteria for inclusion in the study. The training set was composed of the data from 1,200 (69.8%) dogs, while the remaining data from 519 (30.2%) dogs was used for the test set. In total, 135 breeds were represented, with the majority being mixed breed dogs (23.09%), and lesser numbers of Labrador Retrievers (8.14%), Dachshunds (5.22%), Yorkshire Terriers (3.72%), Golden Retrievers (3.14%), Shih Tzus (2.73%), German Shepherd Dog (2.67%), Boxers (2.55%), Beagles (2.21%), and <2% of each remaining breed. The differences in patient characteristics between the total group, test set, and training set are outlined in Table 1. There were no significant differences regarding age, weight, and sex between the training set and test set (p=0.52, 0.26, and 0.97, respectively). There was a significant difference between the dogs of the training set and those of the test set in terms of body condition score (p=0.04), with the dogs of the training set having a slightly higher score than those of the test set. However, this difference was likely not clinically relevant. There was no statistical difference for the proportions of hypocalcemic, normocalcemic, and hypercalcemic dogs between the test set and training set (p=0.56). The distribution of the values of the variables for the test set are shown in Figure 4, and for the training set in Figure 5.

Final Model

The final model included creatinine, albumin, tCa, phosphorus, sodium, potassium, chloride, alkaline phosphatase, triglycerides, and age as independent predictors. Table 2 presents

the 15 hinge functions included in the final model and their corresponding coefficients. An online version of the model is available at: http://vetmed.illinois.edu/study/mars-model/VetMed.php.

Assessment of Predictive Model Performance

The observed-versus-predicted plot is presented in Figure 1. The regression line (miCa = -0.06 + 1.05*piCa) was close to the first bisector, indicating a good fit between measured and predicted ionized calcium. The average difference between piCa and miCa, as estimated via the RMSE, was 0.08 mmol/L. The PI included miCa 94% of the time.

Overall, piCa was better correlated to miCa than tCa or corrected tCa. Pearson's R was 0.78 (CI: 0.74-0.81) for piCa versus 0.64 (CI: 0.59-0.69), 0.7 (CI: 0.65-0.74), and 0.67 (CI: 0.62-0.71) for tCa and the 2 corrected tCa formulae respectively.

The ROC curves for both hypercalcemia and hypocalcemia are shown in Figure 2. The AUC and their CI for piCa, tCa, and the 2 corrected tCa formulae to diagnose hypocalcemia and hypercalcemia are presented in Table 3. The AUC for piCa was significantly higher than for tCa and both corrected tCa equations to diagnose hypocalcemia (0.80 vs 0.68-0.70, $p<0.001$). The AUC for piCa was higher than for tCa and both corrected tCa equations, although not significantly, for diagnosing hypercalcemia (0.95 vs. 0.89-0.92, $p=0.28$).

Sensitivity, specificity, and negative and positive predictive values for piCa and its PI, tCa and the 2 corrected tCa equations for diagnosis of hypocalcemia and hypercalcemia are presented in Table 4A and 4B respectively. Sensitivity of piCa was significantly higher than sensitivity of tCa to detect hypocalcemia (sens=21.8% and 6.4% respectively, $p=0.002$), but significantly lower than those of both corrected tCa (sens=100% for both corrected tCa,

p<0.001). Specificity of piCa was not significantly different from specificity of tCa to diagnose hypocalcemia (spec=98.4% and 98.6% respectively, p=0.76), but was significantly higher than those of both corrected tCa (spec=0% for both corrected tCa, p=0.01). To detect hypercalcemia, piCa was as sensitive as tCa (sens=64% and 71.4% respectively, p=0.5) and both corrected tCa (sens=82.1% and 78.6% for corrected tCa1 and 2 respectively, p=0.06 and 0.12 for both comparisons). Specificity of piCa (spec=99.6%) was significantly higher than those of tCa (spec=98%, p=0.01) and of both corrected tCa (spec=95.9% and 89.6% respectively, p<0.001 for both comparisons) to diagnose hypercalcemia. At the prevalence of hypocalcemia in the test set (i.e.; 15%), positive predictive value of piCa (PPV=70.8%) was much higher than those of tCa (PPV=45.5%) and of both corrected tCa (PPV=15% for both of them). Similarly, at the prevalence of hypercalcemia in the test set (i.e.; 5.4%), positive predictive value of piCa (PPV= 90%) was much higher than those of tCa (PPV= 66.7%) and of both corrected tCa (PPV= 53.5% and 30.1% respectively).

Description of the Relationship between Biochemistry Predictors and Ionized Calcium

The most important variables impacting prediction of miCa were as follows: tCa, chloride, and albumin had the most significant impact. Age, creatinine, alkaline phosphatase, and sodium had a moderate impact while phosphorus, potassium and triglycerides had the lowest influence. Results are summarized in Table 5.

The individual relationships between miCa and the biochemical predictors are presented in Figure 3. The miCa value progressively increases as the levels of tCa and chloride increase. As the albumin level increases, the miCa value decreases. The miCa value decreases as creatinine, sodium, and age increase up to a certain value, but creatinine above 5 mg/dL, sodium

above 154 mmol/L, and age above 2.27 years had no more influence on ionized calcium value. Similarly, the miCa value increases as phosphorus and alkaline phosphatase levels increase up to a certain value, but phosphorus above 4.9 mg/dL and alkaline phosphatase above 848 U/L lose their impact on miCa values. Potassium levels have no influence on miCa value until kalemia reaches 5.1 mmol/L. As potassium level further increases, miCa value decreases. Finally, the relationship between triglycerides and miCa is the most complex. miCa value increases as triglyceride level increases up to 582 mg/dL. When triglyceride level increases further, miCa value progressively decreases.

CHAPTER 5

DISCUSSION

Ionized calcium is considered the gold-standard for evaluating calcium homeostasis but its measurement is not readily available to all veterinarians. A novel formula that predicts ionized calcium with improved accuracy compared to tCa and two commonly used corrected calcium equations was created employing data from routine serum biochemical profiles and patient age, providing veterinarians with an another option for assessing a patient's calcium status when miCa is not available. Additionally, tCa, albumin, and chloride were determined to have the greatest impact on miCa variation.

Several formulae have been developed to try to predict ionized calcium from tCa and other biochemical variables and pH, and were all based on simple or multivariate parametric regression analysis, often linear regression models (D J Meuten 1982; Ladenson, Lewis, and Boyd 1978). All these formulae ended up with poor performance (Schenck and Chew 2005; Ladenson, Lewis, and Boyd 1978). In our study, we used a different approach and utilized a MARS model for several reasons. First, the MARS model is non-linear and therefore took into account the likely complexity of the relationship between ionized calcium and other biochemical variables (Friedman 1991). Second, it is a non-parametric technique and not subject to erroneous assumptions about the distribution of the variables included in the study (Friedman 1991). It also eliminated the need to do any transformations of the variables (Kuhn and Johnson 2013a). Third, no interaction terms were included in order to avoid overfitting to the observed data, which is a recurrent and common problem in predictive modeling (Kuhn and Johnson 2013b). Finally, it allowed us to identify and better define the relationships between ionized calcium and other

biochemical variables. The use of a MARS model might explain why our ionized calcium prediction seems more accurate than the ones that were previously described (Meuten 1982; Ladenson, Lewis, and Boyd 1978).

Nonetheless, it is difficult to ensure accuracy of a predictive model. Ideally, it requires internal validation (i.e., validity of claims for the underlying population from which the data originated, which refers to reproducibility of the model) and external validation (i.e., generalizability of claims to 'plausibly related' but different populations) (Steyerberg and Vergouwe 2014). External validation was not possible in this study because we had access only to data from our hospital. Testing our model on different populations (e.g., from other institutions, in a prospective manner, and with data obtained with different analyzers) would be useful to demonstrate generalizability (Bleeker et al. 2003). Internal validation was performed via data splitting, which allowed assessment of predictive performance on independent data that were not used to build the model (Picard and Berk 1990). On the test set, piCa was strongly to very strongly correlated with miCa, which suggests good potential for piCa to be a marker of calcium homeostasis in dogs (Chan 2003). However, a difference was still identified between piCa and miCa (average 0.08mmol/L). Although this difference was small, it might affect the diagnostic ability of piCa and may explain its poor sensitivity to detect hypocalcemia, for example. In order to enhance its diagnostic performance, a PI was developed, which may help distinguish hypocalcemia and hypercalcemia. For hypocalcemia, the NPV of the lower end of PI was around 98%, allowing exclusion of hypocalcemia if the lower end of PI is above 1.11mmol/L. On the other end, an upper end of PI <1.11mmol/L was associated with 100% PPV, allowing a certain diagnosis of hypocalcemia. Likewise, for hypercalcemia, finding an upper end of PI below 1.37mmol/L allows exclusion of hypercalcemia, while a lower end of PI above

1.37mmol/L is diagnostic for hypercalcemia with 100% certainty. These PPV and NPV may vary with prevalence of hypocalcemia and hypercalcemia, and these results might be interpreted with caution in subpopulation of dogs where the prevalence of hypocalcemia and hypercalcemia is different (Brenner and Gefeller 1997).

A new diagnostic tool is warranted only if it performs better than the tools currently available. NPV of tCa was high for both hypocalcemia and hypercalcemia, which means that a normal tCa was generally useful for excluding the presence of a calcium disorder. On the other hand, PPV of tCa was mild to moderate, which means that use of tCa to assess a calcium disorder may be misleading. This is in agreement with previous studies and indicates the need for further verification via another estimator of calcium homeostasis when tCa is abnormal (Schenck and Chew 2005; Kogika et al. 2006). Correcting tCa with either albumin or total protein formulae resulted in poorer PPV than uncorrected tCa, which means that use of corrected tCa decreased diagnostic accuracy in regards to true calcium status and therefore cannot be recommended. This is also in accordance with another study (Schenck and Chew 2005). Predicted ionized calcium was better correlated to miCa than were tCa and values derived from corrected tCa formulae, and its overall diagnostic performance, as assessed by the AUC of the ROC curve, was better for both hypocalcemia and hypercalcemia. In particular, piCa had a much higher PPV for diagnosis of hypocalcemia, as a piCa < 1.11mmol/L was associated with a 70% chance of true hypocalcemia, as opposed to less than 50% when corrected or uncorrected tCa was used. For hypercalcemia, PPV of piCa was also much higher than those of tCa and corrected tCa formulae, with 90% chance of true hypercalcemia when piCa>1.37mmol/L. These results suggest that piCa would be the most appropriate marker to verify a calcium disorder when tCa is abnormal and miCa is not available.

The MARS model was also able to provide valuable information on the relationship between biochemical variables and miCa. Total calcium and albumin both had a large influence on ionized calcium, which was expected since ionized calcium is the major fraction of total calcium and albumin is the main molecule that binds calcium in the circulation (Schenck, Chew, and Brooks 1996; Moore 1970). Chloride was the second most influential variable on ionized calcium, which was an interesting finding. Overall, miCa steadily increases as chloride increases. The fact that hyperchloremia directly increases plasma ionized calcium is consistent with a previous study performed in cows in which IV administration of $CaCl_2$ resulted in higher plasma ionized calcium concentrations than when the same dose rate of calcium was administered in a calcium gluconate formulation (Hapke and Prigge 1972). The physiologic explanation for this relationship is unclear. Increased plasma chloride is associated with a change in blood pH and pH is known to influence plasma ionized calcium concentration via pH-dependent low affinity electrostatic (salt-type) binding of calcium to negative charges on plasma proteins (Wang et al. 2002a; Fogh-Andersen 1977). The most likely sites for calcium binding on albumin are free carboxyl groups that become available for binding with increased pH (Pedersen 1972). However, no significant correlation was observed between pH and ionized calcium concentration in a study conducted in dogs with chronic kidney disease (Kogika et al. 2006). Also, the lack of contribution of bicarbonate to piCa in this study and in a previous study suggests that the influence of chloride on ionized calcium may not act through changes in blood pH alone (Ladenson, Lewis, and Boyd 1978). A direct change of plasma ionized calcium concentration in response to an increase in plasma chloride, independent from blood pH, may be possible, presumably because the additional chloride displaces calcium that is bound to albumin. Another possible explanation would be that miCa, along with other plasma ions, varies in order to

maintain blood electroneutrality. Indeed, according to our results, miCa consistently increases with increase in anion concentration (phosphorus and chloride) and decreases when cation concentration increases (sodium and potassium). Given that chloride is a major anion in plasma, it is reasonable to consider that miCa, as a cation, moves in equal directions in the serum to maintain electroneutrality.

The moderate influence of age on calcium may be related to skeletal maturation during the growth phase of dogs. Higher ionized calcium values have been documented in puppies compared to adults (O'Brien et al. 2014). Interestingly, chloride levels were also lower in young puppies and slowly increased with aging while ionized calcium was slowly decreasing (O'Brien et al. 2014). The moderate influence of ALP could also be related to skeletal remodeling associated with growth or bone diseases, especially given that other liver enzymes, such as ALT, AST, and GGT, were not significantly influential on miCa. To prove this further, the isoenzymes of ALP could be measured to determine if the bone isoenzyme is the predominately increased value. Creatinine had a moderate effect on calcium and miCa decreased as creatinine increased to a value of 5 mg/dL, above which creatinine no longer had an additional effect on miCa. The relationship between renal disease and disorders of calcium have been studied, and hypocalcemia occurs commonly in patients with renal disease as a consequence of reduced renal calcitriol synthesis and retention of phosphorus in dogs with renal disease (Kogika et al. 2006; Cortadellas et al. 2010). It is surprising that creatinine was no longer influential above 5mg/dL, although ionized calcium concentration was previously shown to become significantly lower in dogs with chronic kidney disease only when IRIS stage 4 is reached (Cortadellas et al. 2010). These results suggest that lower plasma ionized calcium in IRIS stage 4 chronic kidney disease dogs may be referred to other factors, such as pH, rather than directly from the decline of kidney function

(Magner et al. 1988; Bourgoignie et al. 1981; Segev et al. 2010). Interestingly, BUN did not significantly influence ionized calcium concentration, perhaps because BUN is a less specific indicator of decreasing renal function (Concordet et al. 2008). In accordance with previous findings, increasing levels of sodium were associated with decreasing miCa (Akimoto et al. 2001). This association may be explained by sodium-induced conformational change in albumin, which might reduce the capacity of calcium binding (Akimoto et al. 2001). Alternatively, such a relationship might be necessary to maintain serum electroneutrality, but this has not been proven at this time.

Phosphorus had little influence on miCa. This is consistent with the slight negative correlation that has been previously reported between ionized calcium and phosphorus concentration in dogs and the fact that phosphorus concentration cannot be used to predict complexed calcium status (Kogika et al. 2006; Schenck and Chew 2003b). Lastly, triglyceride levels had a unique influence on miCa: miCa increased slightly as triglyceride level increased up to 582 mg/dL, but, when triglyceride level increased further, miCa value decreased. The slight increase in miCa when triglyceride concentration increased up to 582 mg/dL is difficult to explain. However, fatty acids have been demonstrated to induce a conformational change in albumin, which increases its affinity for calcium (Aguanno and Ladenson 1982). Alternatively, the influence of triglycerides on miCa may be due to the association between hypertriglyceridemia and pancreatitis in canines, as hypocalcemia can occur with peripancreatic fat saponification, for instance (Holowaychuk et al. 2009).

There were several limitations to this study. First, due to its retrospective nature, some records were incomplete and there was inconsistent reporting of some of the data, especially pertaining to complete history, medication list, or final diagnosis. It is possible that some patients

were on unrecorded medications that could influence calcium or other biochemical parameters, or had an unrecorded condition that could also cause alterations in calcium homeostasis. Second, proper sample collection and handling cannot be guaranteed due to the retrospective aspect of case recruitment. However, it is standard of care in our hospital to analyze ionized calcium within 15 minutes of collection. It cannot also be guaranteed that all patients were fasted prior to blood analysis, which could influence a variety of factors including total calcium and triglycerides. Third, there were specific variables, such as magnesium and pH, that were not included in the model but that could potentially influence calcium levels. Interestingly, both deficiencies and excesses of magnesium have been shown to directly influence calcium concentrations by a variety of mechanisms, including decreased parathyroid hormone secretion with a consequent decrease in vitamin D activation and end-organ resistance to parathyroid hormone in the kidney and bone, all contributing to hypocalcemia (Rude, Oldham, and Singer 1976; Holowaychuk 2013; Cholst et al. 1984).Treatment of hypomagnesemia has been shown to increase calcium and parathyroid hormone values in both people and dogs (Rude, Oldham, and Singer 1976; Shils 1980; Anast et al. 1976). Serum and whole blood pH have also been shown to be correlated with changes ionized calcium, as calcium will dissociate from protein in acidic environments, thereby increasing the amount of ionized calcium, and the opposite will occur in alkaline environments (Schenck et al. 2012). To compensate for possible changes in pH, an adjustment formula has been used in people but has not yet been validated for use in dogs (Unterer et al. 2004; Schenck and Chew 2008a). For ionized calcium measurements in this study, the normalization equation was not used, therefore allowing potential influence of pH. Inclusion of other predictors such as magnesium levels might further improve the performance of our

model. However, the restriction to utilize only those biochemical variables that are routinely available was maintained in order for the model to be usable by most veterinarians.

In conclusion, a novel formula was created that allows for calculation of a predicted ionized calcium value from readily available biochemical and patient variables. This predicted ionized calcium can be useful in assessing calcium disorders in dogs when measurement of serum ionized calcium is not available. The predicted ionized calcium is a more reliable measure of calcium homeostasis than total calcium and corrected total calcium based on the improved predictive values for both hypercalcemia and hypocalcemia. Factors associated with measured ionized calcium variation were identified, with total calcium, chloride, and albumin being the most influential, and phosphorus having very little impact on ionized calcium. External validation, which implies testing the model on different but related populations of dogs, patients from other institutions, and using different chemistry analyzers would be useful to assure generalizability of these results.

Footnotes

[a] NovaStat CCX, NOVA Biomedical, Waltham, Massachusetts

[b] Roche Hitachi 917, Roche Diagnostics, Indianapolis, Indiana

[c] Olympus AU680, Beckman Coulter, Inc., Brea, California

[d] R version 3.1.1. R Development Core Team (2014). R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

CHAPTER 6

FIGURES AND TABLES

Figure 1: Observed-versus-predicted plot showing the relationship between measured and predicted ionized calcium. The upper and lower limits of normocalcemia (1.11-1.37 mmol/L) are represented by the vertical blue lines for the predicted ionized calcium, and the horizontal solid lines indicate the normal range for measured ionized calcium. The diagonal green line represents the first bisector, on which predicted ionized calcium values that perfectly match measured ionized calcium fall. The thick solid line represents the regression line (miCa = -0.06 + 1.05*piCa). Points that fall within the three boxes along the first bisector were properly classified by predicted ionized calcium, and those points within the four boxes away from the first bisector were misclassified by predicted ionized calcium. ica: measured ionized calcium; predica: predicted ionized calcium.

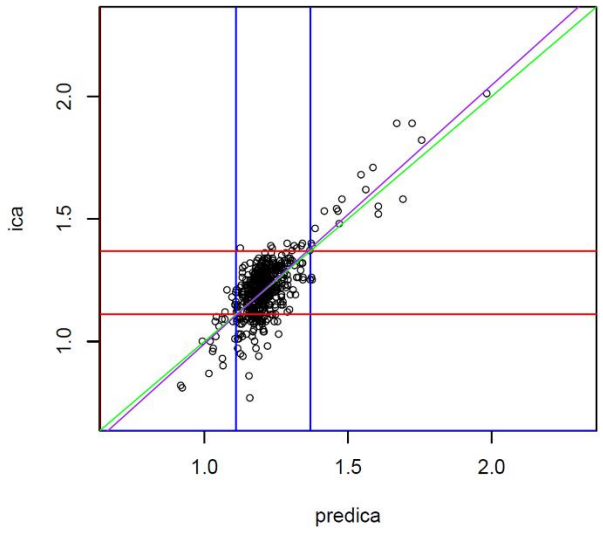

Figure 2: Figure 2. Receiver operator characteristic curves illustrating the overall diagnostic performance of predicted ionized calcium (purple line), total calcium (red line), total calcium corrected with albumin (green line), and total calcium corrected with total protein (blue line), for hypocalcemia (A) and hypercalcemia (B).

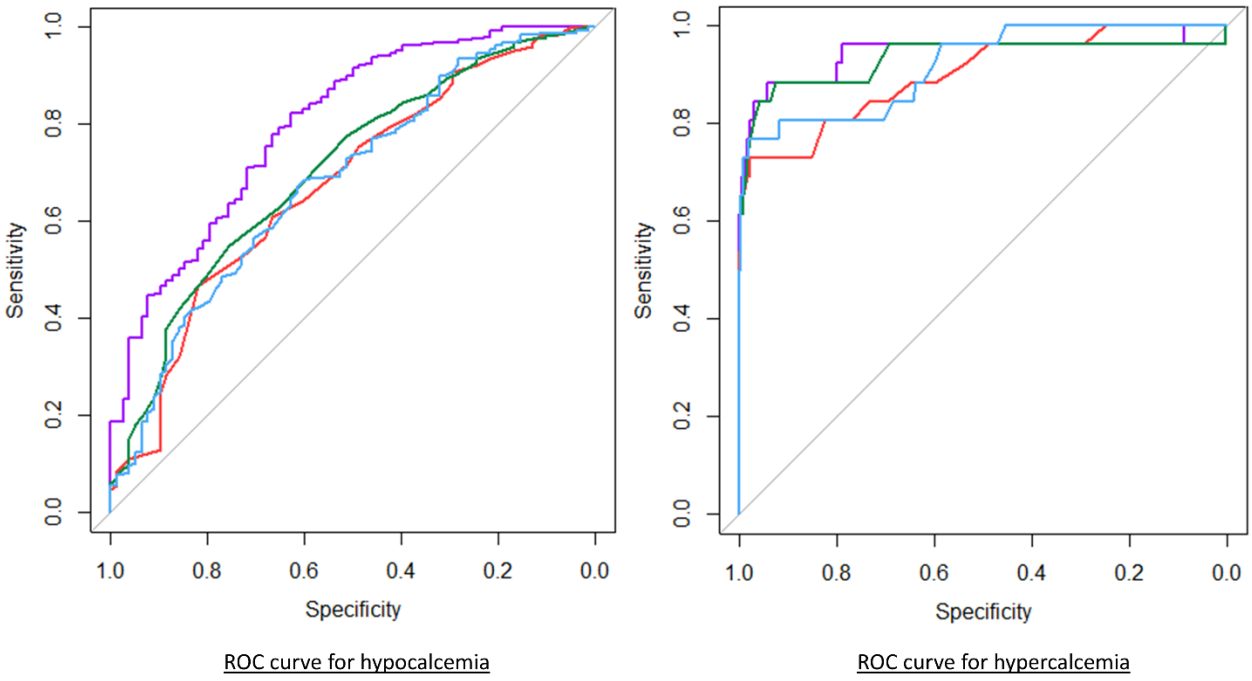

Figure 3: Individual relationships between measured ionized calcium and the biochemical predictors that have been retained in the final predictive model. The measured ionized calcium value is represented on the y axis, against the value of the predictor variables on the x axis. Creat: creatinine; alb: albumin; Tca: total calcium; phos: phosphorus; Na: sodium; K: potassium; Cl: chloride; TALP: total alkaline phosphatase; TG: triglycerides.

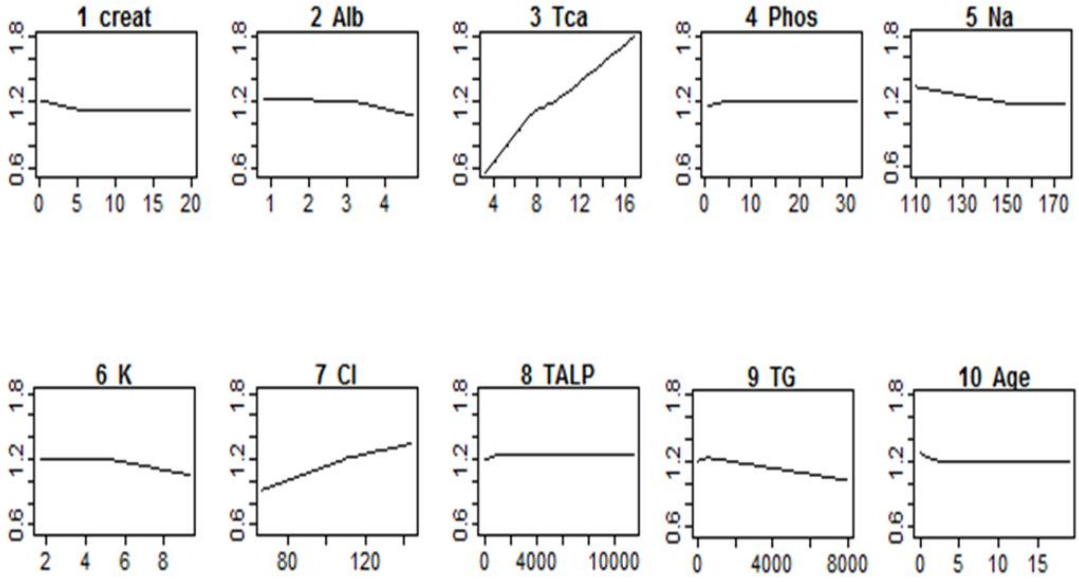

Figure 4. Density curves for the variables included into the model from the test set. The distribution is on the y-axis, and the value of the variable on the x-axis.

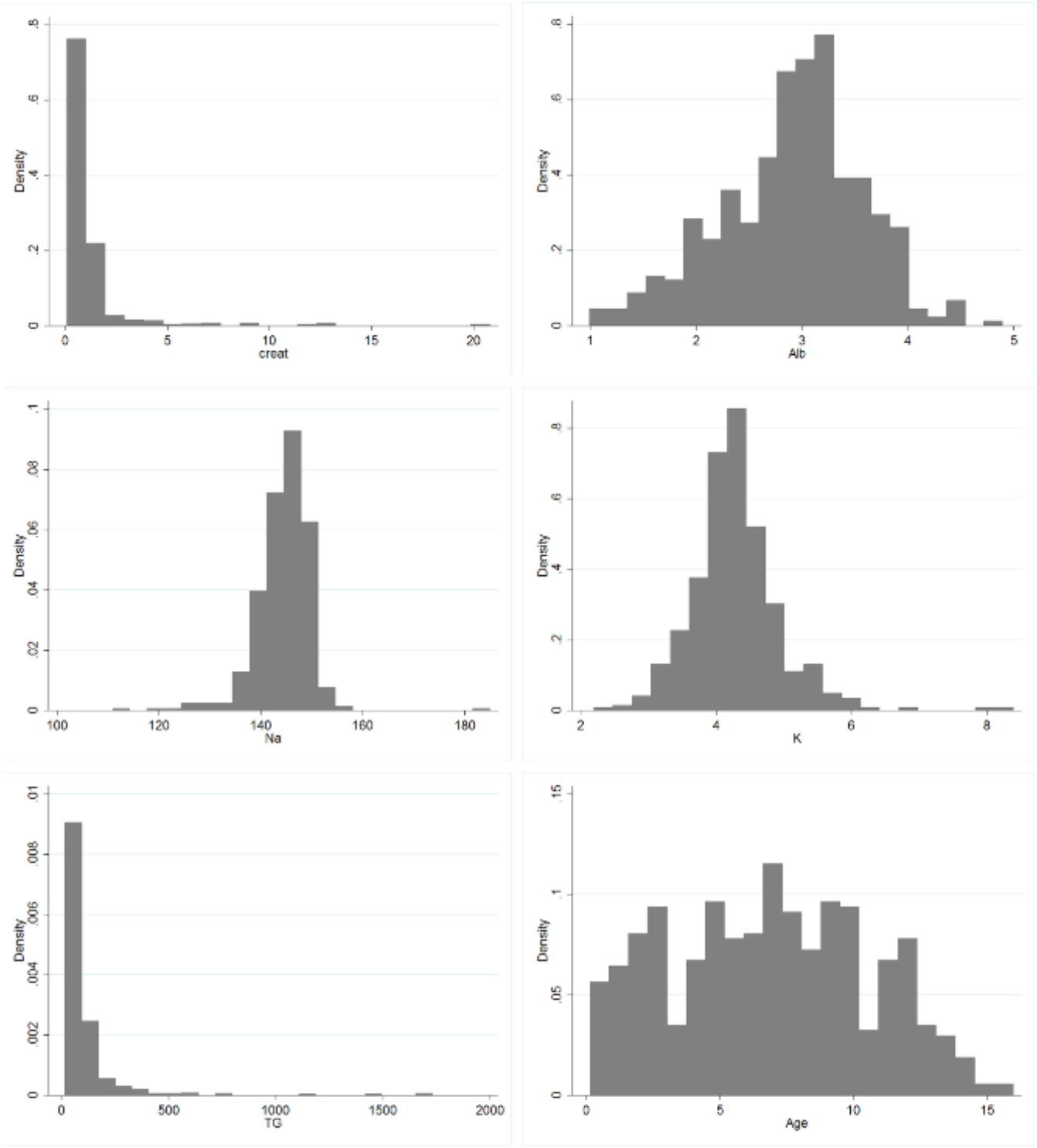

Figure 4 (continued)

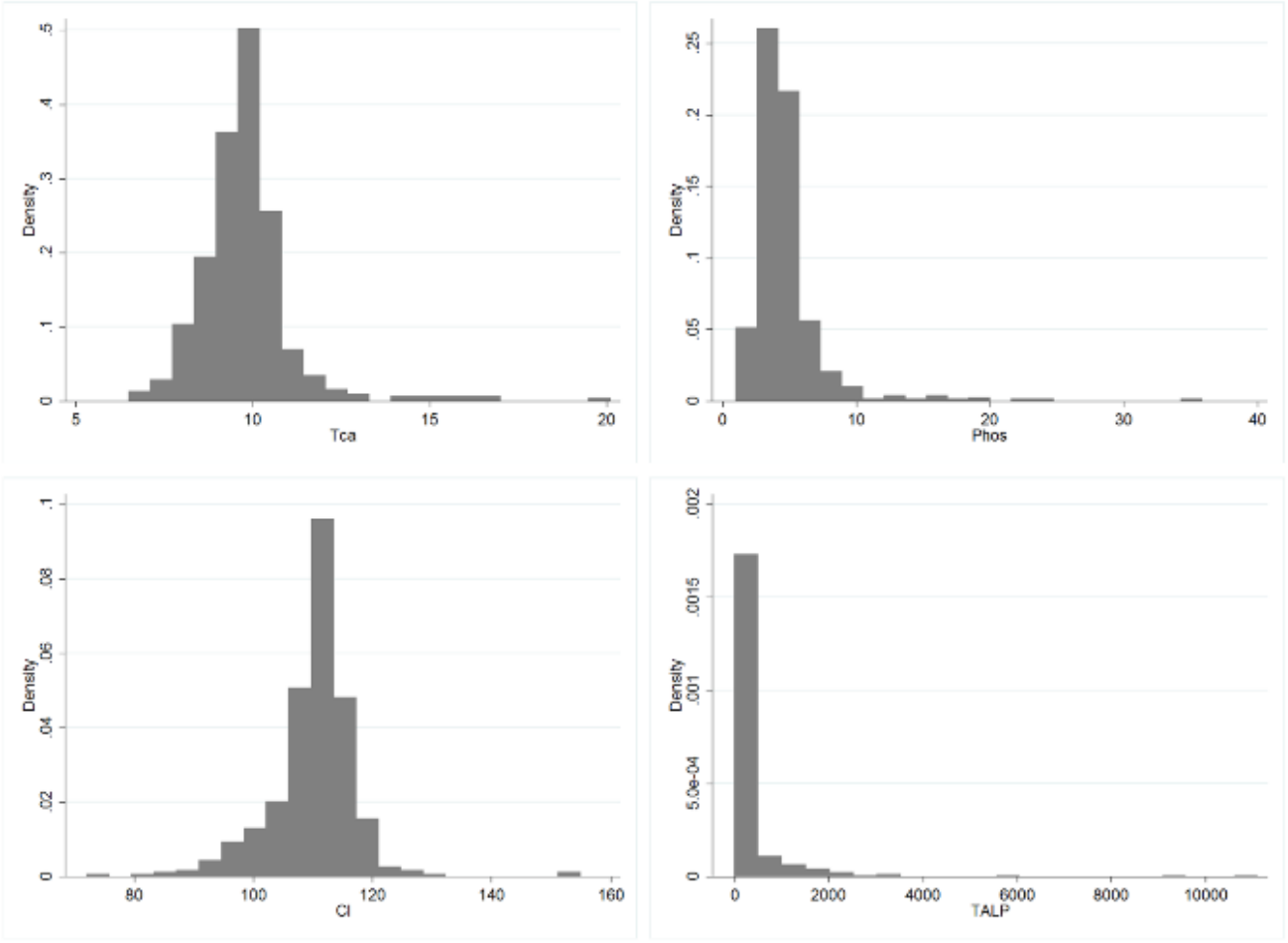

Figure 5. Density curves for the variables included into the model from the training set. The distribution is on the y-axis, and the value of the variable on the x-axis.

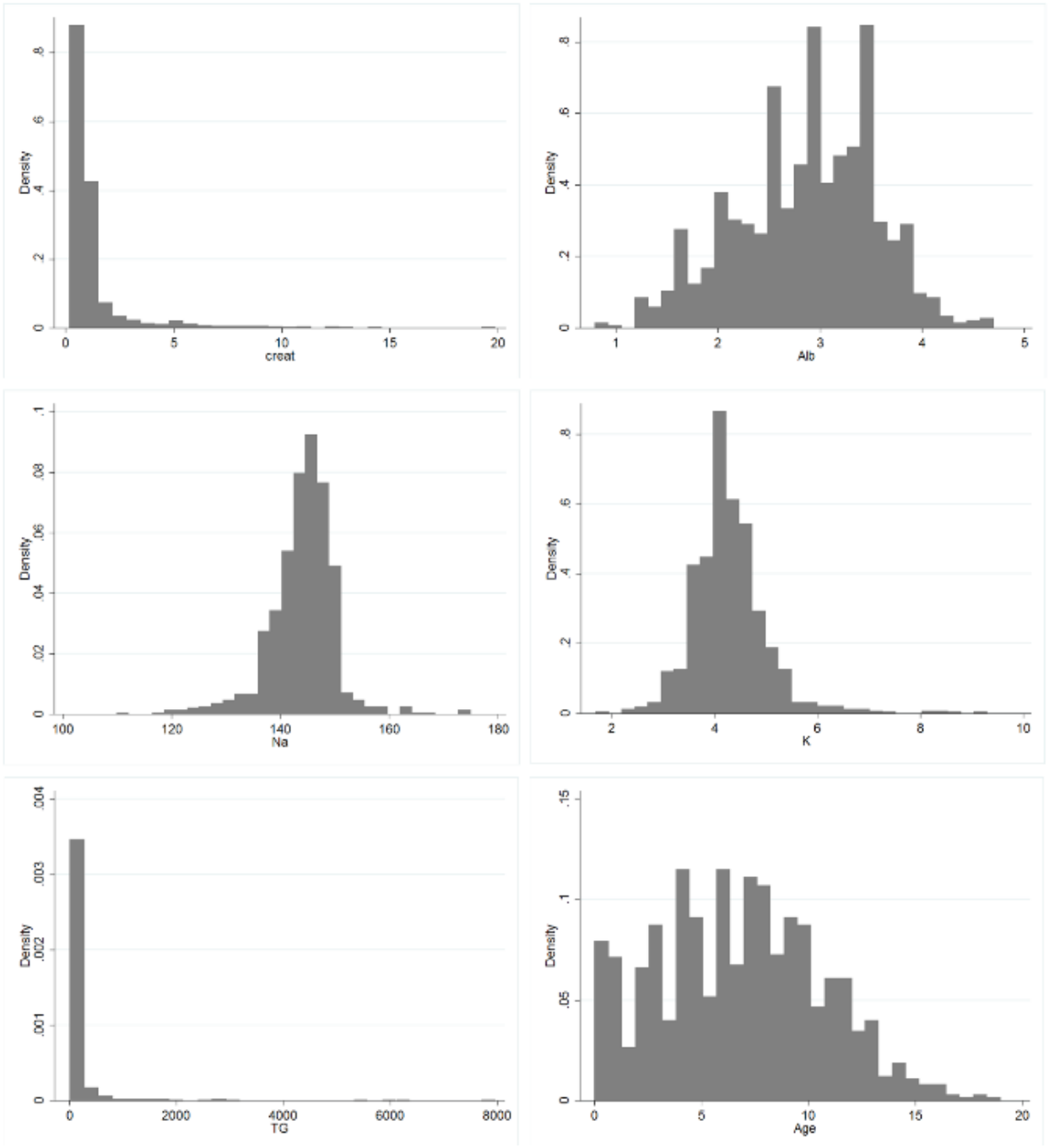

Figure 5 (continued)

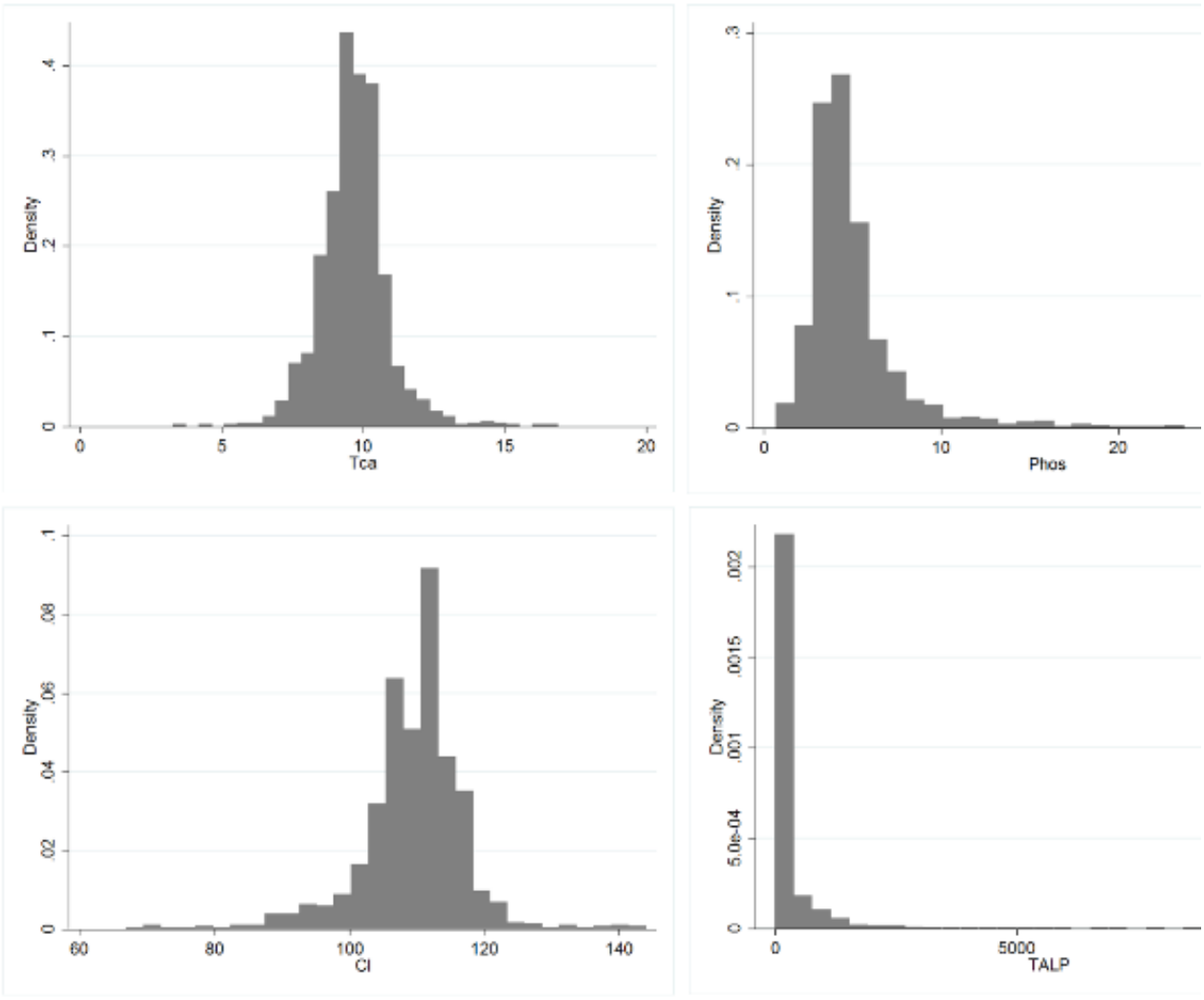

Table 1. Demographic data for the dogs of the total group, test set, and training set.

Variable	Total Group (n=1719)	Training Set (n=1200)	Test Set (n=519)	*P*-Values
Age (years)	6.9 (0.04-19.0)	6.8 (0.04-19.0)	7.0 (.16-16.0)	0.52
Weight (kilograms)	18.0 (0.4-96.0)	17.0 (0.4-94.0)	20.0 (0.8-96.0)	0.26
BCS (9 point scale)	5 (1-9)	5 (1-9)	5 (1-9)	0.04*
Sex				0.97
Neutered Males	723 (42.0%)	509 (42.4%)	214 (41.2%)	
Intact Males	166 (9.7%)	114 (9.5%)	52 (10.1%)	
Spayed Females	718 (41.8%)	499 (41.6%)	219 (42.2%)	
Intact Females	112 (6.5%)	78 (6.5%)	34 (6.5%)	
Calcium Status				0.56
Hypocalcemic	280 (16.3%)	202 (16.8%)	78 (15.0%)	
Hypercalcemic	99 (5.8%)	71 (5.9%)	28 (5.4%)	
Normocalcemic	1340 (77.9%)	927 (77.3%)	413 (79.6%)	

Table entries represent median values (minimum-maximum) for continuous variables (age, weight, and body condition score) and number of dogs (percent of dogs) for categorical variables (sex and calcium status). Significant differences between the dogs of the training set and those of the test set, as assessed via the Mann-Whitney (age, weight, and body condition score) and chi square (sex and calcium status) tests, were defined by a *P*-value<0.05 and are indicated by *

Table 2. Final Multivariate Adaptive Regression Splines Model for prediction of ionized calcium from routine biochemical and patient variables determined from dogs of the training set.

Hinge function of the predictors	Coefficient
(Intercept)	1.05189701
h (5 – creatinine)	0.01707569
h (3.3 – albumin)	0.01479586
h (albumin – 3.3)	-0.08887935
h (7.5 – total calcium)	-0.12828575
h (total calcium – 7.5)	0.04954995
h (total calcium – 9.9)	0.03339810
h (4.9 – phosphorus)	-0.01079317
h (154 – sodium)	0.00371181
h (potassium – 5.1)	-0.03410317
h (111 – chloride)	-0.00672129
h (chloride -111)	0.00390011
h (848 – ALP)	-0.00003635
h (582 – triglycerides)	-0.00005582
h (triglycerides – 582)	-0.00002793
h (2.27 – age)	0.02851557

h() - (hinge function); ALP: alkaline phosphatase

Table 3. Area under the ROC curves and their 95% confidence interval of predicted ionized calcium, total calcium, and two corrected total calcium formulae for diagnosis of hypocalcemia and hypercalcemia in dogs of the test set.

	Hypocalcemia*		Hypercalcemia	
	AUC	95% confidence interval	AUC	95% confidence interval
piCa	0.80	0.74 – 0.85	0.95	0.88 – 1.00
tCa	0.67	0.61 – 0.74	0.89	0.81 – 0.96
Corrected tCa1	0.70	0.64 – 0.77	0.92	0.84 – 0.99
Corrected tCa2	0.68	0.62 – 0.75	0.90	0.83 – 0.97

Hypocalcemia is defined as measured ionized calcium<1.11 mmol/L and hypercalcemia is defined as measured ionized calcium>1.37 mmol/L. For hypocalcemia, there were significant differences for the areas under the curve among the 4 techniques (*p<0.001). piCa: predicted ionized calcium; tCa: total calcium; Corrected tCa1: total calcium corrected with albumin; Corrected tCa2: total calcium corrected with total protein; AUC: area under the curve.

Table 4a. Sensitivity, specificity, and negative and positive predictive values of predicted ionized calcium and its prediction interval, total calcium and two corrected calcium formulae for diagnosis of hypocalcemia in dogs of the test set with a 95% confidence interval (prevalence of hypocalcemia = 15%)

	Sensitivity	Specificity	NPV	PPV
piCa < 1.11 mmol/L	21.8% [13.2-32.6%]	98.4% [96.8-99.4%]	87.7% [84.5-90.4%]	70.8% [48.9-87.4%]
Lower end of PI < 1.11 mmol/L	97.4% [91-99.7%]	21.3% [17.6-25.4%]	97.9% [92.7-99.7%]	18% [14.4-22%]
Upper end of PI < 1.11 mmol/L	2.6% [0.3-9%]	100% [99.2-100%]	85.3% [81.9-88.2%]	100% [15.8-100%]
tCa < 7.6 mg/dL	6.4% [2.1-14.3%]	98.6% [97.1-99.5%]	85.6% [82.3-88.6%]	45.5% [16.7-76.6%]
Corrected tCa1 < 7.6 mg/dL	100% [94.1-100%]	0% [0-0.9%]	ND ND	15% [12.1-18.5%]
Corrected tCa2 < 7.6 mg/dL	100% [94.1-100%]	0% [0-0.9%]	ND ND	15% [12.1-18.5%]

piCa: predicted ionized calcium; PI: prediction interval; tCa: total calcium; Corrected tCa1: total calcium corrected with albumin; Corrected tCa2: total calcium corrected with total protein; NPV: negative predictive value; PPV: positive predictive value; ND: Not determined because denominator is 0.

Table 4b. Sensitivity, specificity, and negative and positive predictive values of predicted calcium and its prediction interval, total calcium and two corrected calcium formulae for diagnosis of hypercalcemia in dogs of the test set with a 95% confidence interval (prevalence of hypercalcemia = 5.4%)

	Sensitivity	Specificity	NPV	PPV
piCa > 1.37 mmol/L	64% [44.1-81.4%]	99.6% [98.5-100%]	98% [96.3-99%]	90% [68.3-98.8%]
Upper end of PI > 1.37 mmol/L	92.9% [76.5-99.1%]	79.2% [75.4-82.7%]	99.5% [98.2-99.9%]	20.3% [13.7-28.3%]
Lower end of PI > 1.37 mmol/L	35.7% [18.6-55.9%]	100% [99.3-100%]	96.5% [94.5-97.9%]	100% [69.2-100%]
tCa > 11.4 mg/dL	71.4% [51.3-86.8%]	98% [96.3-99%]	98.4% [96.8-99.3%]	66.7% [47.2-82.7%]
Corrected tCa1 > 11.4 mg/dL	82.1% [63.1-93.9%]	95.9% [93.8-97.5%]	98.9% [97.6-99.7%]	53.5% [37.7-68.8%]
Corrected tCa2 > 11.4 mg/dL	78.6% [59-91.7%]	89.6% [86.6-92.2%]	98.7% [97.1-99.5%]	20.3% [19.9-42%]

piCa: predicted ionized calcium; PI: prediction interval; tCa: total calcium; Corrected tCa1: total calcium corrected with albumin; Corrected tCa2: total calcium corrected with total protein; NPV: negative predictive value; PPV: positive predictive value.

Table 5. Evaluation of the importance of the predictor variables that form the model for predicting measured ionized calcium changes.

	Number of Subsets	GCV	RSS
Total Calcium	15	100	100
Chloride	14	68.3	69.9
Albumin	13	55.4	57.7
Age	11	40.3	43.5
Creatinine	9	29.9	33.6
Total ALP	8	23.8	28.1
Sodium	6	19.9	23.8
Phosphorus	5	16.6	20.4
Potassium	4	13.6	17.4
Triglycerides	3	9.5	13.4

ALP: alkaline phosphatase; GCV: generalized cross validation; RSS: residual sum of squares. For ease of interpretation, the GCV was scaled so that largest increase was 100, and the RSS was scaled so the largest decrease was 100.

CHAPTER 7

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