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VALIDATION OF AN ADJUSTED CALCIUM FORMULA USING THE ROCHE CALCIUM (NM-BAPTA) AND ALBUMIN (BCG) METHODS AT GROOTE SCHUUR HOSPITAL

BY

DR MBALI NDLOVU

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SUPERVISORS: DR FIERDOZ OMAR AND DR HELENA VREEDÉ

DIVISION OF CHEMICAL PATHOLOGY, UNIVERSITY OF CAPE TOWN

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Date: 31 January 2017

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## ABBREVIATIONS

1,25(OH) <sub>2</sub> D	1,25-dihydroxyvitamin D
aCa	Adjusted calcium
BCG	Bromocresol green
BCP	Bromocresol purple
CAPD	Continuous ambulatory peritoneal dialysis
cCa	Corrected calcium
CCC	Concordance correlation coefficients
CKD	Chronic kidney disease
CMPF	3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid
CPC	Cresolphthalein complexone
ECF	Extracellular fluid
GSH	Groote Schuur Hospital
HREC	Health Sciences Human Research Ethics Committee
Hyper	Hypercalcaemia
Hypo	Hypocalcaemia
iCa	Ionised calcium
ICC	Interclass correlation coefficients
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
ISE	Ion selective electrode
NHLS	National Health Laboratory Service
NKF-K/DOQI	National Kidney Foundation Kidney Disease Outcomes Quality Initiative
NM-BAPTA	5-nitro-5'-methyl-(1,2-bis(o-aminophenoxy) ethan-N,N,N',N'- tetraacetic acid
PTH	Parathyroid hormone
SG	Specific gravity
tCa	Total calcium
tCO <sub>2</sub>	Total carbon dioxide concentration
UCT	University of Cape Town

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## **CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW**

### **1.1. OBJECTIVES OF REVIEW**

This review will aim to provide an overview of calcium homeostasis and its estimation with particular emphasis on:

- Ionised calcium and its measurement
- Total calcium and its measurement
- Derivation of corrected calcium
- Rationale for adjusted calcium including the effect of serum albumin and calcium methodologies in the estimation of calcium status

### **1.2. SEARCH CRITERIA**

The following search criteria were used on Pubmed as the following MeSH terms: adjusted or corrected calcium and serum albumin. References in written papers were also used as part of the literature search.

### **1.3. LITERATURE REVIEW**

#### **A. Introduction**

Calcium is a divalent cation with multiple roles, including a structural role in bone and various metabolic roles. These include blood coagulation, muscle contraction, cell membrane potential, cell permeability as well as hormone secretion, action and signalling (1). Most total body calcium (99%) is contained in the skeleton, where it contributes to its mechanical properties and provides a reservoir for calcium in the extracellular fluid (ECF). The makeup of the remaining 1% is intracellular (0.8%) and extracellular (0.2%). Serum calcium is present in 3 physiological states: protein bound (30-55%), complexed (5-15%) and free (50%) calcium fractions. Approximately 90% protein bound calcium is attached primarily to albumin with the remaining 10% to

globulins. Calcium bound to small organic anions (i.e. citrate, bicarbonate, sulfate, phosphate and lactate) constitutes complexed calcium.

The complexed and free fractions of the serum total calcium (tCa) concentration constitute the diffusible fraction. It is also known as the ultra-filterable calcium as it diffuses through physiological membranes. The protein-bound calcium is the non-diffusible fraction. Each albumin molecule consists of approximately thirty binding sites with only about 10 to 15% being utilized under normal conditions for binding ligands. The free calcium is the biologically active form and is tightly regulated to maintain a narrow physiological range. Excess of calcium in the blood results in a relative increase of the protein-bound fraction to maintain a constant free calcium concentration, and vice versa. All calcium in the human body exists in the ionised form. Non-ionised calcium is a flammable metal and not present in any body tissue (2). However, conventionally, the term “ionised calcium” is used to refer to the biologically active free calcium fraction.

The protein-bound pool acts as reservoir for ionised calcium (iCa). Any transient drop in the free fraction is compensated for by release of calcium ions from the calcium binding sites on the albumin molecule (2). This constitutes the short-term control of iCa. Various factors influence the amount of bound or complexed calcium. These include abnormalities in plasma protein concentration; pH changes; changes in ion concentration (i.e. sodium, magnesium and phosphate); and the presence of chelators such as lactate and citrate. Additionally, variations in the concentration of free fatty acids that bind to albumin can affect the ionised and/or the complexed portion (3).

## **B. Variations in Calcium Homeostasis**

Calcium control is tightly regulated by parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D ( $1,25(\text{OH})_2\text{D}$ ) to maintain a narrow physiological range of iCa. Together they control the resorption of calcium from bone, dietary calcium absorption from the intestine and reabsorption of calcium from the renal tubules (4). Calcitonin, to a lesser extent, plays a role in calcium homeostasis, particularly during hypercalcaemia. Its principal role is to inhibit osteoclast mediated bone resorption to

maintain normal iCa levels. Hormonal regulation of calcium homeostasis constitutes the long-term control of iCa.

Various hypotheses exist regarding the regulation of tCa and iCa. Over and above hormonal influences, calcium levels seem to show variability related to age. This has been well demonstrated in the elderly, post-menopausal women and neonates. Homeostatic control of physiological systems is known to decrease with increasing age (4). The decline in calcium control is further confounded by increased variability of serum iCa, which may be attributed to variations in circadian rhythm; nutritional status; physiological response to exercise; hormonal influences; serum magnesium levels and the renin-aldosterone system (4). In a large epidemiological study, Watson et al not only demonstrated variability in iCa with age but also with gender. This variability occurred earlier in women than in men (4).

Serum tCa has been demonstrated to decline with age (5). This is attributed to an age-related decline in serum albumin concentration. In addition, calcium absorption declines with age, despite an age related increase in PTH levels. Vitamin D deficiency also contributes to decreased calcium absorption, common in the elderly. In contrast, menopause is associated with increased tCa but does not affect iCa and serum albumin concentration (6). The elevation in tCa is likely due to an increase in the complexed or protein-bound calcium fraction. Increased bone resorption secondary to stimulated PTH secretion is unlikely to account for this increase as iCa remains unchanged. Nordin et al demonstrated similar findings in post-menopausal women (7). The increased tCa was accounted for by an elevation in the complexed fraction, as this was associated with an increased anion gap and bicarbonate concentrations. There were no changes in  $1,25(\text{OH})_2\text{D}$  and PTH levels.

A complex process regulates calcium homeostasis in pregnancy, in the foetus and the neonate. This involves endocrine and non-endocrine mechanisms. The latter include dietary calcium and phosphate balance; intestinal calcium secretion; kidney maturation; and acid-base regulation (8). Foetal skeletal development imposes high demands on calcium requirements during pregnancy. This is met by an elevation in maternal PTH secretion secondary to parathyroid gland hyperplasia. Active vitamin D synthesis is also increased leading to increased absorption of calcium (9). Despite

these changes, maternal tCa levels decline to a nadir at 28 to 32 weeks, largely due to a mild hypoalbuminaemia. The iCa levels, however are maintained in the physiological range throughout pregnancy. The abrupt removal of the maternal calcium supply at birth result in alterations in iCa in neonates. As such, adjustments from high to normal calcium levels are observed. Subsequently, an early physiological hypocalcaemia occurring within the first 48 hours has been described in infants born before 37 weeks gestation; in perinatal asphyxia; and those born from diabetic mothers (8).

The observations above highlight the importance of determining the calcium fraction that is not affected by this variability. This will determine the choice of the calcium measurement that will be a more reliable estimate of calcium status. Patient groups affected by variation in tCa will benefit from measurement of iCa to reliably determine calcium homeostasis.

### **C. Ionised Calcium**

Free calcium has a fundamental role in calcium homeostasis in health and is the least affected in the presence of parathyroid abnormalities (1). For this reason, strict maintenance of its equilibrium is essential. In addition to the protein-bound pool (mainly albumin) acting as a reservoir for free calcium to maintain normal levels, albumin also acts as a calcium buffer. The albumin-calcium buffer is highly pH sensitive (2). Changes in hydrogen ion concentration alter the charge on the acidic amino acid residues of the albumin molecule. This affects the number of calcium ions that are bound, and the fraction of tCa that is free. As such, increase in pH results in increased protein binding resulting in a fall in iCa, whereas a decrease in pH has the opposite effect.

The initial method developed for iCa measurement was a bioassay with limited application in clinical practice which required the use of frog tissue. Currently, iCa is measured by potentiometry with a direct ion selective electrode (ISE) (1, 2). This is mostly offered on smaller blood gas analysers that are readily available in most acute care settings. The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) provides recommendations on sampling, transport, storage and an

ISE assay procedure for determination of iCa in heparinised whole blood and plasma as well as serum (10). It emphasises rapid analysis of an anaerobic sample placed on ice to counteract various causes of pH alteration that can alter iCa concentration. Dilution effects from anticoagulant solutions such as heparin should also be avoided.

Albumin binds a relatively predictable proportion of calcium at a given pH, therefore multiple algorithms have been developed to derive estimates of iCa (1, 2, 11). However, derived iCa estimates are only approximations as calcium binding is also affected by several other variables including albumin, globulin, bicarbonate, citrate, pH and phosphate. In clinical practice, formulae predicting iCa are no longer used (1, 11).

As the relationship between iCa and pH is well defined and predictable, iCa can be mathematically “corrected” to a pH of 7.40 (2). This may produce clinically unacceptable estimates that do not reflect iCa in relation to the actual clinical pH regulation. This is particularly important in scenarios where disturbances in acid-base regulation are likely to occur for example chronic renal failure (12). Other scenarios where there are variations in albumin and/or globulin levels may also affect the ability of pH to predict calcium binding (2, 11). Variations in posture and venous stasis with prolonged use of a tourniquet may result in changes in the total protein concentration and pH. Consequently differences in the protein-bound and free calcium fractions may be observed (4). It is therefore preferable to report the uncorrected iCa to reflect the iCa present in the body at the time of analysis.

The best assessment of calcium status is by the direct measurement of iCa. Nonetheless, its use is limited due to technical challenges relating to equipment maintenance (including frequent electrode replacement), analytical performance and lack of measurement standardisation. Another limiting factor, particularly in the outpatient setting is the requirement for rapid analysis of an anaerobic sample (1, 13).

Despite the challenges associated with iCa measurement, iCa remains the most physiologically important measure of calcium homeostasis. Its measurement should be used whenever possible to diagnose disorders of calcium homeostasis (2, 14). Preanalytical factors that may lead to alteration in pH need to be minimised. There is

also a need for assay standardisation and improvement in analytical performance and availability of this relevant measure of calcium status in the outpatient setting.

#### **D. Total Calcium**

The measurement of tCa is widely available and most frequently requested to assess blood calcium status. Several methods are used to measure tCa – spectrophotometry of dye-calcium complexes, ISE's and atomic absorption spectrophotometry. The most routinely used methods in clinical practice are dye binding methods such as 5-nitro-5'-methyl-(1,2-bis(o-aminophenoxy) ethan-N,N,N',N'-tetraacetic acid (NM-BAPTA), cresophthalein complexone (CPC) and Arsenazo III. Measurement of tCa relies on a shift in the absorption of dyes upon binding to tCa (2). These methods are standardised and demonstrate acceptable precision and accuracy (15, 16).

The tCa is highly correlated with iCa in the absence of abnormalities in serum proteins or blood pH. It is therefore a reasonable substitute measurement in these patients (2). Conversely, alteration of calcium distribution between the bound and unbound fractions in some patients is responsible for the poor correlation between tCa and iCa. Examples are changes in serum proteins, pH; presence of certain anions that chelate calcium (i.e. lactate, citrate); and drugs or fatty acids that bind albumin. Specifically, derangements in serum proteins, particularly albumin affect tCa, in the absence of abnormalities in iCa. Hence the implementation of formulae to “correct” measured tCa for the actual concentration of albumin (2, 17). However, it remains inadequate in a number of clinical scenarios where iCa is the preferred measurement. These include: critical illness, blood transfusion containing citrate; advanced chronic kidney disease (CKD); hyperparathyroidism; hypercalcaemia of malignancy; and all neonates (1, 8).

Critically ill patients have associated hypoalbuminaemia in response to stress. In addition, altered affinity of albumin to calcium, acid-base abnormalities and acute elevations of free fatty acids with heparin sulphate administration, are common in this setting. Interestingly, Dickerson et al demonstrated that the provision of intravenous lipid emulsion is not associated with hypocalcaemia, and that iCa did not correlate with serum triglyceride concentrations (18). In light of the multiple alterations in

calcium homeostasis observed in critically ill patients, tCa is not suitable for monitoring calcium status in critically ill patients.

Late stage CKD is associated with variations in pH and albumin, which may variably change the different calcium fractions. Despite derivation of algorithms to predict iCa from tCa and albumin measurements, these tend to overestimate tCa, leading to inappropriate use of calcium containing phosphate binders or vitamin D and its analogues (19). Citrate complexes free calcium thus reducing the iCa fraction whilst tCa remains constant. This may occur following transfusion with citrated blood (1). In addition, citrate is administered as an anticoagulant for continuous renal replacement therapy. Here iCa is also measured in post-filter blood samples, to evaluate the adequacy of anticoagulation and identify citrate toxicity (20). Since direct citrate measurement is not routinely performed, it is not possible to estimate the reduced iCa fraction that occurs following binding to citrate. It is therefore essential to measure iCa directly in both these scenarios to identify disturbances in calcium homeostasis (1).

In primary hyperparathyroidism, hypercalcaemia of malignancy and neonatal hypocalcaemia, iCa has a greater diagnostic accuracy since tCa is often normal or only intermittently elevated. Multiple endocrine neoplasia type 1 mostly presents with hypercalcaemia and this is frequently used to screen asymptomatic individuals in affected families (1). False negative results may have clinical consequences in these affected individuals.

In neonates and children, critical illness alters the calcium binding to albumin, and other proteins may play a greater role in calcium binding. In addition, measurement of iCa provides a more reliable estimation of calcium status especially with the sudden adjustments that occurs at birth. Therefore, tCa is a poor predictor of calcium status in sick neonates and children compared to iCa (21).

The effect of posture on serum protein and tCa has been extensively studied. A decrease in the hydrostatic pressure of the vascular system is associated with fluid shifts from the ECF into the intravascular space. A change from an upright to a supine position is associated with a decrease in tCa greater than its biological variation (22).

This is related to a concomitant decline in serum proteins, particularly albumin (23). Prolonged venous stasis is associated with an increase in the protein bound fraction, causing an increase in tCa (24-26). Positioning either arm from a horizontal point to below the plane of the supine body also results in increased serum protein in the blood in that arm (23). The iCa, being diffusible, should not alter appreciably but significant differences have been observed with alterations in posture (22, 25). The iCa is only expected to change with alterations in pH which may occur after prolonged periods of venous status associated with forearm exercise. These manoeuvres may introduce variability in laboratory results that are inconsistent with clinical findings and should be avoided.

Where iCa calcium measurements are not available, tCa testing allows for screening of patients suspected of disorders of calcium homeostasis. However, its reliability relies on the absence of abnormalities in serum protein or pH. Since tCa binds primarily to albumin, variation in serum albumin levels alters the tCa concentration. As a means of improving the clinical utility of tCa measurements in patients with alterations in albumin concentration, equations to “correct” the tCa for serum albumin were derived. The following section will discuss the derivation of corrected calcium and its limitations.

### **E. Corrected Calcium**

Several factors have been described as having a possible influence on tCa in serum including specific gravity (SG), total protein and albumin concentration. This led to derivation of formulae to “correct” the measured tCa, taking into account these factors. Although SG varies mainly with total protein, other factors are known to affect it (i.e. abnormally high urea, glucose and lipid levels). Despite an assumption that a similar linear relationship exists between SG and tCa, the two correlate poorly. In addition, poor precision and lack of widespread availability of manual SG methods, do not advocate its use for deriving correction factors (25, 27). Total protein and albumin have shown significant correlations despite low correlation coefficients (25, 28). Corrections based on the total protein concentration are inaccurate when serum protein distribution is abnormal, particularly in hypergammaglobulinaemia, commonly encountered in patients with disorders of calcium homeostasis (13, 28).

Since tCa binds predominantly to albumin, a factor based on albumin was suggested to correct the tCa. This factor is also likely to remain valid in the presence of pathological elevations in globulin levels (25).

Various approaches have been adopted to derive algorithms or formulae to correct measured tCa for albumin in patients deemed free of calcium disorders. Most are based on linear regression of serum tCa concentration on albumin concentration using specific methods for tCa and albumin measurements. The most commonly cited formulae in the literature used to derive cCa include (11):

$$\text{Orrell (1971): } cCa = tCa \text{ (mmol/L)} + 0.0176 (34 - [\text{albumin}] \text{ (g/L)})$$

$$\text{Payne et al (1973): } cCa = tCa \text{ (mmol/L)} + 0.0246 (40.4 - [\text{albumin}] \text{ (g/L)})$$

$$\text{Berry et al (1973): } cCa = tCa \text{ (mmol/L)} + 0.0227 (46 - [\text{albumin}] \text{ (g/L)})$$

After exclusion of abnormal tCa values, linear regression of tCa on albumin was performed and the regression coefficient calculated (28, 29). Ideally, cCa formulae should be derived from patients with a wide range of albumin concentrations and without any disturbances in calcium homeostasis. In some, the calcium values were corrected to the mean albumin concentration using a formula established on their regression. This allowed for an arbitrary reference point for albumin concentration with which all individual cCa values can be compared (25). When this approach is applied over a wide range of albumin concentrations to derive cCa, confidence limits of corrected values are comparable with tCa ranges. Using linear regression, the cCa formula is determined as; *total calcium – (slope x albumin) + (mean normal total calcium – intercept calcium)*. Additionally, most formulae apply a correction of mean calcium values to the mean of the normal reference interval. The most widely used formula in clinical practice is based on the formula derived by Payne et al. While the original formula derived by Payne had a factor of 0.0246, the formula commonly implemented used a factor of 0.02 (originated from an anonymous author in 1977, British Medical Journal). When albumin concentrations are substantially lower than 30 g/L, cCa values can significantly affect clinical interpretation.

Herewith follows a description of the derivation of cCa formulae and their limitations. The Payne cCa formula was derived using specific calcium (CPC) and albumin (bromocresol green (BCG) dye binding) methods performed in a single laboratory.

Two hundred samples received by the laboratory for “liver function test” were used in this study. The distribution of their serum tCa and albumin concentrations was 1.77 to 2.70 mmol/L (reference range: 2.25 to 2.60 mmol/L) and 20 to 48 g/L (reference range: 37 to 47 g/L), respectively. Although the distribution of albumin values was adequate particularly in the low range, 24.5% of samples were hypocalcaemic (28). Since these abnormal calcium values were removed prior to performing linear regression, this significantly reduced the sample size. In addition, the BCG albumin method used is known to react with alpha globulins resulting in overestimation of albumin levels. Hypergammaglobulinaemia is common in patients with hypoalbuminaemia. This may have introduced an error in the regression coefficient.

In contrast, Orrell included nine hundred and fifty four specimens for the derivation of cCa comprising inpatients and outpatients. However, the exclusion of patients with disorders of calcium homeostasis was not considered when selecting patients from acute wards. These patients were therefore more likely to have abnormal tCa and albumin concentrations. The inpatients had a mean tCa and albumin concentration of 2.31 mmol/L and 34 g/L, respectively, whereas the outpatients were 2.42 mmol/L and 42 g/L, respectively. The mean albumin concentration was significantly different between the two groups. This difference may have been attributed to the postural differences in these patients. Using the mean of the tCa and albumin concentrations for all patients may have introduced an error in the relationship between tCa and albumin. The correlation coefficient of tCa on albumin on linear regression observed by Payne (0.989) et al was significantly higher than that determined by Orrell (0.707) despite a larger sample size (28). This was accounted for by the inclusion of patients with nephrotic syndrome. Evidence suggests that these patients have higher tCa for the degree of hypoalbuminaemia, as there may be other macromolecules that bind calcium more effectively than albumin. Furthermore, these patients have higher tCa than those with a similar degree of hypoalbuminaemia of another cause (28). Overall, these formulae demonstrate inadequacies during their derivation. In addition, they were derived in a single laboratory using specific tCa and albumin methods despite their widespread use in clinical laboratories (17). The use of regression coefficients derived from these studies, are therefore questionable for application in clinical practice.

The use of an average regression coefficient to correct tCa has been challenged through the application of certain techniques (i.e. alteration in posture and prolonged venous stasis). The resultant differences between regressions coefficients following these techniques have been used to suggest the unsuitability of using an average coefficient. However, the variable relationship between tCa and albumin is only observed following these techniques. Hence corrections cannot be made in their presence. A free flow technique should be used when obtaining blood for tCa and albumin measurements. Sudden changes in posture should be avoided and noted at the time of sample collection in case variability is introduced by these changes (23). In addition, the effect of venous occlusion on plasma albumin is not comparable to the steady and slow decline in plasma albumin due to disease (24, 26). Overall, average regression coefficients can be used to correct tCa in patients with hypoalbuminaemia with suspicion of calcium disturbance. This is provided they are derived from patients without disturbances in calcium metabolism (13).

Some cCa formulae have shown poor correlation coefficients between tCa and albumin with limited ranges observed over the regression lines. The calcium-albumin binding ratio cannot be assumed to be constant, making extrapolation at low albumin levels problematic. There may also be increased protein binding of calcium in the presence of hypoalbuminaemia. Hence the application of an average correlation coefficient, based on a constant linear relationship, may lead to inaccurate estimation of tCa at abnormally low albumin levels. This may mask the presence of hypocalcaemia (3). Payne et al noted that the cCa equation could not be applied in patients with nephrotic syndrome and hypoalbuminaemia (28). However, the albumin level at which these effects are most likely to occur is uncertain.

Whilst the majority of these cCa formulae were previously thought to have been derived from clinically appropriate data, none were validated using an independent dataset (consisting of a wide range of tCa and albumin concentrations) and failed to produce similar tCa estimates (30). They also assumed that the average correlation coefficient relating tCa to total protein or albumin was applicable across all patients (3). Importantly, none of the formulae were compared to iCa to assess their performance. In general, use of albumin-cCa tends to underestimate the incidence of hypocalcaemia and overestimate the incidence of hypercalcaemia compared to measured tCa. This is well documented in patients with renal failure (12, 31, 32).

Since a strong linear relationship exists between variables that affect cCa estimations, it is likely that correlation coefficients will falsely overestimate the degree of agreement between the iCa and cCa (11). Use of cCa in critically ill surgical patients is also associated with significant underestimation of hypocalcaemia and overestimation of hypercalcaemia (33, 34). In multiple trauma patients receiving specialized nutritional support, cCa was overestimated in patients with hypocalcaemia (18).

The validity of corrected calcium formulae has also been evaluated in several patient groups including the elderly, neonates and CKD patients. Albumin-cCa in elderly patients suffers from underestimation of hypocalcaemia despite being less common than hypercalcaemia. Hypercalcaemia is attributed to the higher incidence of malignant disorders and disturbances in calcium homeostasis common in these patients. It has been suggested that cCa formulae are only valuable in excluding hypercalcaemia in very old, bedbound patients (35, 36). In neonates, the relationship between tCa and albumin is significantly different compared to adults. The effect is clinically significant when correcting calcium using adult-derived cCa equations. The varying degrees of renal dysfunction and altered binding of calcium to albumin, is responsible for the poor performance of cCa in sick neonates (21). In addition, the higher incidence of hypoalbuminaemia does not advocate correction of tCa using a fixed binding ratio of tCa to albumin since it increases in hypoalbuminaemia. Derivation of neonate-specific equations also shows poor prediction of calcium status compared with iCa, particularly in detecting hypocalcaemia. Despite these limitations, adult-derived cCa equations are suggested for use in children one year and older (21). Below this age, the derivation of neonate-specific equations is limited by the impracticality of collecting data in the specific age groups.

The National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF-K/DOQI) clinical guidelines of 2003 advised maintenance of normal serum tCa, to avoid adverse effects on bone mineralization and increased mortality (19, 37). The formula derived by Orrell most closely approximates cCa in stable haemodialysis patients and was recommended where iCa is inaccessible (11). However, cCa demonstrates poor diagnostic performance in correctly classifying hypocalcaemia and hypercalcaemia. The risk of underestimation is significantly increased by

hypoalbuminaemia (less than 30 g/L) and lower total carbon dioxide concentrations (tCO<sub>2</sub>), particularly in later stages of CKD. Hypoalbuminaemia is also a risk factor of overestimating calcium status (1, 19, 32). Despite the potential adverse outcomes associated with false negative and false positive results, lack of availability of iCa measurements limit its general use in these patients.

Despite these concerns, these formulae are still applied widely in clinical practice to screen patients with disturbances in calcium homeostasis whilst acknowledging their limitations. Assays for the measurement of tCa and albumin are readily available on automated analysers, allowing for estimations of albumin-cCa where iCa is not readily available. The costs of providing cCa tend to be lower than those associated with iCa, justifying the use of cCa formulae. Despite their poor performance in different patient groups, improvement of cCa formulae can substantially increase their clinical performance (2).

## **F. Adjusted Calcium**

In March 2015 the Association for Clinical Biochemistry and Laboratory Medicine published a position paper with the recommendation that laboratories should develop and use locally derived formulae specific to their calcium/albumin methods, to report the “adjusted calcium”. (17). The term “adjusted calcium” was recommended for use rather than “corrected calcium”. Note that in this literature review the term “corrected calcium” (cCa) has been used consistently to refer to non-method-specific formulae, while the term “adjusted calcium” (aCa) has been used consistently to refer to method-specific formulae.

Similar to cCa, aCa formulae are based on the linear regression of serum tCa concentration on albumin concentration using specific methods for tCa and albumin measurements. Numerous adjusted calcium (aCa) formulae are available. Their validity is restricted to the specific combination of tCa and albumin methods and analytical platform. Therefore their application should be limited to use in laboratories using the same methodology and same analytic techniques as the laboratory at which they are derived.

Most laboratories measure serum or plasma albumin by automated colorimetric dye binding methods. These dyes, BCG and bromocresol purple (BCP) bind albumin resulting in a shift in the absorption spectrum. Although automated immunochemical methods show better performance, they are expensive and time consuming, limiting their use in clinical laboratories (38). BCG albumin closely correlates with albumin measured by specific immunological methods in the range of 5 to 48 g/L (13). Therefore use of any albumin method is suggested for aCa provided it is used to both define and make the adjustment.

The impact of a change in the method used for the calcium assay on aCa levels has been shown to be small and not clinically significant (39). In contrast, systemic differences between BCG and BCP methods are recognised (30). As previously noted, measurement of albumin by the BCG method is flawed by the overestimation of albumin concentration in the presence of increased alpha globulins. Increased alpha globulins are usually associated with low albumin levels. Hence, the adjustment varies as the positive bias in albumin levels becomes higher in hypoalbuminaemia. This has been minimised by use of a rapid reaction technique with newer methods thus reducing the effect of the globulin fraction (30). The relationship between serum tCa and albumin levels has been shown to be linear at albumin concentration of 32 to 50 g/L. However, this correlation deviated at albumin levels less than 32 g/L. This is where the globulin fraction is likely to have a greater impact when using the BCG albumin method (40). Although BCP methods are more specific, albumin levels are underestimated in patients receiving haemodialysis, particularly continuous ambulatory peritoneal dialysis (CAPD). This is due an endogenous non-dialyzable uraemic toxin, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), that inhibits only BCP binding to albumin (41). In addition, the redox status of thiol groups attached to albumin molecules affect its reactivity with BCP (32). The effect of the albumin assay on aCa values has been well demonstrated in patients with CKD (30, 32, 38). The change between BCG and BCP albumin methods has demonstrated disparity in the classification of calcium status. The change in analytical platform may also influence the relationship between tCa and albumin. This suggests that the regression coefficient of tCa on albumin depends on the analytical technique used, irrespective of similarities in analytical principles (40, 42). The aCa is most clinically useful in patients with mild disturbances in calcium homeostasis (i.e. borderline

hypocalcaemia and hypercalcaemia). It is therefore important that a correct regression coefficient is applied to avoid misclassification in calcium status.

Given the discrepancy in serum albumin depends on the method and analytical platform used, it is suggested that laboratories derive their own suitable aCa formulae using their own data collected over an extended period of time. This also extends to laboratories using other albumin-specific methods. These formulae must also be monitored regularly for their validity (27, 28, 40, 42). Current recommendations by the Association for Clinical Biochemistry and Laboratory Medicine (ACB) are that laboratories determine their own aCa formulae specific for their population data and analytical methodology for tCa and albumin and platform. Laboratories may choose to derive their formula from the regression of tCa on albumin using local data, or validate an existing published formula (17). Their validity should be reviewed regularly, particularly with a change in the analytical methodology or performance (39, 40, 42).

Several studies have reported the derivation and validation of aCa formulae that are appropriate for the local population and analytical methodologies. Although these formulae demonstrated good internal validity, like the traditional cCa formula by Payne et al, these aCa formulae had a tendency to underestimate hypocalcaemia and overestimate hypercalcaemia (27, 43, 44). It is postulated that use of the mean tCa of the local dataset rather than harmonized reference intervals may improve classification of calcium status (21, 42). Also, their performance was investigated only against the Payne cCa formula and not against iCa as the gold standard. Comparison of aCa against the gold standard marker of calcium homeostasis is required to confirm the true validity of locally derived aCa formulae as the iCa concentration is the most physiologically appropriate measure of calcium homeostasis. The ACB recommendation therefore prompted the derivation of an aCa formula specific to our tCa and albumin methods and analytical platform at GSH, as well as validation against iCa.

*Word count: 5204*

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## **CHAPTER 2: PUBLICATION READY MANUSCRIPT**

This manuscript meets all the requirements set out by the “Instructions for Authors” of Clinical Chemistry (Part 3).

### **2.1. JOURNAL CATEGORY: Evidence-Based Laboratory Medicine and Test Utilization**

### **2.2. TITLE: Validation of an adjusted calcium formula using the Roche calcium (NM-BAPTA) and albumin (BCG) methods at Groote Schuur Hospital**

Mbali Ndlovu<sup>1</sup>, Heleen Vreede<sup>1</sup> and Fierdoz Omar<sup>1</sup>

1. Division of Chemical Pathology, Groote Schuur Hospital, National Health Laboratory Service and University of Cape Town, South Africa

Corresponding author

Mbali Ndlovu, B.Pharm, MBBCh, FCPATH SA (Chem)

Division of Chemical Pathology

C17, Groote Schuur Hospital

Anzio Road, Observatory, 7925

Telephone: 27 21 404 6330

Fax number: 27 21 404 4050

Email: [Mbali.ndlovu@uct.ac.za](mailto:Mbali.ndlovu@uct.ac.za) or [Mbali.ndlovu@nhls.ac.za](mailto:Mbali.ndlovu@nhls.ac.za)

Keywords

Adjusted calcium formula, Roche, NM-BAPTA, BCG

This manuscript has not been previously presented.

### 2.3. ABSTRACT

**Introduction:** Most laboratories continue to adjust serum total calcium (tCa) concentration for serum albumin as a surrogate marker of calcium status, despite the availability of ionised calcium (iCa) measurement. Current recommendations by the Association for Clinical Biochemistry and Laboratory Medicine (ACB) advocate that laboratories should use formulae specific for their tCa and albumin methods and analytical platforms. The National Health Laboratory Service at Groote Schuur Hospital (GSH) undertook to investigate this recommendation. An adjusted calcium (aCa) formula specific for the Roche serum tCa and albumin methods was derived from 3131 patients. We investigated the validity and clinical utility of this locally derived aCa formula.

**Methods:** The tCa, albumin and iCa were analysed in blood from 162 inpatients and outpatients at GSH. Corrected calcium (cCa) was calculated using the Payne cCa formula, and aCa was calculated with the new aCa formula. Patients were classified as hypo-, normo- or hypercalcaemic using iCa, tCa, cCa and aCa measurements. Cohen's kappa statistic, log-linear and logistic regression models and interclass and concordance correlation coefficients were used to assess agreement between tCa, Payne cCa and aCa against iCa (gold standard). Agreement was further assessed according to renal status and albumin concentrations.

**Results:** The aCa demonstrated good correlation with iCa, but its performance was not significantly better than tCa or Payne cCa in correctly classifying calcium status. Furthermore, albumin concentration was demonstrated to predict the performance of the calcium status classification by the aCa and cCa formulae, irrespective of renal status.

**Conclusion:** The laboratory-specific aCa formula did not perform significantly better than tCa and the Payne cCa formula. This implies that aCa does not add value over tCa where iCa measurements are not readily available.

*Word count: 278*

## 2.4. LIST OF ABBREVIATIONS

**aCa**, adjusted calcium; **ACB**, Association for Clinical Biochemistry and Laboratory Medicine; **BCG**, bromocresol green; **BCE**, bromocresol purple; **cCa**, corrected calcium; **CCC**, concordance correlation coefficient; **CPC**, O-cresolphthalein complexone; **CKD**, chronic kidney disease; **GSH**, Groote Schuur Hospital; **iCa**, ionised calcium; **ICC**, interclass correlation coefficient; **NHLS**, National Health Laboratory Service; **NM-BAPTA**, 5-nitro-5'-methyl-1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; **tCa**, total calcium;

## 2.5. INTRODUCTION

In clinical practice, calcium status is investigated by the direct measurement of ionised calcium (iCa) or its estimation from measurements of total calcium (tCa). Despite being the most accurate measure of calcium status, iCa measurement is not universally utilised due to the technical challenges associated with equipment maintenance, analytical performance and lack of measurement standardisation. In addition, the requirement for rapid analysis of an anaerobic sample to avoid alterations in pH, particularly limits its application in the outpatient setting (1, 2). Measurement of tCa remains a suitable alternative in patients without abnormalities in serum proteins and pH (3). Since tCa binds predominantly to albumin, formulae were developed to correct or adjust tCa for serum albumin (4). Consequently, most laboratories continue to use albumin-corrected tCa as a surrogate marker of calcium status (5).

The validity and usefulness of adjusted or corrected calcium (cCa) formulae have been questioned in recent years due to wide inter-laboratory differences in cCa values obtained by different analytical methods and platforms used to measure serum tCa and albumin (4, 6). In addition, none of these formulae were validated on an independent dataset to assess their performance, thus indicating insufficient evidence for their application in clinical practice (7). The cCa formulae are mostly based on the linear regression of tCa on albumin using specific methods for the measurement of tCa and albumin (8). Payne et al described the most frequently used formula to correct for tCa in 1973. This formula was derived from the analysis of the linear regression relationship between tCa and albumin, measured using O-cresolphthalein complexone (CPC) and bromocresol green (BCG) dye binding methods, respectively. Two hundred samples received by the laboratory for “liver function test” were used in this study. The distribution of the serum tCa and albumin concentrations was 1.77 to 2.70 mmol/L (reference interval: 2.25 to 2.60 mmol/L) and 20 to 48 g/L (reference interval: 37 to 47 g/L), respectively. Although the distribution of albumin values was adequate particularly in the low range, 24.5% of the samples were hypocalcaemic. An additional correction of mean calcium values to the mean of the normal reference range was also applied. The cCa formula was determined as  $cCa = tCa \text{ (mmol/L)} + 0.0246 (40.4 - \text{albumin (g/L)})$ . Ideally, a cCa formula should be derived from patients

with a wide range of albumin concentrations and without any disturbances in calcium homeostasis. The exclusion of abnormal calcium values prior to performing linear regression analysis significantly reduced the sample size. In addition, the BCG albumin method used is known to react with alpha globulins resulting in the overestimation of albumin levels. Hypergammaglobulinaemia is common in patients with hypoalbuminaemia. This may have introduced an error in the linear regression analysis. An important limitation in this study is that the performance of this cCa formula was not tested against iCa to confirm its validity.

This formula has been used extensively in clinical practice, but was later shown to perform poorly in the following clinical scenarios: critical illness, nephrotic syndrome and hypoalbuminaemia; blood transfusions containing citrate; advanced chronic kidney disease (CKD); hyperparathyroidism; hypercalcaemia of malignancy; as well as all neonates (1, 9-13). The effect of hypoalbuminaemia on the performance of cCa formulae has been particularly well demonstrated in patients with chronic kidney disease. Use of cCa in this group tends to underestimate the incidence of hypocalcaemia and overestimate the incidence of hypercalcaemia (13-16).

In recent years, with the development of newer methodologies for albumin measurement and the shift from BCG to bromocresol purple (BCP) albumin methods, the performance of this formula has fallen into further question (6, 12). As these methods measure albumin variably, the relationship between tCa and albumin also varies depending on the method used (6, 15). Several studies have demonstrated misclassification of calcium status, particularly in hypoalbuminaemic patients with use of these adjustment equations (8, 17). This implies that a single cCa formula cannot be used across analytical methods or platforms (12). The Association for Clinical Biochemistry and Laboratory Medicine (ACB) therefore recommends that laboratories determine their own adjusted calcium (aCa) formula specific for their analytical methodology and platform for tCa and albumin (8). Laboratories may choose to derive their formula from the regression of tCa on albumin using local population data, or validate an existing published formula (8). Ideally its validity should be reviewed regularly, particularly with a change in the analytical methodology or performance (6, 12, 18).

The National Health Laboratory Service (NHLS) at Groote Schuur Hospital (GSH) undertook this recommendation to derive an aCa formula specific to their tCa and albumin methods. The following aCa formula was derived according to guidelines described by the ACB (8):  $aCa \text{ (mmol/L)} = tCa \text{ (mmol/L)} - (0.0149 \times [\text{albumin}] \text{ (g/L)}) + 0.6216$  (unpublished data). This aCa formula was derived using simultaneously run albumin and tCa data from 3131 GSH patients, with the following exclusion criteria: patients with renal impairment (creatinine  $>200 \mu\text{mol/L}$  or urea  $>15 \text{ mmol/L}$ ); hypomagnesaemia (using hypokalaemia as a surrogate marker); liver disease (using alanine transaminase and alkaline phosphatase  $>$ upper reference limit); tCa concentration  $<2.0$  and  $>2.7 \text{ mmol/L}$ ; hypo/hyperparathyroidism; vitamin D deficiency or toxicity (excluded if vitamin D was requested); hypoadrenalism (excluded if cortisol was requested); patients on parenteral nutrition and patients with malignancy (excluded if tumour markers were requested).

The aim of this study is therefore to validate the performance of this newly derived aCa formula against iCa, as the gold standard, in correctly categorising calcium status and to compare its performance to that of tCa and cCa, respectively.

## **2.6. MATERIALS AND METHODS**

### **I. SUBJECT SELECTION**

A total of 162 patients (medical and surgical) were recruited from inpatient (n=10) and outpatient (n=152) departments at GSH over a one-year period (June 2015 to July 2016). Exclusion criteria included: younger than 18 years of age, critically ill patients and patients who could not provide written informed consent. The inclusion criteria included patients older than 18 years of age, outpatients from medical (endocrine, cardiac, renal, liver and diabetic) and surgical clinics as well as healthy controls. Written informed consent was obtained prior to blood collection. The aim was to obtain a wide range of tCa and albumin concentrations. The outpatients were recruited on routine follow-up appointments.

Venous blood was collected without (or minimal) venous stasis into calcium-balanced heparinised syringes (BD “calcium balanced”), transported on ice and analysed within

15 minutes, for iCa. In addition, blood was collected into serum separator tubes and allowed to clot at room temperature, after which they were centrifuged. These were processed for serum tCa and albumin. Prior to commencement of the study, institutional and research ethics approval was obtained for this study (HREC REF: 724/2015).

## **II. MEASUREMENT**

The concentration of serum tCa and albumin were measured on the Roche Cobas 6000 using the 5-nitro-5'-methyl-1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (NM-BAPTA) and BCG albumin dye binding methods, respectively. The analytical precision for tCa was 1.47 and 1.66% at 2.22 and 3.38 mmol/L, respectively. The analytical precision for albumin was 1.75 and 1.83% at 33 and 49 g/L, respectively. The iCa was measured on the Roche Cobas b123 point of care system, by ion selective electrode. The analytical precision for iCa was 2.99, 1.51 and 1.34% at 0.70, 1.27 and 1.75 mmol/L, respectively.

## **III. STATISTICAL ANALYSIS**

Descriptive and comparative analyses were performed using STATA Release 14 (StataCorp 2015, College Station, TX: StataCorp LP) to assess the agreement between tCa, cCa and aCa against iCa. Agreement was further assessed according to renal status and albumin concentrations (with albumin lower than 35 g/L considered hypoalbuminaemic) (19). The categorisation of calcium status as hypo-, normo- or hypercalcaemic was based on a published reference interval for tCa (2.15-2.50 mmol/L) (the same reference interval was used for cCa and aCa). (19) The reference interval for iCa was provided by the manufacturer (1.12-1.32 mmol/L).

Data was assessed using the following three methodologies: (a) Agreement between iCa and the three calcium measurements (tCa, cCa and aCa) in classifying patients according to calcium status (i.e., hypo, normo and hyper) was assessed by weighted Cohen kappa statistic, the log-linear model of independence as well as the quasi-independence model. The quasi-independence model provided the best fit for our data

and was therefore used to further delineate findings. (b) Quantitative agreement between the different calcium measurements in placing patients at the same level relative to their respective reference intervals was assessed using interclass and concordance correlation coefficients. For this analysis, normalised results expressed as z-scores of the reference interval (calculated as  $[\text{calcium} - \text{mean calcium}]/\text{standard deviation of 95\% confidence limits}$ ) were used. (c) To assess whether albumin concentration and renal status influence the agreement between iCa and the different calcium measurements in correctly categorising calcium status, logistic regression modelling was used.

Explanation of statistical tools used:

- a) Weighted Cohen kappa statistic, and log-linear models of independence and quasi-independence:

These models present another way of evaluating agreement by comparing the observed counts to the values predicted by the log-linear models. Subsequently, they provide insight into the patterns of agreement and disagreement. The fit of these models are evaluated by the Pearson and Deviance statistics. The quasi-independence model includes an additional coefficient that describes agreement beyond that expected under independence. In addition, it calculates the odds of a calcium result being in agreement with the iCa.

- b) Interclass and concordance correlation coefficients:

The interclass correlation coefficient (ICC) was used to assess the agreement of quantitative measurements of iCa and the three calcium measurements. The ICC has been demonstrated to be a more appropriate measure of agreement than linear regression analysis. The consistency of agreement and the absolute agreement are assessed in the form of a ratio of variance on a scale of 0-1, where 0 indicates complete disagreement and 1 complete agreement. To allow for direct comparison between iCa; and tCa, cCa and aCa values, calcium values were normalised to z-scores of their respective reference intervals (the reference interval for iCa differs substantially from that of these other calcium measurements) (7, 20).

The concordance correlation coefficient (CCC),  $\rho_c$  was also used to assess the agreement between the iCa and the three calcium measurements. It is supplemented

by the Pearson's coefficient ( $r$ ) and bias-correcting factor ( $C_b$ ), which evaluate precision (linear fit of the relationship) and accuracy (measures how far the best-fit (45-degree) line deviates from the line of identity), respectively.  $C_b$  of 1 implies no deviation from the 45-degree line and hence good agreement. The CCC therefore assesses both the correlation and agreement between iCa and the three calcium measurements. The CCC satisfies  $-1 \leq \rho_c \leq +1$ , where 0 corresponds to no agreement and; -1 and +1 corresponds to complete negative and positive agreement, respectively. The CCC is complementary to the ICC in measuring agreement between iCa; and tCa, cCa and aCa values. Under these coefficients, agreement will be further assessed according to renal status and albumin concentrations.

c) Logistic regression model:

Using this model, we evaluated the proportion of calcium values classified correctly between iCa and the three calcium measurements based on renal status and albumin concentration. We further sought to evaluate the albumin concentration at which the agreement between iCa and the three calcium measurements performed poorly. This will assist in determining an albumin concentration cut-off whereby use of tCa or aCa would provide an unreliable estimation of calcium status. A p-value of  $<0.05$  was considered statistically significant.

## **2.7. RESULTS**

### **I. DESCRIPTIVE ANALYSES**

Patients were categorised according to renal status based on a diagnosis of renal disease by the nephrology clinic. Patients with renal disease are referred to as the renal patients and patients without confirmed renal disease are referred to as non-renal patients. The renal group comprised of 24 patients with the remaining classified as non-renal (n=138). The diagnoses of the renal group included lupus nephritis, post-renal transplant, and polycystic kidney disease. None of the patients were receiving renal dialysis. Their median (interquartile range) serum creatinine concentration was 113  $\mu\text{mol/L}$  (77 to 205  $\mu\text{mol/L}$ ). The patient characteristics of the renal and no-renal groups are presented in Table 1. A serum albumin concentration of 35 g/L was used to stratify patients as normoalbuminaemic (n=148) and hypoalbuminaemic (n=14). The

median (interquartile range) for serum albumin was 43 g/L (40 to 45 g/L). The range, median and interquartile ranges of the calcium values are presented in Table 2.

## II. COMPARATIVE ANALYSES

The comparison of classification of calcium status between iCa and; tCa, cCa and aCa, respectively, are presented in Table 3. The percentage agreement between iCa; and tCa, cCa and aCa was 56%, 58% and 57%, respectively. The percentage agreement between iCa and the three calcium measurements did not differ significantly between non-renal and renal patients (Table 4). The percentage agreement between iCa and tCa was higher in hypoalbuminaemia (71%) than in normoalbuminaemia (55%). In contrast, percentage agreement between iCa; and cCa and aCa were higher in normoalbuminaemia than in hypoalbuminaemia (Table 4). The most disagreement in calcium classification was demonstrated when iCa was hypocalcaemic (the corresponding tCa, cCa and aCa classified these patients as normocalcaemic in a large number of cases); or normocalcaemic (where tCa, cCa and aCa classified a fair number of these as hypercalcaemic). This pattern of disagreement in iCa-classified hypocalcaemia was apparent irrespective of renal status or albumin concentration. However, disagreement when iCa-classified normocalcaemia occurred more frequently in non-renal and normoalbuminaemic cases.

- a) Agreement assessment using weighted Cohen kappa statistic, log-linear model of independence and quasi-independence model:

The weighted Cohen's kappa statistic demonstrated poor agreement between iCa and the three calcium measurements (Table 5). The log-linear independence model demonstrated a poor fit for our data and the quasi-independence model was therefore used. The coefficients and odds ratio using the quasi-independence model for comparison between iCa; and tCa, cCa and aCa categories are presented in Table 5. This model demonstrates strong agreement between iCa and tCa in hypocalcaemia and hypercalcaemia with positive coefficients, while the normocalcaemic group demonstrated less agreement. A similar pattern of agreement was demonstrated for cCa and aCa when compared with iCa. Furthermore, aCa was more likely to correctly

identify hypocalcaemic cases in true hypocalcaemia (estimated odds  $2.84 \times 10^7$ ) than not. For tCa and cCa, the estimated likelihood odds of correctly classifying true hypocalcaemia were 18.34 and 3.21, respectively. A similar pattern was observed when identifying normocalcaemic cases in true normocalcaemia (see estimated odds in Table 5). There were insufficient patients in the hypercalcaemia category to assess performance in that category.

b) Quantitative agreement using interclass and concordance correlation coefficients:

The data associated with the different calcium measurements (iCa, tCa, cCa and aCa) followed normal distributions. The consistency of agreement and absolute agreement between iCa and the three calcium measurements are described in Table 5. Consistency of agreement between iCa; and tCa and aCa, were demonstrated to be better than that between iCa and cCa. Renal patients demonstrated the best consistency of agreement, with better ICC for all calcium measurements.

Furthermore, the consistency of agreement between iCa and tCa, cCa and aCa, respectively, was better in patients with normoalbuminaemia. While hypoalbuminaemic patients had better ICC's between iCa; and cCa and aCa, their confidence intervals were wide.

The relationship between iCa and the three calcium measurements by CCC is described in Table 5. Evaluation of the agreement by CCC demonstrated similar findings to the ICC. The Pearson's coefficient demonstrated reasonable correlations between iCa and the various calcium measurements, with the best correlation observed between iCa and aCa. However, there was marked deviation from the line of identity and therefore poor agreement (see rho\_c in Table 5) for all calcium measurements. The correlation and bias between iCa and tCa, cCa and aCa are shown in Figures 1 (a), (b) and (c), respectively. There is a tendency to overestimate at low serum calcium and underestimate at high calcium concentrations. The best agreement was demonstrated in the renal group, with fairly good correlation (demonstrated by the Pearson's coefficients) and less deviation from the line of identity across all measurements (see rho\_c in Table 5).

The agreement between iCa and tCa did not differ significantly between normoalbuminaemic and hypoalbuminaemic groups. However, less deviation from the line of identity ( $C_b$  of 0.887) was seen in the hypoalbuminaemic group. In contrast, cCa and aCa demonstrated better agreement and less deviation from the line of identity in the normoalbuminaemic group, but better correlation in the hypoalbuminaemic group (demonstrated by the Pearson's coefficients).

- c) Logistic regression model to assess agreement of calcium classification with respect to albumin concentration or renal status:

This model included albumin concentration and renal status as predictors of consistent calcium classification. Overall, albumin concentration and renal status were statistically significant predictors of agreement between iCa and aCa classification (LR  $\chi^2= 6.10$ ,  $p=0.047$ ). The same was true for cCa (LR  $\chi^2 = 6.10$ ,  $p=0.047$ ). Agreement between the iCa and tCa classification was related to albumin concentration and renal status (LR  $\chi^2 = 3,61$ ,  $p=0.164$ ) but not statistically significant. Therefore, further evaluation of albumin concentration and renal status as predictors of agreement between iCa and tCa classification was not performed.

The coefficient for albumin concentration when holding renal status constant was statistically significant ( $p=0.018$ ), with a one-unit increase of albumin being associated with increased log odds of 1.073 that the classification of calcium status between iCa and aCa would be the same. The presence of renal disease was associated with increased log odds (0.348) that classification of calcium status between iCa and aCa would be the same, but was not statistically significant ( $p=0.462$ ). This implies that renal status does not significantly influence the performance of this calcium measurement in correctly classifying calcium status. This model demonstrated better agreement in the calcium classifications for cCa and aCa at higher albumin concentrations. However we could not demonstrate the serum albumin concentration at which tCa or aCa become unreliable indicators of calcium status.

## 2.8. DISCUSSION

### I. DISCUSSION

The traditional marker of calcium status remains the corrected or adjusted tCa concentration, despite the availability of an alternative method of measurement, iCa. The NHLS laboratory at GSH undertook to develop and validate an albumin-aCa formula derived from local laboratory data. This was based on the recommendations by the ACB. Its performance was investigated against iCa (gold standard). Comparison of aCa against the gold standard marker of calcium homeostasis is required to confirm the true validity of a locally derived aCa formula. The pH-uncorrected iCa, reflecting the iCa relative to the actual clinical pH, was used to prevent the underestimation of the true iCa concentration.

The overall pattern of disagreement demonstrated in our data is consistent with previous studies (7, 15). There is generally poor agreement between iCa and the different calcium measurements. The cCa or aCa tend to underestimate the incidence of hypocalcaemia and overestimate the incidence of hypercalcaemia compared to the measured iCa. This is well demonstrated in stable CKD patients receiving haemodialysis with hypoalbuminaemia (13-15). Hypoalbuminaemia is an additional risk factor of overestimating calcium status (1, 15, 16). However, we were unable to demonstrate a greater incidence of misclassification specifically in renal patients. In fact, the pattern of disagreement when iCa was hypocalcaemic occurred irrespective of renal status. This difference may have been accounted for by the difference in the renal group in our study to that previously investigated. Only four patients in the renal group were hypoalbuminaemic, compared to those that were normoalbuminaemic (n=20). In addition, none of our renal patients were undergoing renal dialysis. The small number of hypoalbuminaemic and renal patients may have been insufficient in demonstrating the previously noted findings (7, 15).

The best consistency of agreement and correlation with iCa was shown with aCa. The aCa also demonstrated the best agreement in the presence of renal disease. In contrast, several studies have showed unsatisfactory performance of the Payne formula and other correction formulae when compared to iCa in stable CKD patients on haemodialysis (7, 13, 14). Our renal group consisted of only 24 patients with a fairly

narrow range of serum creatinine concentrations. The renal group may have been more homogenous and thus shown better agreement compared to the non-renal group. The non-renal group varied widely from healthy controls to diabetic, endocrine, liver, cardiac and surgical patients. The heterogeneity of this patient group may have influenced the performance of the aCa formula. Nephropathy is a common complication in diabetic patients. The presence of renal damage was not assessed in these patients. Therefore we are uncertain whether this was a confounding factor to our results. Additionally, the performance of aCa formulae has not been previously investigated in renal patients not undergoing renal dialysis as in our study group. Hence the ability of these adjustment equations in correctly classifying calcium status specifically in this group of renal patients has not been reported. Clearly defined parameters for renal disease in a larger patient group would convincingly distinguish renal and non-renal patients and aid in delineating these findings. As these findings are conflicting to those previously reported in renal patients, our study does not permit for a more cohesive interpretation of these results.

The correlation between iCa and tCa in the hypoalbuminaemia and normoalbuminaemia was not significantly different. This was an unexpected finding since tCa correlates with iCa in patients with normal serum proteins (2, 3). Overall, normoalbuminaemia showed better performance between iCa and the three calcium measurements. The aCa demonstrated the best correlation with iCa in normoalbuminaemic patients. However, this was not significantly different from the tCa and cCa measurements. The inconsistencies in the agreement and correlation between iCa and the three calcium measurements when stratifying by albumin concentration suggest that these findings are inconclusive. A larger number of hypoalbuminaemic patients with varying albumin concentrations is required to more adequately describe the agreement of tCa and aCa with iCa measurements in our population.

Normally, serum albumin concentrations have a limited range in the non-critically ill, making it difficult to assess the linear relationship between tCa and albumin at these levels. However, Ashby et al demonstrated a deviation in the correlation at albumin concentrations less than 32 g/L. This is where the globulin fraction is likely to have a greater impact on the albumin concentration when using the BCG albumin method

(6). Abnormally low albumin concentrations may therefore result in clinically significant changes in calcium status classification (9). We were able to demonstrate improved agreement between iCa; and cCa and aCa classifications with higher albumin concentrations. Nonetheless, we were unable to demonstrate the albumin level at which the correlation between tCa or aCa, and albumin will deviate. Hence we could not establish the serum albumin concentration at which tCa or aCa are unreliable indicators of calcium status. However, the above findings suggest that the use of this aCa formula in patients with abnormally low albumin concentration may lead to discordant classification between iCa and aCa. This may lead to clinically inappropriate estimates of calcium status.

Differences between the BCG and BCP albumin methods are well recognised, particularly in patients with CKD (15, 21, 22). Since both formulae were derived from serum albumin measured by the BCG method, marked differences in their performance were not observed.

Overall, aCa demonstrated the best consistency of agreement and correlation with iCa. However, these findings were not significantly different from those shown by tCa and cCa. Although the quasi independence model demonstrated increased odds of aCa of identifying true hypocalcaemia and normocalcaemia than tCa and cCa, our other findings did not show better performance. The large estimated likelihood odds of aCa classifying true hypocalcaemia do suggest that there is a strong outcome. However, due to the large values in the hypocalcaemia category (aCa) relative to other values, the odds obtained cannot be sensibly interpreted.

Previous studies have demonstrated good agreement of tCa with iCa to an extent that is similar to various cCa formulae (7, 23, 24). This has brought into dispute the value of correction or adjustment formulae. Jain et al demonstrated similar performance of a new cCa formula and tCa in stable haemodialysis patients using the ICC to compare its agreement with iCa (14). Despite demonstrating similar performance of their new cCa formula to our aCa formula, they recommended their formula for use in stable haemodialysis patients. Furthermore, agreement improved when an alternative reference interval was applied to iCa values. The clinical impact of using a harmonised reference interval for aCa when validating a local aCa formula has been previously investigated Davies et al. They showed that the use of a harmonised tCa

reference interval would have resulted in misclassification of calcium status in hypocalcaemia and normocalcaemia, compared to a locally derived tCa reference interval (18). We therefore postulate that use of a reference interval derived from our population data may have improved the agreement between iCa; and tCa, cCa and aCa. Further investigation of the agreement between iCa and aCa with the use of a locally derived reference interval is warranted.

## **II. LIMITATIONS**

Serum creatinine levels for non-renal patients were not available and therefore presence of underlying renal disease could not be evaluated. This was particularly important since this group had a higher median for age than the renal group. The relatively small sample size (n=162) with a limited spread of calcium and serum albumin concentrations, particularly the hypercalcaemic and hypoalbuminaemic patients was a further limitation in our study. A larger sample size is required to confirm the findings in the renal and hypoalbuminaemic patients. A free flow technique should be used when obtaining blood for tCa and albumin measurements. Some patients necessitated the use of a tourniquet but we avoided prolonged venous stasis. This may have introduced variability in tCa and albumin measurements.

### **2.9. CONCLUSION**

This study demonstrates that the laboratory-specific aCa formula does not perform significantly better than tCa and the Payne cCa formula. We demonstrated that albumin concentration is a predictor of correct calcium classification when correction or adjustments are made. These results imply that adjusted calcium does not add value over tCa, where iCa measurements are not readily available. Further investigation into the agreement of iCa and aCa is warranted following the application of population specific tCa reference intervals.

*Word count: 4312*

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## 2.11. TABLES

<b>Renal Patients</b>	
<b>Serum creatinine (<math>\mu\text{mol/L}</math>)</b>	
Median	113
Range	46 – 1173
[Interquartile range]	[77 – 205]
<b>Age (years)</b>	
Median	33
Range	21 – 75
[Interquartile range]	[31 – 50]
<b>Gender</b>	
Male	10
Female	14
<b>Non-renal Patients</b>	
<b>Age (years)</b>	
Median	49
Range	18 – 81
[Interquartile range]	[35 – 60]
<b>Gender</b>	
Male	52
Female	86

**Table 1: Demographic data of patients in the renal and non-renal groups.**

	<b>Ionised calcium (mmol/L)</b>	<b>Total calcium (mmol/L)</b>	<b>Corrected calcium (mmol/L)</b>	<b>Adjusted calcium (mmol/L)</b>
<b>All patients</b>				
<b>Median</b>	1.14	2.39	2.31	2.36
<b>Range</b>	0.64 – 1.51	1.45 – 2.83	1.45 – 2.81	1.48 – 2.84
<b>[Interquartile range]</b>	[1.10 – 1.18]	[2.30 – 2.44]	[2.25 – 2.37]	[2.30 – 2.42]
<b>Non-renal patients</b>				
<b>Median</b>	1.14	2.39	2.31	2.36
<b>Range</b>	0.95 – 1.35	1.89 – 2.81	2.12 – 2.59	2.14 – 2.66
<b>[Interquartile range]</b>	[1.10 – 1.17]	[2.31 – 2.45]	[2.25 – 2.37]	[2.30 – 2.42]
<b>Renal patients</b>				
<b>Median</b>	1.14	2.33	2.32	2.36
<b>Range</b>	0.64 – 1.51	1.45 – 2.83	1.45 – 2.81	1.48 – 2.84
<b>[Interquartile range]</b>	[1.10 – 1.19]	[2.26 – 2.43]	[2.25 – 2.43]	[2.30 – 2.42]
<b>Normoalbuminaemia</b>				
<b>Median</b>	1.14	2.39	2.30	2.36
<b>Range</b>	0.64 – 1.51	1.45 – 2.83	1.45 – 2.81	1.48 – 2.84
<b>[Interquartile range]</b>	[1.11 – 1.18]	[2.31 – 2.45]	[2.24 – 2.37]	[2.30 – 2.41]
<b>Hypoalbuminaemia</b>				
<b>Median</b>	1.09	2.12	2.44	2.31
<b>Range</b>	0.95 – 1.20	1.89 – 2.81	2.27 – 2.66	2.14 – 2.66
<b>[Interquartile range]</b>	[1.00 – 1.16]	[1.98 – 2.22]	[2.35 – 2.51]	[2.26 – 2.45]

**Table 2: The median, range and interquartile range for ionised, total, corrected and adjusted calcium values.**

			TOTAL CALCIUM			CORRECTED CALCIUM			ADJUSTED CALCIUM		
			Hypo	Normo	Hyper	Hypo	Normo	Hyper	Hypo	Normo	Hyper
IONISED CALCIUM	Hypo	62	11	47	4	4	56	2	3	57	2
	Normo	97	1	78	18	2	89	6	0	87	10
	Hyper	3	0	1	2	0	2	1	0	1	2
	Total	162	12	126	24	6	147	9	3	145	14

**Table 3: 3 x 3 tables comparing the classification of calcium status by (a) total, (b) corrected and (c) adjusted calcium to ionised calcium.**

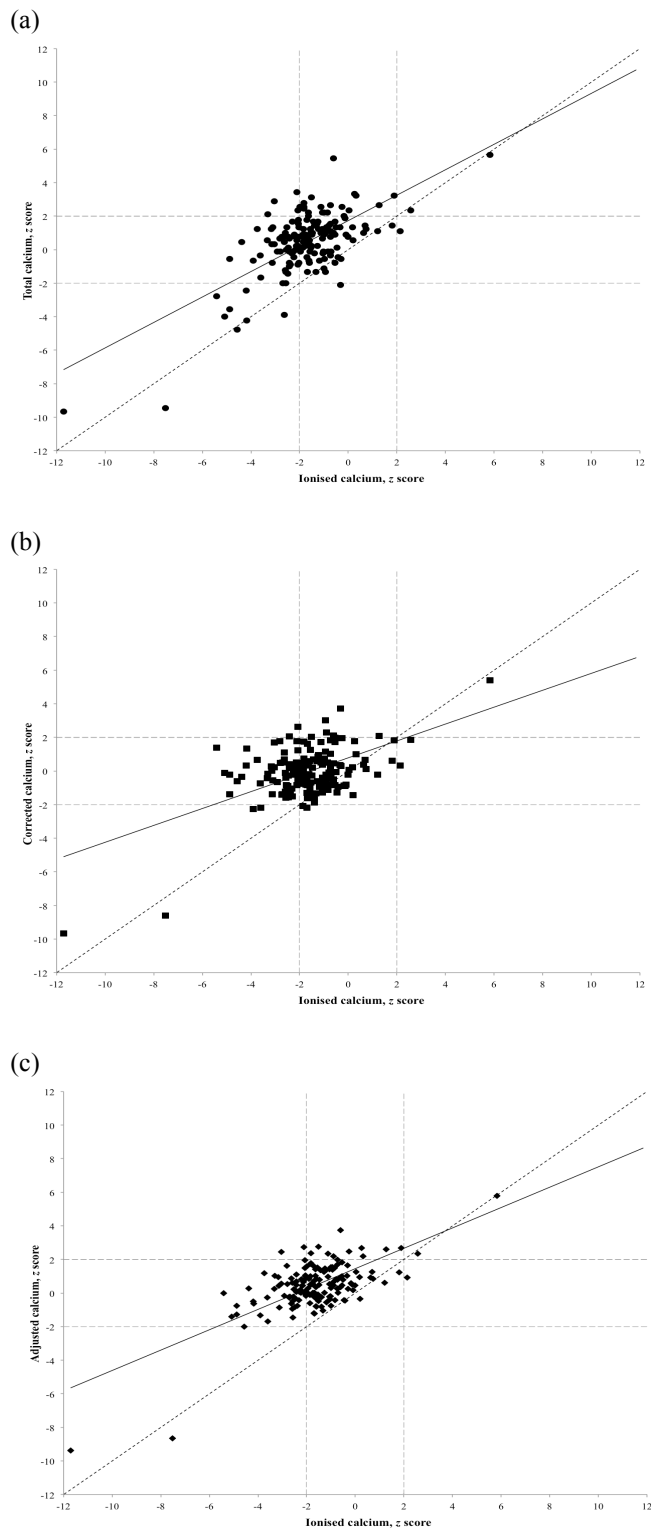
	<b>Total calcium</b>	<b>Corrected calcium</b>	<b>Adjusted calcium</b>
<b>All patients</b>	56%	58%	57%
<b>Non-renal patients</b>	55%	58%	57%
<b>Renal patients</b>	62%	58%	58%
<b>Normoalbuminaemia</b>	55%	63%	59%
<b>Hypoalbuminaemia</b>	71%	7%	29%

**Table 4: Percentage agreement between total, corrected and adjusted calcium with ionised calcium.**

	Total Calcium	Corrected calcium	Adjusted calcium
<b>Cohen kappa statistic (weighted)</b>			
	0.244	0.100	0.156
<b>Quasi-independence model</b>			
<b>Coefficient – hypo</b>	3.908	1.818	18.34
<b>Coefficient – normal</b>	-0.999	-0.651	-1.186
<b>Coefficient – hyper</b>	3.163	2.662	4.043
<b>Estimated Odds</b>			
<b>Hypo</b>	18.34	3.21	2.84 x 10 <sup>7</sup>
<b>Normal</b>	8.70	7.47	17.40
<b>Interclass correlation coefficient</b>			
	ICC [95% CI]	ICC [95% CI]	ICC [95% CI]
<b>Consistency of agreement</b>	0.672 [0.578 – 0.748]	0.550 [0.433 – 0.649]	0.682 [0.590 – 0.756]
Renal	0.887 [0.757 – 0.949]	0.870 [0.723 – 0.942]	0.918 [0.820 – 0.964]
Non-renal	0.506 [0.370 – 0.620]	0.193 [0.028 – 0.349]	0.414 [0.265 – 0.543]
Normoalbuminaemia	0.665 [0.565 – 0.746]	0.612 [0.585 – 0.759]	0.643 [0.537 – 0.728]
Hypoalbuminaemia	0.616 [0.149 – 0.858]	0.811 [0.509 – 0.935]	0.744 [0.372 – 0.910]
<b>Absolute agreement</b>	0.411[-0.0935 – 0.709]	0.384 [-0.020 – 0.635]	0.382 [-0.099 – 0.697]
Renal	0.835 [0.460 – 0.939]	0.776 [0.183 – 0.924]	0.799 [0.013 – 0.943]
Non-renal	0.231 [-0.093 – 0.524]	0.109 [-0.049 – 0.269]	0.164 [-0.083 – 0.420]
<b>Concordance correlation coefficient</b>			
	Rho_c [95% CI] Pearson's r C_b	Rho_c [95% CI] Pearson's r C_b	Rho_c [95% CI] Pearson's r C_b
<b>Overall</b>	0.410 [0.330 – 0.490] 0.679 0.604	0.383 [0.288 – 0.476] 0.551 0.693	0.380 [0.304 – 0.457] 0.685 0.555
<b>Renal</b>	0.829 [0.709 – 0.948] 0.890 0.931	0.768 [0.621 – 0.916] 0.871 0.882	0.793 [0.666 – 0.919] 0.918 0.863
<b>Non-renal</b>	0.230 [0.155 – 0.303] 0.511 0.449	0.109 [0.016 – 0.201] 0.196 0.553	0.163 [0.097 – 0.228] 0.425 0.383
<b>Normoalbuminaemia</b>	0.359 [0.280 – 0.439] 0.666 0.540	0.485 [0.390 – 0.580] 0.644 0.753	0.392 [0.310 – 0.473] 0.683 0.573
<b>Hypoalbuminaemia</b>	0.581 [0.256 – 0.905] 0.654 0.887	0.156 [0.030 – 0.281] 0.783 0.199	0.292 [0.093 – 0.490] 0.818 0.356

**Table 5: Weighted Cohen kappa statistic and quasi-independence model data. The Interclass correlation coefficient (ICC) and concordance correlation coefficient (rho\_c) are represented with their 95% confidence intervals (95% CI). The consistency of agreement and absolute agreement are represented for normalized data (zCa). The concordance correlation coefficient includes the Pearson's coefficient (r) as a measure of precision and bias-correcting factor (Cb), as a measure of accuracy.**

## 2.12. FIGURES



**Figure 1: Concordance plots demonstrating the correlation and agreement between normalised (a) iCa and tCa, (b) iCa and cCa, and, (c) iCa and aCa values expressed as z scores. The dashed line represents the line of perfect concordance (line of identity). The solid line represents the line of best fit. The dashed grey lines represent  $\pm 2$  standard deviations of the 95% confidence interval.**

## CHAPTER 3: SUPPORTING DOCUMENTS

### 3.1. ETHICS APPROVAL



UNIVERSITY OF CAPE TOWN  
Faculty of Health Sciences  
Human Research Ethics Committee



Room E52-24 Old Main Building  
Groote Schuur Hospital  
Observatory 7925  
Telephone [021] 406 6338 • Facsimile [021] 406 6411  
Email: [shuretta.thomas@uct.ac.za](mailto:shuretta.thomas@uct.ac.za)  
Website: [www.health.uct.ac.za/fhs/research/humanethics/forms](http://www.health.uct.ac.za/fhs/research/humanethics/forms)

01 October 2015

**HREC REF: 724/2015**

**Dr F Omar**  
Chemical Pathology  
C17  
NGSH

Dear Dr Omar

**PROJECT TITLE: VALIDATION OF AN ADJUSTED CALCIUM FORMULA USING THE ROCHE CALCIUM (NM-BAPTA) AND ALBUMIN (BCP) METHODS AT GROOTE SCHUUR HOSPITAL (MMed-Dr M Ndlovu) sub-study-linked to 288/2015**

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

**Approval is granted for one year until the 30<sup>th</sup> October 2016.**

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.

(Forms can be found on our website: [www.health.uct.ac.za/fhs/research/humanethics/forms](http://www.health.uct.ac.za/fhs/research/humanethics/forms))

**Please quote the HREC REF in all your correspondence.**

***We acknowledge that MMed student, Dr Mbali Ndlovu will also be involved in this study.***

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Yours sincerely

**PROFESSOR M BLOCKMAN**  
**CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE**

Federal Wide Assurance Number: FWA00001637.

Institutional Review Board (IRB) number: IRB00001938

This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP), South African Good Clinical Practice Guidelines (DoH

HREC 724/2015

## 3.2. INSTITUTIONAL APPROVAL



### GROOTE SCHUUR HOSPITAL

Enquiries: Dr Bernadette Eick

E-mail : [Bernadette.Eick@westerncape.gov.za](mailto:Bernadette.Eick@westerncape.gov.za)

Dr F. Omar  
Division of Chemical Pathology  
NHLS – C-Floor  
NEW MAIN BUILDING

E-mail: [Fierdoz.omar@nhls.ac.za](mailto:Fierdoz.omar@nhls.ac.za) / [Mbali.Ndlovu@nhls.ac.za](mailto:Mbali.Ndlovu@nhls.ac.za)

Dear Dr Omar

**RESEARCH PROJECT: Validation Of An Adjusted Calcium Formula Using The Roche Calcium (NM-BAPTA) And Albumin (BCP) Methods At Groote Schuur Hospital (Mmed Dr M. Ndlovu) – Sub Study Linked To 288/2015**

Your recent letter to the hospital refers.

You are hereby granted permission to proceed with your research and is valid until **30 October 2016**.

Please note the following:

- a) Your research may not interfere with normal patient care.
- b) Hospital staff may not be asked to assist with the research.
- c) No additional costs to the hospital should be incurred i.e. Lab, consumables or stationary may be used.
- d) **No patient folders may be removed from the premises or be inaccessible.**
- e) Please introduce yourself to the person in charge of an area before commencing.
- f) Please discuss the study with the HOD before commencing.
- g) Please provide the research assistant/field worker with a copy of this letter as verification of approval.
- h) Confidentiality must be maintained at all times.
- i) Should you require additional research time beyond the stipulated expiry date, please apply for an extension.
- j) **Once research is complete, please submit a copy of the publication or report.**

I would like to wish you every success with the project.

Yours sincerely

**DR BERNADETTE EICK**  
**CHIEF OPERATIONAL OFFICER**

**Date:** 30 March 2016

BE/vms

C.C. Mr. L. Naidoo, Dr B. Jacobs, Dr H. Vreede  
G46 Management Suite, Old Main Building,  
Observatory 7925

Tel: +27 21 404 6288 fax: +27 21 404 6125

Private Bag X,  
Observatory, 7935

[www.capegateway.gov.za](http://www.capegateway.gov.za)

### 3.3. DEPARTMENTAL APPROVAL (MEDICINE)



## DEPARTMENT OF MEDICINE

ACTIVE IN CLINICAL SERVICE, HEALTH EDUCATION AND RESEARCH



09 February 2016

Attention: Dr Bernadette Eick ,  
G46, Old Main Building  
Groote Schuur Hospital  
Observatory.

**Re: Dr M Ndlovu's permission to collect patient samples for MMed project.**

Dear Dr Eick.

Dr Ndlovu is conducting a study as part of her MMED dissertation.

- Project title: Validation of an adjusted calcium formula using the Roche Calcium (NM-BAPTA) and albumin (BCP) methods at Groote Schuur Hospital.
- This Study has been approved by Ethics ref no. **HREC ref: 724/2015** .

I hereby grant Dr Ndlovu permission to conduct the above-mentioned study in the relevant divisions of the Department of Medicine. This includes the collection of samples from inpatients of the general medical wards and clinics listed below:

- Endocrine
- Liver
- Renal

Yours sincerely

**PROFESSOR GARY MAARTENS**  
**ACTING HEAD: DEPARTMENT OF MEDICINE**

ACTING HEAD: PROFESSOR GARY MAARTENS  
J46, J floor, Old Main Building, Groote Schuur Hospital, Observatory, 7925, Cape Town, South Africa  
Phone: +27 21 4066200 Fax: +27 21 4486815 URL: <http://web.uct.ac.za/depts/medicine/> Email: Gary.Maartens@uct.ac.za

### 3.4. DEPARTMENTAL APPROVAL (SURGERY)

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**From:** Michelle Ahsing [mailto:michelle.ahsing@uct.ac.za]

**Sent:** 11 February 2016 07:54 AM

**To:** Mbali Ndlovu

**Cc:** Eduard Jonas; Eugenio Panieri; Sean Burmeister; Marc Bernon; Paul Goldberg; Adam Boutall (boutall@icloud.com); Claire Warden; 'Lydia Cairncross' (lydiacairn@gmail.com); Francois Malherbe; Nadraj Naidoo; galyachinnery@gmail.com; Andrew Nicol; Pradeep Navsaria; Sorin Edu (sorinedu@yahoo.com); Elmi Muller; David Thomson; Juan Klopper; Christos Kloppers (jckloppers@gmail.com); Shreya Rayamajhi; Tinus du Toit; jj.venter50@gmail.com; 'Heather Bougard'; fazlin noor; Anders Grotte; wendydawnchristian@gmail.com; Mark Hampton (drmarkhampton@icloud.com); lisa kaestner

**Subject:** MMed Project

11<sup>th</sup> February 2016

Dr Mbali Ndlovu

Dear Dr Ndlovu

I have no objection to you having access to patients in the Surgical Wards at Groote Schuur Hospital. Obviously you have to ensure Ethics clearance and permission from the Hospital administration.

I also suggest that, if possible, you collect the samples when patients are having blood taken for other reasons.

Yours sincerely

PROFESSOR DEL KAHN  
HEAD: DIVISION OF GENERAL SURGERY

**Michelle Ahsing**

---

Department of Surgery

PA to Professor D Kahn

Room J45.23 | J Floor | Old Main Building

### 3.5. RESEARCH PROTOCOL

#### I. INTRODUCTION

##### 1. Background

Most laboratories estimate total serum calcium concentration to assess calcium status. Calcium is a divalent cation with multiple roles, i.e. structural role in the skeleton. It also has numerous metabolic roles in muscle contraction, signalling, hormone secretion and action, coagulation, cell membrane potential and cell permeability (1). The majority of total body calcium (99%) is contained in the skeleton, contributing to the mechanical properties of the skeleton as well as providing a reservoir for calcium in the extracellular fluid (ECF). Extracellular calcium, including intracellular calcium constitutes approximately 1% to the total body calcium content (2). Calcium is present in serum in 3 physiological states namely,

- Protein bound calcium – 30-55% (mostly bound to albumin with the remainder binding to globulins)
- Calcium complexed with small anions (i.e. bicarbonate, citrate, sulfate, phosphate, lactate) - 5-15%
- Free ionised calcium – 50%

The free ionised calcium accounts for the biologically active form and plays a crucial role in calcium homeostasis in health individuals and patients with parathyroid abnormalities (2). In clinical practice, calcium status is investigated by the direct measurement of ionised calcium or its estimation from measurements of total calcium. Unfortunately, its direct measurement is not universally available and requires the rapid analysis of an anaerobic sample to avoid alterations in pH. This particularly continues to plague its application in the outpatient setting. However, there are specific clinical situations that warrant the direct measurement of ionised calcium. These include patients receiving transfusions with citrated blood, critically ill patients, and in patients with late stages of chronic kidney disease (CKD), hyperparathyroidism and hypercalcaemia of malignancy (2).

The concentration of total serum calcium varies with serum albumin concentrations while the concentration of the physiologically active ionised calcium concentration is tightly controlled by stringent endocrine regulation (3). Therefore, the derivation of an equation to adjust the measured total serum calcium concentration for the serum albumin concentration. The most commonly used equation in clinical practise is demonstrated below:

$$\text{Corrected [calcium] (mmol/L)} = \text{total [calcium] (mmol/L)} + 0.02 (40 - [\text{albumin}] \text{ (g/L)})$$

It is based on linear regression of serum calcium on albumin to derive the corrected calcium equation. It was also derived in a single laboratory using specific calcium (O-cresolphthalein complexone) and albumin (bromocresol green (BCG) binding dye reagent) methods, in patients deemed free of calcium disorders. The original equation derived by Payne et al, i.e. *corrected [calcium] (mmol/L) = total [calcium] (mmol/L) + 0.025 (40 - [albumin] (g/L))*, was also based on the linear regression of serum calcium on serum albumin with an additional correction of the calcium values to the mean normal calcium concentration. The authors showed a good correlation between serum total calcium and albumin concentrations, however, noted that the adjustment could not be applied to calcium values on patients with nephrotic syndrome and hypoalbuminaemia (4).

The serum total calcium to albumin relationship may be affected by changes in laboratory techniques, in particular, the shift from the BCG binding dye reagent to the bromocresol purple (BCP) binding dye reagent for serum albumin measurement. The different analytical platforms, on which the assays are performed, may also influence this relationship and impact the assessment of calcium status (1, 3-5). In addition, application of this equation in patients with abnormally low albumin concentrations may classify patients differently as hypocalcaemia, normal, and hypercalcaemia. In the presence of renal failure, corrected calcium tends to underestimate hypocalcaemia and overestimate hypercalcaemia compared with ionised calcium (3, 6).

Several other equations have been described based on the linear regression of serum calcium on serum albumin mostly based on BCG determinations of serum albumin, a method with variable positive bias relative to the BCP method. Subsequently, they all suffer from differences in the classification of calcium status.

## 2. Significance

Given the variability of adjustment equations between different calcium and albumin analytical methods and platforms, the Association for Clinical Biochemistry and Laboratory Medicine (ACB), recommended that laboratories should use equations based on the regression of calcium on albumin concentration specific for their calcium and albumin methods and analytical platforms. This may be performed either by locally deriving an equation or by validation of an existing equation (1).

The National Health Laboratory Service (NHLS) at Groote Schuur Hospital (GSH) undertook this recommendation and derived an equation based on the guidelines from the ACB. The new adjusted calcium formula was derived using data from 3131 GSH patients, as per these recommendations and included the following exclusions (1):

- Patients with renal impairment [creatinine >200  $\mu\text{mol/L}$  or urea >15  $\text{mmol/L}$ ]
- Hypomagnesaemia [hypokalaemia as a surrogate marker]
- Liver disease [ALT/ALP > upper reference limit]
- Total calcium concentration <2.0 and >2.7  $\text{mmol/L}$
- Hypo/hyperparathyroidism i.e. PTH outside the healthy population reference range
- Vitamin D deficiency/ toxicity (excluded if vitamin D was requested)
- Hypoadrenalism (excluded if cortisol was requested)
- Patients on parenteral nutrition
- Patients with malignancy (excluded if tumour markers were requested)

Analyses of serum calcium and albumin were performed using the Roche Cobas 6000 5-nitro-5'-methyl-1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (NM-BAPTA) calcium method and the BCG albumin dye binding method respectively. The newly derived adjusted calcium formula:

*Adjusted [calcium] (mmol/L)*

$$= \text{total [calcium]} (\text{mmol/L}) - (0.0149 \times [\text{albumin}] (\text{g/L})) + 0.6216$$

Hence, this study to assess the performance of the adjusted calcium formula against

the ionised calcium concentration, as the gold standard, in correctly categorizing patients as hypocalcaemic, normal and hypercalcaemic. Furthermore, a comparison will be made between ionised calcium; and total serum calcium Payne corrected calcium. This will assess the performance and relevance of the Payne corrected calcium formula. If the performance of the adjusted calcium formula is deemed adequate, it would be a feasible alternative to the current Payne corrected calcium formula. In addition, the NHLS undertook to develop several other formulae for the different combination of serum calcium and serum albumin methods in use within the NHLS laboratories nationally, using the same guidelines recommended by the ACB. The same mathematical methodology was applied to derive these formulae and in principle, it may be possible that validation of two formulae would be adequate in assessing the performance of the other formulae developed similarly.

### **3. Aims and objectives**

- a) Testing of the performance adjusted calcium formula for the Roche Cobas 6000 NM-BAPTA serum calcium and BCG serum albumin methods on a larger sample size (n=150).
- b) Categorisation of calcium status as hypocalcaemia, normal and hypercalcaemia, based on the ionised calcium, total serum calcium, Payne corrected calcium and adjusted calcium.
- c) Comparison of the performance of the total serum calcium, corrected calcium (Payne) and the adjusted calcium against ionised calcium (gold standard), in correctly classifying calcium status.
- d) Testing the performance of the adjusted calcium formula for the Siemens ADVIA 1800 *o*-Cresolphthalein complexone (CPC) serum calcium and BCG serum albumin methods on the same samples by performing similar analyses of the total calcium, corrected calcium (Payne) and adjusted calcium against the ionised calcium.<sup>1</sup>

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<sup>1</sup> The analysis of the serum total calcium and albumin was not performed adequately on the

## II. METHODS

### 1. Study design

The study is a comparative analytical study that will be an extension of a previously approved study by the University of Cape Town Human Research Ethics Committee (HREC/REF: 288/2015 - Derivation and testing of adjusted calcium formula for the Roche calcium (BAPTA method) and albumin methods (BCG method)). The present study will aim to compare the performance of the new adjusted calcium formula against the ionised calcium using a larger sample size. The previous study was limited by a smaller sample size and inability to assess performance of the new adjusted calcium formula in patients with low albumin concentrations. In addition to the above-mentioned adjusted calcium formula, several other formulae were developed for the different combinations of serum calcium and albumin methods used within the NHLS. In principle, validation of another formula derived mathematically similarly to the GSH formula, may not necessitate validation of the other formulae. Testing of the adjusted calcium formula for the Siemens ADVIA 1800 calcium (CPC method) and albumin methods (BCG method) against the ionised calcium to assess its performance.

### 2. Subject selection

For the present study, we aim to investigate the performance and suitability of the new formula in 200 patients selected for a wide range of serum albumin and calcium concentrations. The following exclusion criteria will apply:

- Patients < 18 years
- Critically ill patients
- Patients who do not provide written consent to participate in the study

Patients will be recruited from the outpatients department (i.e. endocrine, liver, renal clinics) as well as inpatients in the medical and surgical wards from GSH<sup>2</sup>.

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<sup>2</sup> Patients from the wards were excluded to avoid their discomfort from multiple blood draws. Patients in the outpatient departments would only have one blood draw including bloods requested for their routine visit.

Serum samples for calcium and albumin as well as whole blood for ionised calcium will be obtained after informed consent. Access restricted computer records will only be available to staff involved in the study to ensure patient privacy and confidentiality. Outpatients will not be compensated and the visit, at which bloods are drawn, would be part of their routine follow-up appointment. The attending clinician for the patient will be informed of any abnormal results.

### **3. Measurement**

Venous blood will be collected without venous stasis, into calcium-balanced heparinised syringes and transported on ice and analysed within 15 minutes, for ionised calcium determination. In addition, blood will be collected into serum separator tubes, centrifuged and processed for serum albumin and calcium determinations. A trained person using an aseptic technique will collect a total volume of 10 ml of blood. The concentration of serum total calcium and serum albumin will be measured using the NM-BAPTA calcium method and BCG albumin method respectively on the Roche Cobas 6000 analyser at Chemical Pathology Laboratory, NHLS GSH. Ionised calcium testing will be performed on the Roche Cobas b 123 point of care (POC) system, by ion selective electrode. Furthermore, the concentration of serum calcium and serum albumin will also be measured using the CPC calcium method and the BCG albumin method respectively on the Siemens ADVIA 1800 platform at Chemical Pathology Laboratory, NHLS Tygerberg Hospital. This will enable the validation of another formula derived mathematically similarly to the formula developed at GSH.<sup>1</sup>

In addition, data will be extracted from the laboratory information system (LIS) in patients where serum calcium and albumin were measured, for comparative analyses between the Payne corrected and adjusted calcium formulae (ethics approval obtained HREC/REF: 288/2015).<sup>3</sup>

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<sup>3</sup> This analysis was not performed for this study due to time constraints, however will be reported as part of the findings from the previous study.

### **III. DATA ANALYSIS**

Comparative and descriptive analyses will be performed using Microsoft Excel and Statistica to assess the performance of total calcium; Payne corrected calcium and adjusted calcium against ionised calcium.

- Categorisation of data will be performed using ionised calcium as the gold standard. Published reference ranges will be used as well as the medical decision limits in correctly classifying patients as hypocalcaemic, normal and hypercalcaemic as follows:
  - Total serum calcium: 2.15-2.50 mmol/L (Adult reference range, Tietz Clinical Guide to Laboratory Tests, 4<sup>th</sup> Edition, page 202)
  - Ionised calcium: 1.12 – 1.32 mmol/L (Roche Cobas b 123 POC system)
- The agreement will be described for the total calcium, corrected calcium and adjusted calcium against the ionised calcium, as well as against each other.<sup>3</sup>
- The corrected and adjusted calcium will be compared using ordinary linear regression analysis and the Wilcoxon test (p-value < 0.05 will be considered statistically significant).<sup>3</sup>
- Results will be stratified according to the albumin concentration to assess at which albumin levels the formulae perform adequately.

### **IV. ETHICS AND COMMUNICATION**

#### **1. Ethics**

Prior to commencing the study, this protocol will be presented to the Department of Clinical Laboratory Sciences Research Committee followed by submission to the Health Sciences Human Research Ethics Committee (HREC) of the Faculty of Health Sciences University of Cape Town (UCT), for ethics approval.

#### **2. Reporting**

The results will be used for the purpose of obtaining an MMed qualification through the University of Cape Town. In addition, this study will act as a pilot study for the application of the new adjusted calcium formula in clinical practise. Should there be obvious findings from this study, it will determine changes in clinical practise and failure to do so, may necessitate further studies to validate the adjusted calcium formula. This study will also be for presentation at the annual Pathology congress (local and international) and will form part of a publication in a peer-reviewed journal (HREC/REF: 288/2015).

## **V. LOGISTICS**

### **1. Timeline**

August 2015: Protocol submission for approval by the Department of Clinical Laboratory Sciences Research Committee followed by submission to the UCT HREC for approval.

September – December 2015: Sample collection and data analysis; as well as preparation of an abstract for presentation at the 2016 American Association for Clinical Chemistry Annual Meeting & Clinical Lab Expo – pending approval by the HREC.

June - August 2016: Writing up of data and preparing mini-dissertation as well as article for submission for publication.<sup>4</sup>

### **2. Budget**

Expenses anticipated will be for analysis of serum total calcium and albumin as well as ionised calcium. These expenses will be covered by the Division of Chemical Pathology, NHLS, GSH.

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<sup>4</sup> Timelines were delayed due to preparation for the FC PathSA (Chem) exit exam.

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(7-12)

### **3.6. INFORMED CONSENT FORM**

**DEPARTMENT OF PATHOLOGY  
DIVISION OF CHEMICAL PATHOLOGY  
CONSENT FORM**

**Investigators: Dr M Ndlovu, Dr F Omar & Dr H Vreede**

**VALIDATION OF AN ADJUSTED CALCIUM FORMULA USING THE  
ROCHE CALCIUM (NM-BAPTA) AND ALBUMIN (BCG) METHODS AT  
GROOTE SCHUUR HOSPITAL**

Calcium is the most abundant mineral in the body that is an essential part of bones and teeth. The heart, nerves and blood clotting systems also need calcium to work. In the blood, calcium is present as a free form, known as the ionised calcium. This is the active calcium and its levels therefore reflect calcium balance in the body. In addition, calcium is also bound to a protein in the blood that is made by the liver called albumin.

Free calcium is affected by a many factors such as exposure of the blood sample to air, time taken for the blood to be drawn from your arm to when it is measured in the laboratory; and high temperatures. This means that special precautions need to be taken in order to ensure a correct laboratory result. As such, most laboratories offer an alternate method for its estimation. This involves measuring all the calcium in the blood (known as the total calcium) and the albumin level. Both the total calcium and albumin values are then applied into a formula to calculate an “adjusted” calcium level. This adjusted calcium is thought to provide a good indication of the free calcium level in the blood.

This study aims to evaluate the newly developed adjusted calcium formula at Groote Schuur Hospital, NHLS laboratory. The adjusted calcium derived from this new formula will be compared to the free calcium as well as the total calcium in order to assess the ability of this formula to correctly reflect calcium balance in the body.

For this purpose we would like to obtain blood from you. A maximum of 10 ml (~2 teaspoons) blood will be taken in order to measure the levels of total calcium, free calcium and albumin. Blood will be taken by inserting a needle into a vein in your arm. This may cause some discomfort. There is also a theoretical risk of infection but sterile procedures will be used to minimise this risk and only qualified persons will draw blood. The blood will then be taken to the laboratory for measurement of total calcium, free calcium and albumin.

Participant: I, .....(name and surname) agree to participate in the above study and hereby give permission that my blood may be taken and analysed.

I understand that I will undergo the following:

1. Venous blood will be taken:
  - a. Blood will be drawn directly into a balanced-heparinised syringe for ionised calcium measurement.
  - b. Blood will also be drawn into a gel separator tube (5 ml) for albumin and total calcium measurement.
  - c. Risk of infection will be minimized by using sterile procedures, and a suitably qualified person will take all blood samples.
2. I further understand that a portion of these blood samples may be stored for:
  - a. Possible reanalysis.
  - b. Further research studies, subject to the approval of the University of Cape Town Human Research Ethics Committee and that any information from such research will remain confidential.
  - c. However, I can ask that my blood be not stored and destroyed after this use.

Regarding storage of your blood, please tick the appropriate block below:

- I am happy to have my blood stored:
- I am **NOT** happy to have my blood stored:

3. I understand that any abnormal results will be provided to the clinician in charge of my treatment and may therefore be used in my medical management.

4. I have been informed that:

- a. No blood will be stored for DNA / genetic analysis
- b. The investigators are under the obligation to respect medical confidentiality and every effort will be made to protect my confidentiality. My name will not be used in any study report.

I UNDERSTAND THAT I MAY WITHDRAW MY CONSENT FOR ANY ASPECT OF THE ABOVE STUDY AT ANY TIME WITHOUT AFFECTING MY FUTURE MEDICAL CARE.

ALL OF THE ABOVE HAVE BEEN EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND MY QUESTIONS HAVE BEEN ADEQUATELY ANSWERED

**Contact details:** For any queries related to the research study, you can call the UCT Faculty of Health Sciences Human Research Ethics Committee contact number: 021 406 6492 or the investigators at the Division of Chemical Pathology: 021 404 4135/4118/5429

Participant Name:.....

Participant Sign:.....

Date:.....

Informed consent obtained by:

Name: .....

Sign: .....

Witness:.....

Sign:.....

# Clinical Chemistry

## Information for Authors

*Revised August 2016*

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### **OVERVIEW**

*Clinical Chemistry*, issued monthly, is published in print and electronically by the American Association for Clinical Chemistry. The journal welcomes contributions, either experimental or theoretical, in the field of laboratory medicine. It is the leading forum for peer-reviewed, original research on innovative practices in today's clinical laboratory. In addition to being the most cited journal in the field, *Clinical Chemistry* has the highest Impact Factor among journals of clinical chemistry, clinical (or anatomic) pathology, analytical chemistry, and the subspecialties, such as transfusion medicine and clinical microbiology.

Submissions of the following nature are welcomed:

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- Analytical techniques
- Molecular diagnostics
- Test utilization or testing-related health or financial outcomes
- Instrumentation

- Data processing
- Statistical analyses of data
- Clinical investigations in which laboratory testing has played a major role
- Laboratory animal studies of chemically oriented problems of human disease

Contributions should consist of subject matter that is original and significantly advances the state of knowledge of clinical chemistry, and conclusions that are justified from the design of the experiments and the data presented. The information must be sufficiently detailed to permit replication of the work by a competent worker in the field. Lastly, the writing must be clear, concise, and grammatically correct.

Equal consideration is given to original manuscripts in English from any country, regardless of membership in the Association. It is, however, advised that all non-English speaking authors enlist the aid of a native-English speaking colleague to correct English language usage before submission. Submissions must adhere to the “Uniform Requirements for Manuscripts Submitted to Biomedical Journals” (1).

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References:

- 1 International Committee of Medical Journal Editors. Uniform requirements for manuscripts submitted to biomedical journals. *Ann Intern Med* 1997;126:36-47. [\[Full Text\]](#)

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## **STANDARDS FOR REPORTING SCIENTIFIC DATA**

- [Description of Analytical Methods and Results](#)
- [Statistics](#)
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- [Checklist for the Description of Sequence Variants at the Human Genome Variation Society](#)

## **DESCRIPTION OF ANALYTICAL METHODS AND RESULTS**

Manuscripts describing the development and evaluation of the performance of methods and instruments should discuss linearity, imprecision, analytical specificity, recovery, lower limit of detection, comparability with other analytical methods, lower limit of quantification and reference interval(s). Some clinical data are usually needed.

Document the analytical advantages of the new or modified method over existing methods.

Analytical method validations should conform to the protocols and requirements in the Center for Drug Evaluation and Research (CDER) Guidance for Industry: Bioanalytical Method Evaluation, 2001 (1).

Calibration curves and linearity: Data for these studies should be analyzed by linear regression analysis (if a linear response is obtained) and should include the slope, intercept,  $r^2$ , standard deviation of residuals, and the standard deviations of the slope and intercept.

Standard deviations of repeated points may be included.

In preparing nonlinear calibration curves, authors may use any objective, statistically valid method but must specify the method used (see, e.g., (2)).

Imprecision: Studies must include estimates of "within-run" and "total" standard deviations (2). Each should be determined at low,

normal, and above-normal concentrations with use of specimens that are in an appropriate biological matrix.

One method for estimating both within-run and total standard deviations is the analysis of variance experiment described in NCCLS EP5-T (3), which calls for two replicates per specimen per run and two runs per day for 20 days. This permits separate estimation of between-day and between-run, within-day standard deviations, as well as within-run and total standard deviations. For acceptable alternatives that include only one run per day, see the cited document.

Indicators of Accuracy ("Trueness"): Accuracy (or "trueness" in the recent nomenclature) of a new method can be estimated by (a) analyses of certified Reference Materials by the new method or (b) comparisons of results of a new method with results of a Reference Method. These are the only accepted approaches to trueness. When neither is available, other evidence relevant to the ability of the method to measure the analyte (measurand) is needed. Recovery studies involve analyses after known amounts of analyte are added to the biological fluid on which the determination will be performed. Recovery of added analyte should be calculated  $[(\text{final concentration} - \text{initial concentration}) / \text{added concentration}]$ , not the observed final concentration as a proportion of expected final concentration.

Interference studies should be performed to assess the effects of common interferents, including lipid particles, hemoglobin, bilirubin, and components of uremic plasma. Exogenous materials, such as ingredients of blood collection containers (tubes) and commonly used or commonly coadministered drugs that might interfere with the determination, should also be tested for interferences.

Selection of materials to test should be guided by an understanding of the chemistry and physics of the measuring system. Thus chemicals that are structurally similar to the analyte should be tested to assess the selectivity of the method. (The term "selectivity" is preferred over specificity; selectivity can be quantified.) In characterizing non-spectrophotometric methods, chemicals that may interfere in the detection system should be studied more intensively than chemicals that are historically important for interference in spectrophotometric methods.

Comparison-of-methods studies should compare results by the new or proposed method with those by a reference-quality method or other generally accepted analytical method for which assay performance is documented (4, 5).

It is desirable to test 100 to 200 different samples from patients who have been selected to include a wide variety of pathologic conditions and to present a range of values for the analyte that includes those likely to be encountered in routine application. For a table of the required number of samples, see Linnet (6). If regression analysis is used for statistical evaluation of the data, supply slopes and intercepts (and their standard deviations) and standard deviations of residuals (Sy<sub>x</sub>, often called standard errors of estimates). Unbiased (e.g., Deming) regression is typically required (7). A program to perform Deming regression is available online as a supplement from this journal (8).

The correlation coefficient has limited utility. Residuals plots [e.g., Bland-Altman (9, 10)] are often useful. On the horizontal axis, plot the mean of results by the two studied methods, not the result of one method.

Analytical sensitivity and detection limit: These terms are commonly confused. The International Union of Pure and Applied Chemistry defines analytical sensitivity as the ability of an analytical procedure to produce a change in signal for a defined change of the quantity. This is often visualized as the slope of the calibration curve.

The limit of detection (LOD) is defined as the lowest concentration or amount of an analyte that can be reliably identified as being qualitatively present in the sample. The limit of quantification (LOQ) is defined as the lowest concentration or amount of analyte that can be reproducibly quantified in a sample. The most acceptable criteria for ascertaining the LOQ is the concentration of analyte that can be measured with an imprecision of less than 20% and a deviation from target of greater or less than 20% (1). The operational definition of the LOD and LOQ must be supplied by the author. Additional considerations related to this topic are presented by Linnet (11).

Analytical quality: Results obtained for the performance characteristics should be compared objectively with well-documented quality specifications, e.g., published data on the state-of-the-art performance required by regulatory bodies such as CLIA 88, or recommendations documented by expert professional groups (12).

Reference interval (normal range): Depending on the conclusions of the accuracy studies, modification of an accepted reference interval may be indicated. Description of the reference interval study should include details about sampling; selection of subjects,

including their number, age, and sex distribution; the statistical method for summarizing the results (13); and other factors that would influence the values obtained.

Mass spectrometric assays must be evaluated for matrix effects (ion suppression or enhancement) (14, 15).

Chromatograms: Chromatograms from gas-liquid and liquid chromatography should usually be presented so that readers can see the efficiency of the separation and observe the resolution from interferences in the matrix. Similar images are often needed for electrophoretic separations.

Enzyme activities: Enzyme activities may be expressed in international units (U) or katal. Temperature and other key assay features must be described in the text or by reference to a published method.

When first mentioned in the text, enzymes (whether measured by activity or mass assays) must be numbered (EC no.) in accordance with the recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology on the Nomenclature and Classification of Enzymes (16).

References:

- 1 Center for Drug Evaluation and Research (CDER) Guidance for Industry: Bioanalytical Method Evaluation, 2001.
- 2 Linnet K, Boyd JC. Selection and analytical evaluation of methods – with statistical techniques. In: Burtis CA, Ashwood ER, Bruns DE. Tietz textbook of clinical chemistry and molecular diagnostics. 4th ed. St. Louis: Saunders, 2006: 353-407.
- 3 NCCLS Tentative Guideline EP5-T. User evaluation of precision performance of clinical chemistry devices. Wayne, PA: National Committee for Clinical Laboratory Standards, June 1984.
- 4 Carey RN, Garber CC. Evaluation of methods. In: Kaplan LA, Pesce AJ, eds. Clinical chemistry. Theory, practice and correlation, 2nd ed. St. Louis: CV Mosby, 1989:290–310.
- 5 Koch DO, Peters T Jr. Selection and evaluation of methods. In: Burtis CA, Ashwood ER, eds. Tietz textbook of clinical chemistry, 2nd ed. Philadelphia: WB Saunders, 1994:508–25.
- 6 Linnet K. Necessary sample size for method comparison studies based on regression analysis. Clin Chem 1999;45:882–94. [\[Abstract/Full Text\]](#)

- 7 Linnet K. Evaluation of regression procedures for methods comparison studies. Clin Chem 1993;39:424–32. [\[Full Text\]](#)
- 8 Martin RF. General Deming regression for estimating systematic bias and its confidence interval in method-comparison studies. Data supplement. GDR: Executable program for general Deming regression calculations and graphics. [\[Abstract/Full Text/Data Supplement\]](#)
- 9 Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1986;1:307–10. [\[Abstract\]](#)
- 10 Altman DG, Bland JM. Commentary on quantifying agreement between two methods of measurement. Clin Chem 2002;48:801-2. [\[Extract/Full Text\]](#)
- 11 Linnet K, Kondratovich M. Partly nonparametric approach for determining the limit of detection. Clin Chem 2004;50:732-40. [\[Extract/Full Text\]](#)
- 12 Fraser CG, Petersen PH. Analytical performance characteristics should be judged against objective quality specifications. Clin Chem 1999;45:321–3. [\[Extract/Full Text\]](#)
- 13 Solberg HE. Establishment and use of reference values. In: Burtis CA, Ashwood ER, eds. Tietz textbook of clinical chemistry, 4th ed. St. Louis: Saunders, 2006:425–48.
- 14 Annesley TM. Ion Suppression in Mass Spectrometry. Clin Chem 2003;49:1041-44. [\[Full Text\]](#)
- 15 Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal Chem 2003;75:3019-30. [\[Abstract\]](#)
- 16 International Union of Biochemistry and Molecular Biology, Nomenclature Committee. Enzyme nomenclature 1992. San Diego: Academic Press, 1992:862pp.

## **STATISTICS**

Describe statistical methods with enough detail to enable a knowledgeable reader with access to the original data to verify the reported results.

When possible, quantify findings and present them with appropriate indicators of measurement error or uncertainty.

Avoid sole reliance on statistical hypothesis testing, such as the use of P values, which fails to convey important quantitative information.

When appropriate, confidence intervals should be presented; see,

e.g., Harris (1), Henderson (2), and references therein.

References:

- 1 Harris EK. On P values and confidence intervals (why can't we P with more confidence?) [Editorial]. *Clin Chem* 1993;39:927-8. [\[Full Text\]](#)
- 2 Henderson AR. Chemistry with confidence: should Clinical Chemistry require confidence intervals for analytical and other data? [Opinion]. *Clin Chem* 1993;39:929-35. [\[Abstract/Full Text\]](#)

### ***STUDIES WITH HUMAN SUBJECTS***

Authors are responsible for ensuring compliance of human studies with the Helsinki Declaration of 1975 as revised in 2008:

<http://www.wma.net/en/30publications/10policies/b3/index.html>.

Approval by the appropriate institutional committee on human research (Institutional Review Board) must be documented in the manuscript and, unless excepted by that committee, informed consent of all participants studied for the report must be included.

### ***ANIMAL STUDIES***

Authors are responsible for ensuring that studies of animals are in accordance with the Committee on Care and Use of Laboratory Animals. The information can be found in the [Guide for the care and use of laboratory animals](#). Washington, DC: Institute of Laboratory Animal Resources, National Research Council, 1985:83pp.

### ***NIH FUNDING/OPEN ACCESS REQUIREMENTS***

The National Institutes of Health (NIH) has implemented its "Policy on Enhancing Public Access to Archived Publications Resulting from NIH Funded Research." This policy requests that authors reporting research that is funded (in whole or part) by a current NIH grant submit to NIH PubMed Central (PMC) manuscripts after they have been peer reviewed and accepted for publication in scientific journals. The "accepted manuscript" is the version of the manuscript after the author's final revision but before post-acceptance editing and copyediting. The Policy is available online at <http://grants.nih.gov/grants/guide/notice-files/NOT-OD-05-045.html>. The policy applies to manuscripts accepted for publication in *Clinical Chemistry* on or after May 2, 2005. The policy requests that the release date to the public be within 12 months after the official date of final publication. *Clinical*

*Chemistry's* existing policy that has been in effect since the Journal first appeared online in 1998 is to make all content publicly available 12 months after publication date. Authors have the option to request that their papers be made freely available immediately at the time of online publication, for a fee of \$3500. For more information or to request open access publication, contact the Editorial Office at [clinchemed@aacc.org](mailto:clinchemed@aacc.org).

If an author chooses to submit an accepted manuscript to PMC, he or she will be asked to indicate when that manuscript should be made available to the public. Consistent with our existing policy, the American Association for Clinical Chemistry (AACC) grants the author permission to allow public release of the accepted manuscript through PMC 12 months after publication in *Clinical Chemistry*.

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The AACC requires that authors add the following disclaimer to the manuscript before sending it to PMC:

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Authors may contact *Clinical Chemistry* via e-mail at [clinchemed@aacc.org](mailto:clinchemed@aacc.org) with any questions related to submitting their accepted manuscripts to PMC. More information from NIH can be found at <http://publicaccess.nih.gov/FAQ.htm>.

## **CHECKLIST FOR THE DESCRIPTION OF SEQUENCE VARIANTS AT THE HUMAN GENOME VARIATION SOCIETY**

Requirements for the description of sequence variants can be found at <http://www.hgvs.org/mutnomen/checklist.html>.

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## **TOOLS FOR DIAGNOSTIC ACCURACY**

- [Studies of Diagnostic Accuracy \(STARD\)](#)
- [Outcomes Studies \(CONSORT\)](#)
- [Minimum Information about a Microarray Experiment \(MIAME\)](#)
- [Minimum Information about Quantitative Real-Time PCR Experiments \(MIQE\)](#)

## **STUDIES OF DIAGNOSTIC ACCURACY (STARD)**

[STARD Checklist \[PDF\]](#)

[STARD Flowchart \[PDF\]](#)

Explanatory document, with examples:

<http://www.clinchem.org/cgi/content/full/49/1/7>

STARD guidelines: <http://www.clinchem.org/cgi/content/full/49/1/1>

For studies of diagnostic accuracy of tests, complete the STARD Checklist for Evaluations of Diagnostic Accuracy (1) electronically upon submission. Do not send the checklist via e-mail or upload it as supplemental material.

The STARD statement (1) and explanatory document (2) provide guidance helping authors to modify their manuscript as needed to provide the requested information. Guidelines include:

-Provide literature reference(s) describing the evaluated test(s) and criterion "gold standard" test(s) or include detailed descriptions of them.

-Follow accepted methodologic standards including the following:

- 1 Specify spectrum of evaluated patients (age and sex distributions, eligibility criteria, and summary of symptoms or disease stage).
- 2 Analyze pertinent subgroups of subjects (e.g., symptomatic and asymptomatic patients).
- 3 Avoid verification bias (usually by application of a "gold-standard" test to all subjects rather than to a clinically selected subset).
- 4 Categorize test results and patients independently to avoid reviewer bias (usually by performance of tests with blinding to patient information and vice versa).
- 5 Provide confidence intervals (or SE) for indices of diagnostic

accuracy such as sensitivity/specificity, likelihood ratios, and areas under receiver-operating characteristic (ROC) curves (3).

6 Indicate the number of indeterminate test results and their use (if any) in further data analysis.

7 Provide laboratory data on analytical imprecision of the test (usually day-to-day CV at two or more concentrations) or reproducibility of observer interpretation (e.g., for a visually read, dichotomous [positive/negative] test).

-A flow diagram is strongly recommended (1, 2).

-When evaluating diagnostic accuracy in clinical studies, simple testing of the significance of differences between mean values of patient groups (e.g., by Student's t-test) provides insufficient information to assess diagnostic accuracy.

-Scatter plots of data, calculations of diagnostic sensitivities and specificities and their confidence intervals (3), and use of approaches such as ROC curves (4), cumulative distribution analyses (5), likelihood ratios (6), and discriminant analysis (7) provide information that is appropriate to specific situations.

-Confidence intervals should be provided (1).

-Discussions of predictive values in illustrative settings may be useful additions to assess the potential clinical utility of tests.

-Analysis of serial measurements requires special attention (8).

References:

1 STARD Group. Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD statement. Clin Chem 2003;49:1-6. [\[Abstract/Full Text\]](#)

2 Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM, et al. The STARD statement for reporting studies of diagnostic accuracy: explanation and elaboration. Clin Chem 2003;49:7-18. [\[Abstract/Full Text\]](#)

3 Harper R, Reeves B. Reporting of precision of estimates for diagnostic accuracy: a review. BMJ 1999;318:1322-3. [\[Full Text\]](#)

4 Zweig MH, Campbell G. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine [Review]. Note that in Figs. 4-12 in this paper, the labels for the x-axis at the top and bottom are reversed. The (correct) dual labeling of the x-axis solves the problem of whether to plot specificity or 1 - specificity on the x-axis. Clin Chem 1993;39:561-77. [\[Abstract/Full Text\]](#)

5 Krouwer JS. Cumulative distribution analysis graphs-an

- alternative to ROC curves [Technical Brief]. Clin Chem 1987;33:2305-6.
- 6 Albert A. On the use and computation of likelihood ratios in Clinical Chemistry. Clin Chem 1982;28:1113-9. [[Abstract/Full Text](#)]
- 7 Solberg HE. Discriminant analysis. Crit Rev Clin Lab Sci 1978;9:209-42.
- 8 Matthews JNS, Altman DG, Campbell MJ, Royston P. Analysis of serial measurements in medical research. Br Med J 1990;300:230-5.

### **OUTCOMES STUDIES (CONSORT)**

[Outcomes studies \(CONSORT\)](#)

[CONSORT Checklist \[PDF\]](#)

[CONSORT Flowchart \[PDF\]](#)

The CONSORT statement (1), although designed for randomized controlled trials and used for therapeutic rather than diagnostic interventions, is recommended as an often-useful guide.

For questions, contact *Clinical Chemistry* via e-mail at [clinchemed@aacc.org](mailto:clinchemed@aacc.org).

References:

- 1 Moher D, Schulz KF, Altman DG for the CONSORT Group. The CONSORT statement: revised recommendations for improving the quality of reports of parallel group randomized trials. [www.consort-statement.org](http://www.consort-statement.org).

### **MINIMUM INFORMATION ABOUT A MICROARRAY EXPERIMENT (MIAME)**

The MIAME Checklist can be found at

<http://fged.org/projects/miame/>.

MIAME describes the minimum information about a microarray experiment that is needed to enable interpretation of the results of the experiment unambiguously and potentially to reproduce the experiment.

The MIAME checklist is a description of MIAME principles designed to help authors, reviewers, and editors of scientific journals meet MIAME requirements and to make microarray data available to the community in a useful way.

MIAME is neither a dogma nor a legal document - it assumes a cooperative data provider and a fair reviewer.

### **MINIMUM INFORMATION ABOUT QUANTITATIVE REAL-TIME**

## **PCR EXPERIMENTS (MIQE)**

### **MIQE Checklist [PDF]**

For studies that include quantitative real-time PCR experiments, complete the MIQE checklist for evaluation of qPCR experiments during electronic submission. Do not send the checklist via e-mail or upload it as supplemental material. The full text of the MIQE guidelines is available online:

<http://www.clinchem.org/cgi/content/short/55/4/611>. A PDF version for all essential components of the checklist can be obtained by the link above.

Recommended nomenclature should be used, including:

- **qPCR** for quantitative real-time PCR
- **RT-qPCR** for reverse transcription quantitative real-time PCR
- **reference genes** instead of housekeeping genes
- **hydrolysis probes** instead of TaqMan® probes
- **dual hybridization probes** for HybProbes® (LightCycler®) probes
- **quantification** instead of quantitation
- **Cq** instead of Ct, Cp, or TOP
- **quantification cycle** instead of threshold cycle or crossing point

Authors are also encouraged, but not required, to include the additional desirable items of the MIQE guidelines (1). The MIQE guidelines are intended to help authors plan, perform and present qPCR experiments. They are also a guide for reviewers and editors to judge the quality of qPCR data. Incomplete information may be grounds for manuscript rejection. Use of Supplemental Data is encouraged as necessary. The most common errors in performing and reporting qPCR data include:

- 1 Not enough information for others to replicate the experiment, including how the nucleic acid was prepared, reverse transcribed, and amplified. Primer sequences are required. Probe sequences are strongly encouraged especially in methods manuscripts, but their omission may be acceptable in clinical manuscripts if they are commercially available as products.
- 2 Inadequate storage and/or nucleic acid preparation, leading to poor nucleic acid quality and variable results.
- 3 Suboptimal primers for reverse transcription and/or PCR resulting in low yield, specificity and/or PCR efficiency.
- 4 Inappropriate analysis of data.
- 5 Use of a single reference gene in RT-qPCR without justification (2).

The guidelines require not only delineation of what was done, but presentation of evidence that validates the method used. For example, these include evidence of RNA integrity and purity, PCR specificity, calibration curves and calculations of PCR efficiency and limits of detection. At the option of the editor, MIQE requirements may be relaxed in reports using qPCR arrays, although all manuscripts will be judged on their relative merit, and the relative merit of a manuscript using qPCR increases as compliance with the MIQE guidelines increases.

References:

- 1 Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem*. 2009 Apr;55(4):611-22.
- 2 Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*. 2002 Jun 18;3(7):RESEARCH0034.

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### ***ETHICAL CONSIDERATIONS***

In order to ensure the highest ethical standards, *Clinical Chemistry* adheres to the recommendations from the [International Committee of Medical Journal Editors \(ICMJE\)](#) and the [Committee on Publication Ethics \(COPE\)](#) for guidance on policies and procedures regarding scientific misconduct. Misconduct including, but not limited to, the following is strictly prohibited and will result in immediate removal of a paper from consideration by the journal.

#### **Duplicate Submissions**

Submissions that are under review or that have been previously published with another journal.

#### **Duplicate Publications and Plagiarism**

Manuscripts that have substantial overlap with a manuscript that has already been published, without proper citation.

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### ***TYPES OF SUBMISSIONS***

- |                                    |                                      |
|------------------------------------|--------------------------------------|
| • <a href="#">Article</a>          | • <a href="#">Opinion</a>            |
| • <a href="#">Citation Classic</a> | • <a href="#">Perspective</a>        |
| • <a href="#">Clinical Chemist</a> | • <a href="#">Point/Counterpoint</a> |
| • <a href="#">Clinical Case</a>    | • <a href="#">Q&amp;A</a>            |

- |   |   |
|---|---|
| <ul style="list-style-type: none"> <li>• Study / Commentary</li> <li>• Correction</li> <li>• Editorial</li> <li>• Genetic Metabolic Series</li> <li>• Inspiring Minds</li> <li>• Letter to the Editor / Reply</li> <li>• Mini-Review</li> <li>• Obituary</li> </ul> | <ul style="list-style-type: none"> <li>• Reflection</li> <li>• Review</li> <li>• Special Report</li> <li>• Unveiling the Right Side</li> <li>• What Is Your Guess?</li> </ul> |
|---|---|

## **ARTICLE**

Research or scientific articles are submitted directly from authors. There are no restrictions on topics that are considered for publication, as long as the subject matter is original and relates experimentally or theoretically to the field of laboratory medicine. The information must be sufficiently detailed not only to enable readers to understand and appreciate the material presented, but also to permit replication of the work by other scientists in the field. Articles should contain a structured abstract limited to 250 words and formatted to include separate headings of: Background, Methods, Results, and Conclusions. The main text should not exceed 3,500 words. The manuscript should have no more than 40 references and a total of 6 tables and/or figures. Supplemental data are permitted for Articles.

Articles should list no more than 20 authors, with any additional contributors listed in the Acknowledgments. Although exceptions are rare, you may email [clinchemed@aacc.org](mailto:clinchemed@aacc.org) detailing each author's contribution to your submission, which will be forwarded to the editor.

Authors may submit short videos to complement their Articles. Videos can be used to illustrate a laboratory technique, hardware prototype, or clinical presentation that would benefit from such an addition, or to visually communicate to the reader novel features, special steps in a procedure, pitfalls, or other information that may not be easily conveyed through text or a figure. Videos should be of high quality, no more than 5 minutes in length, and submitted as .mp4 files. Please do not send proprietary file types such as .wmv (Windows Media) and .rm (Real Media) files.

Authors of Articles will be prompted at submission to provide a

brief summary of their work, not to exceed 100 words. In the event of acceptance, this will be recorded and included as an audio file in all online versions of the table of contents. The Audio Summary should address the following questions:

- 1 What was the paper about/what was the rationale for the study?
- 2 How did you approach the problem?
- 3 What were your findings?
- 4 What are the implications of the findings and how do they add to the field?

A simple phonetic spelling of the first author's full name should also be provided.

### ***CITATION CLASSIC***

Citation Classics are typically invited submissions that highlight a landmark article in the field of clinical chemistry. In this feature, one of the authors of the original article provides some historical insights and anecdotal stories surrounding its publication.

Citation Classics should not include an abstract and are limited to 600 words, no more than 6 references, and no more than 5 authors. Generally, tables and figures are not permitted; however, if the text does not exceed 500 words, one table or figure will be allowed. Also, supplemental data are not permitted for Citation Classics.

### ***CLINICAL CHEMIST***

This monthly feature provides a forum for informing readers about general items of interest. Topics might include announcements for upcoming conferences, awards received by members of the AACC, announcements of new features in the journal, humorous items, artwork or photographs from readers, or general scientific news. Readers may submit items for consideration in the following categories: [Unveiling the Right Side](#), [What Is Your Guess?](#), and [Genetic Metabolic Series](#), following the specific guidelines for each. The editors will make the final decision on the appropriateness and priority for inclusion in this section of the journal.

Please note that the journal is not currently considering unsolicited submissions to the Technology Corner, Science in the Arts, and Best of *Clinical Chemistry* sections.

### ***CLINICAL CASE STUDY / COMMENTARY***

Clinical Case Studies are submitted directly from authors. These articles are intended to be educational, with the goal of helping to develop or improve problem-solving skills. Clinical Case Studies may report unusual (although not necessarily rare) biochemical manifestations of disease, atypical presentation of disease, situations where the laboratory helped in making or clarifying a diagnosis, or information that would be helpful in understanding the pathophysiology of a disease.

Two accompanying commentaries will introduce additional concepts that may be useful to readers, discuss confounding factors that might affect a diagnosis or analytical result, and/or provide comments about the case itself. Commentaries are invited and authored by clinical chemists, physicians, or scientists with expertise in the area.

A Clinical Case Study should not include an abstract. It should, however, include a case description followed by the case discussion, limited to 1,500 words total. References are limited to 10, and the tables and figures are limited to 2 in total. Authors should include 3-5 brief questions regarding the case that would stimulate discussion and learning about the disease state. These questions will be circulated to educational centers before publication. The author should also list up to 5 points to remember at the end of the manuscript. The questions and points are not included in the manuscript count of 1,500 words. The case should read as a bit of a mystery or diagnostic dilemma. The case title, case description, and questions should not disclose the diagnosis. Supplemental data are not permitted for Clinical Case Studies. No more than 5 authors should be listed.

Commentaries are limited to 300 words. They should not include an abstract, references, or tables and figures and should have no more than 5 authors. Supplemental data are not permitted for Commentaries.

### ***CORRECTION***

Corrections are unique and will be considered on a case-by-case basis. Authors are encouraged to contact the Editorial Office at [clinchemed@aacc.org](mailto:clinchemed@aacc.org) should they wish to submit a Correction or should they find a printer error that needs correcting.

### ***EDITORIAL***

These are typically invited submissions. Editorials provide opinions and observations by an expert in the field about the subject matter

or content of a scientific paper published in *Clinical Chemistry*. In addition to further educating readers on a selected topic, Editorials are designed to stimulate readers to formulate their own opinions about a paper and its value to the field. In some cases, Editorials may also be independent opinions and observations about a controversial topic or changes taking place in the field. Editorials are limited to 1,500 words. They should not include an abstract. References are limited to 15, and tables and figures are not permitted. No more than 5 authors should be listed. Supplemental data are not permitted for Editorials.

### ***GENETIC METABOLIC SERIES***

The Genetic Metabolic Series falls under the Clinical Chemist and contains short clinical cases in biochemical genetics. Each article will include the actual case presentation, a chromatogram, the biochemical pathway of the defect, and a short case discussion. Submissions should not exceed 600 words in length and should include no more than 2 tables or figures and 1 reference.

### ***INSPIRING MINDS***

These biographical articles are commissioned to present the achievements of distinguished clinical chemists, as well as their philosophical views on their professional life and the field of clinical chemistry.

### ***LETTER TO THE EDITOR / REPLY***

Letters are submitted directly from authors and can be used to report an observation or to discuss a previously published [original Article](#). Letters in response to papers other than [Articles](#) will not be considered for publication. Letters report observations on interferences, suggestions to improve test performance, or other observations that are of importance to the wider audience. A Reply to a Letter may also be solicited by the editors. The one figure or table provided should be concise and should not be multipart (i.e., Fig. 1A, 1B, 1C, Part 1, Part 2).

A Letter to the Editor is limited to 750 words. It should not include an abstract. The references are limited to 5, and only 1 table or figure is permitted. Supplemental data are not permitted for Letters to the Editor.

A Reply is limited to 750 words. It should not include an abstract. The references are limited to 5, and only 1 table or figure is permitted. Supplemental data are not permitted for Replies.

Letters to the Editor and Replies should list no more than 8 authors, with any additional contributors listed in the Acknowledgments. Although exceptions are rare, you may email [clinchemed@aacc.org](mailto:clinchemed@aacc.org) detailing each author's contribution to your submission, which will be forwarded to the editor. In some instances, editors may request that a submission of another article type to *Clinical Chemistry* be decreased to meet the requirements of a Letter to the Editor.

### **MINI-REVIEW**

Mini-Review articles are typically invited submissions. Mini-Reviews are intended to provide a general overview of a topic. Basic information is provided, along with selected references that can aid the reader in obtaining additional information about the subject. The use of illustrative figures or tables is encouraged. A Mini-Review article should consist of a structured abstract limited to 250 words with headings of Background, Content, and Summary. The text should not exceed 3,500 words. The manuscript should have no more than 40 references and a total of 4 tables and/or figures. Supplemental data are permitted for Mini-Review articles.

Mini-Reviews should list no more than 5 authors, with any additional contributors listed in the Acknowledgments. Although exceptions are rare, you may email [clinchemed@aacc.org](mailto:clinchemed@aacc.org) detailing each author's contribution to your submission, which will be forwarded to the editor.

### **OBITUARY**

Obituary announcements and associated biographies can be commissioned by the Journal or submitted by authors. Prior to submission of an Obituary, authors should contact the Journal with information about the person who has passed away and with a description of the individual's career achievements and unique contributions. A decision will then be made on proceeding with the Obituary.

Obituaries should include personal information about the deceased (birthplace, education, place of residence, employment), highlights of this person's achievements (research accomplishments, awards, elected positions, committees, service to the profession), and anecdotal information about what made the person unique.

Obituaries are limited to 600 words with no abstract, references, or tables. One figure/image file is permitted. Supplemental data are not permitted for Obituaries.

### **OPINION**

Opinion articles are invited submissions that present the belief or personal view of the author(s) on a specific topic. An opinion implies a conclusion thought out yet open to dispute. Opinion articles are often a formal expression by an expert of his/her judgment or advice. Unlike Editorials and Letters to the Editor, Opinion articles do not comment on, or refer to, specific papers published in the journal.

An Opinion should not include an abstract and is limited to 1,500 words, 15 references, 1 table or figure, and no more than 5 authors. Supplemental data are not permitted for Opinions.

### **PERSPECTIVE**

These articles are invited submissions. Perspectives highlight a clinical, analytical, or basic science report that was published in a journal other than *Clinical Chemistry* but has implications for the practice of clinical chemistry.

Perspectives should not include an abstract. They are limited to 1,500 words, 5 references, 1 table or figure, and no more than 5 authors. Supplemental data are not permitted for Perspectives.

### **POINT/COUNTERPOINT**

These articles are typically invited submissions from experts in a selected discipline and provide different viewpoints on a topic that may be controversial, lacks consensus in the scientific community, or may be of high public interest. In most cases an author or group of authors is asked to write the first half of the article, describing the importance of the topic, challenges to be addressed, current limitations, and/or unmet needs. A second author or group of authors is invited to provide a "Counterpoint" discussion of a different viewpoint or critical factors.

Point/Counterpoint submissions should not include an abstract. The manuscript is limited to 1,500 words, 15 references, and 1 table or figure. Supplemental data are not permitted for Point/Counterpoint.

### **Q&A**

This invited feature is meant to highlight a timely and important

issue, either clinical or analytical, through a series of questions posed to leaders in the field by a moderator. The moderator is required to include an introductory paragraph and photographs of each expert. Five to 8 questions may be posed to 3 to 5 experts with the moderator documenting the answers. Submissions are limited to 3,500 words.

### ***REFLECTION***

These articles are invited submissions. Reflections are authored by highly accomplished scientists in their field who have greatly contributed to science. Reflections will be reserved for special issues and will focus on the specific advancements the individual has made in his or her field.

Reflections are limited to 2,000 words and should not include an abstract. The references are limited to 20, and 1 table or figure is permitted. Supplemental data are not permitted for Reflections.

### ***REVIEW***

Review articles are typically invited submissions. Reviews are intended to provide comprehensive coverage of a topic, including background clinical or analytical information, the relevance and importance of the subject matter, and potential future directions. The use of illustrative figures or tables is encouraged.

A Review article should consist of a structured abstract with headings of Background, Content, and Summary limited to 250 words. The text should not exceed 5,000 words. The manuscript should have no more than 75 references and a total of 6 tables and/or figures. Supplemental data are permitted for Review articles.

Reviews should list no more than 15 authors, with any additional contributors listed in the Acknowledgments. Although exceptions are rare, you may email [clinchemed@aacc.org](mailto:clinchemed@aacc.org) detailing each author's contribution to your submission, which will be forwarded to the editor.

### ***SPECIAL REPORT***

Special Reports may be submitted directly by authors or invited by the journal. The types of papers that would be considered include consensus reports, guideline development, position statements, or evidence-based recommendations on test utilization or quality specifications. The editors may also decide to classify other

miscellaneous submissions under this heading.

A Special Report should consist of a structured or unstructured abstract limited to 250 words. The main text should be no more than 5,000 words. The manuscript should have no more than 40 references and a total of 4 tables and/or figures. Supplemental data are permitted for Special Reports.

Special Reports should list no more than 20 authors, with any additional contributors listed in the Acknowledgments. Although exceptions are rare, you may email [clinchemed@aacc.org](mailto:clinchemed@aacc.org) detailing each author's contribution to your submission, which will be forwarded to the editor.

### ***UNVEILING THE RIGHT SIDE***

Submissions should highlight the creative side of someone in the field of chemistry. This can be poetry, a short story, photographs, or other creative artwork. Submissions are limited to 400 words and/or one image, photograph, or poem. All submissions are subject to review. Cover letter should state interest in contributing to Unveiling the Right Side and must be submitted under the category of Clinical Chemist.

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### ***MANUSCRIPT PREPARATION***

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*Clinical Chemistry* follows the [recommendations](#) for authorship set out by [The International Committee of Medical Journal Editors \(ICMJE\)](#). In accordance with these recommendations, manuscripts are considered for publication with the understanding that each listed author must meet the following criteria:

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- 1 International Committee of Medical Journal Editors. Uniform requirements for manuscripts submitted to biomedical journals. *Ann Intern Med* 1997;126:36-47. [[Full Text](#)]

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- 2. Fiechtner M, Ramp J, England B, Knudson MA, Little RR, England JD, et al. Affinity binding assay of glycohemoglobin by two-dimensional centrifugation referenced to hemoglobin Alc. Clin Chem 1992;38:2372–9.

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- *Abstract:*

- 3. Hortin GL, King C, Kopp J. Quantification of rhesus monkey albumin with assays for human microalbumin [Abstract]. Clin Chem 2000;46:A140-1.

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- *Editorial:*

- 4. Demers LM. New biochemical marker for bone disease: is it a breakthrough? [Editorial]. Clin Chem 1992;38:2169–70.

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- *Letter to the Editor:*
- 5. Davey L, Naidoo L. Urinary screen for acetaminophen (paracetamol) in the presence of N-acetylcysteine [Letter]. Clin Chem 1993;39:2348–9.
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- *Book Chapter:*
- 6. Rifai N, Warnick GR. Lipids, lipoproteins, apolipoproteins, and other cardiovascular risk factors. In: Burtis CA, Ashwood ER, Bruns DE, editors. Tietz textbook of clinical chemistry and molecular diagnostics. 4th Ed. St. Louis (MO): Elsevier Saunders; 2006. p. 903-81.
- 
- *Thesis:*
- 7. Haughton MA. Immunonephelometric measurement of vitamin D binding protein [MAppSci thesis]. Sydney, Australia: University of Technology, 1989:87pp.
- 
- *Books and Monographs (serial volumes should be treated as journals):*
- 8. Harrell FE Jr. Regression modeling strategies. New York (NY): Springer; 2001.
- 9. Bailar JC III, Mosteller F, editors. Medical uses of statistics. 2nd Ed. Boston (MA): NEJM Books; 1992:449 p.
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- *Published Ahead of Print:*
- 10. Milbury CA, Li J, Makrigiorgos GM. PCR-based methods for the enrichment of minority alleles and mutations. [Epub ahead of print] Clin Chem February 6, 2009 as doi:10.1373/clinchem.2008.113035.
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- *Supplement:*
- 11. Castelli WP. Lipids, risk factors and ischaemic heart disease. Atherosclerosis 1996;124 Suppl:S1-9.
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- *Internet Source:*
- 12. American Association for Clinical Chemistry. AACC continuing education. <http://www.aacc.org/education-and-career/continuing-education> (Accessed June 2016).
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- *Clinical Chemistry Trainee Council Webcasts and Pearls of Laboratory Medicine:*

- 14. Browning T. Vitamin D. April 2012. Available from: <http://www.traineecouncil.org/pearl> (*Clinical Chemistry Trainee Council, Pearls of Laboratory Medicine*).

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If you have registered and have not received a verification e-mail, this may be due to a spam blocking filter associated with your institutional server. You may need to manually register [clinchemed@aacc.org](mailto:clinchemed@aacc.org) so as to accept mail from *Clinical Chemistry*. Your Systems Manager should be able to help you with this problem.

#### Java Difficulties:

If you are using Internet Explorer as your browser and are having trouble accessing pages, you might want to try the following:

### Tools

### Internet Options

### Advanced

Under heading Microsoft VM, make sure all 3 boxes are checked:

*Java console*

*Java logging*

*JIT Compiler*

Click okay.

### Restart machine

You may check to see if the site works properly by going to <http://submit.clinchem.org/>.

Please note that login information (e-mail and password) for the submission and review site will not be the same login information you use if you subscribe to the online journal.

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Often, due to problems with the initial registration or infrequent use, an author may have two accounts listed. Multiple accounts lead to future system access problems and therefore must be merged.

If an author or reviewer suspects this is the problem, send a request with the preferred e-mail address, current contact information, and the suspected invalid e-mail address to the *Clinical Chemistry* editorial office at [clinchemed@aacc.org](mailto:clinchemed@aacc.org).

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Bench>Press requires users to have their browser set to accept cookies in order to maintain their logged-in status throughout the system. A "cookie" is a message given to a browser by a server. The browser stores the message, and the message is sent back to the server each time the browser requests a page from the server. If the user does not have cookies enabled, the user will be required to repeatedly sign in to the system.

Most browsers will allow the user to manually alter the cookie settings from the Tools or Internet Options bar.

#### Expertise Terms:

The Area of Expertise list was developed to help editors choose reviewers who possess specific knowledge in a given area.

Our tracking system has been set to allow this page to appear---as a gentle reminder---until the reviewer has selected at least one item on the list. As soon as the reviewer selects a topic and saves it, it will no longer appear.

Changing these areas of expertise or adding more detailed information can be accomplished by simply logging into <http://submit.clinchem.org/> at any time and clicking the Personal Info area at the top of the page.

On the lower right side of the page, the user will find "Expertise Terms: Edit Expertise Terms." Click on this link and change the data accordingly.

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*1000 Kilobytes (KB) = 1 Megabyte (MB)*

*1000 Megabytes (MB) = 1 Gigabyte (GB)*

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### **CLINICAL CHEMISTRY EDITORIAL OFFICE**

*Clinical Chemistry*

900 Seventh Street, NW

Suite 400

Washington, DC 20001

United States

[clinchemed@aacc.org](mailto:clinchemed@aacc.org)

(202) 420-7678

(202) 833-4576 [FAX]

Sheehan Misko, Associate Director: [smisko@aacc.org](mailto:smisko@aacc.org)

Kristen Hauck, Assistant Managing Editor: [khauck@aacc.org](mailto:khauck@aacc.org)

Sarah Lake, Editorial Specialist: [slake@aacc.org](mailto:slake@aacc.org)

Patty Brady, Editorial Specialist: [pbrady@aacc.org](mailto:pbrady@aacc.org)

Erin Roberts, Editorial Specialist: [eroberts@aacc.org](mailto:eroberts@aacc.org)

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