ORIGINAL ARTICLE

Derivation and Internal Validation of an Equation for Albumin-adjusted Calcium at a Tertiary Hospital in Selangor, Malaysia

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ABSTRACT

Introduction: Total calcium concentration is widely used to assess body calcium status although limited by many confounding factors. Thus, this study aimed to derive and internally validate an albumin-adjusted calcium equation for a selected Malaysian population. **Method:** This cross-sectional study involved 1011 adults at an emergency department of a tertiary hospital. Patients who had total calcium, ionised calcium and albumin measurements taken simultaneously were included. Derivation of the albumin-adjusted calcium equation was based on the adjustment equation obtained from the Association for Clinical Biochemistry and Laboratory Medicine 2015 position paper. Additionally, the equation was internally validated and compared with ionised calcium (gold standard) and the conventional Payne's equation. **Results:** The newly derived equation = total calcium + 0.017 (41.35 – albumin). Internal validation exhibited the amount of shrinkage of 0.049. It tends to overestimate the adjusted calcium by a mean difference of 0.029 mmol/L compared to Payne's equation. The comparison between Payne's equation and the new equation with ionised calcium reclassified 402 and 486 patients, respectively into different calcium status. When both equations were compared, calcium status classification significantly differed in all and hypoalbuminaemic subjects by 90 and 16 patients, respectively. **Conclusion:** Locally derived albumin-adjusted calcium equation differed statistically in calcium status classification when compared to the Payne's equation. However, to confirm this significance, the result must be compared to ionised calcium under strict, controlled preanalytical conditions. In terms of clinical significance, there was no difference in classification of calcium status between Payne's and the new equation at medical decision limits.

Keywords: Albumin-adjusted calcium, Corrected calcium, Total Calcium, Ionised calcium, Hypoalbuminaemia

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INTRODUCTION

Disorders of calcium homeostasis are one of the commonest encountered problems in hospitalised patients. In a study done in Italy on hospitalised patients, 27.7% were hypocalcaemic (1) while a review literature quoted that 15% to 88% of them were hypocalcaemic (2). In Malaysia, the prevalence of hypocalcaemia and hypercalcaemia in hospitalised patients in 1995 were 18% and 2.4%, respectively (3). However, there is no recent data available. Calcium disorders have severe implications on the health of the patient, increasing morbidity, mortality and health costs.

Measurement of serum calcium is typically used to assess body's calcium status, which provides supporting biochemical information whether a patient needs treatment. In current practice, total calcium is often used as the primary measurement method. However, total calcium measurement has several limitations since many confounding factors can affect the equilibrium between the free ionised and the bound calcium. These include an abnormality in plasma protein concentrations such as in multiple myeloma and disturbances in acid-base status, which is common in acutely ill patients

(4). Since 40% of total calcium is protein-bound, variation in protein levels especially albumin will consequently alter the concentration of measured total calcium. This variation does not affect the level of free ionised calcium (5). Thus ideally, the free ionised calcium measurement has been suggested as a gold standard for calcium status (6).

Considering the effect of albumin concentration on serum total calcium measurement, researchers have developed equations to compensate for this effect to get 'corrected calcium'. Several equations are currently available such as by Payne (7) and Orrell (8). These equations were developed many decades ago using linear regression analysis based on their respective laboratory's local population results, hence based on Western population and specific methods. Thus, these equations may be invalid when calcium and albumin results are generated by alternative assays, rendering the serum total calcium to albumin relationship unreliable (9). For example, a new method for serum calcium measurement uses 5-nitro-5′-methyl-(1,2-bis(o-aminophenoxy)ethan-N,N,N′,N′-tetraacetic acid (NM-BAPTA) method while Payne's equation was developed using calcium O-cresophthalein complexone (O-CPC) method. Currently, the customarily cited equation by Payne in 1973, which is 'corrected calcium (mmol/L) = total calcium (mmol/L) + 0.02 (40 - [albumin] (g/L) is routinely used worldwide by various clinical laboratories to provide an approximation of calcium concentration in hypoalbuminaemia patients (10). The difference in methods may contribute to significantly different calcium results using the formerly established Payne's equation when applied to the analysers using a different method from Payne et al. (9). Hence, in 2015, the Association for Clinical Biochemistry and Laboratory Medicine (ACB) suggested the term 'corrected' to be changed to 'adjusted' and that each laboratory should develop its own adjusted calcium equation (10).

In view that the albumin-adjusted calcium equation is used as a clinical decision point, it is important that each laboratory establishes its locally derived albumin-adjusted calcium equation for its population. This will reduce morbidity and mortality associated with inaccurate classification of calcium status (hypo-, normo-, and hyper-calcaemia). Hence, this study aimed to derive and internally validate an albumin-adjusted calcium equation for total calcium and serum albumin measurements in a multi-racial Malaysian population using local laboratory data. Total calcium and albumin were also compared according to gender, race and age as well as the difference in classification of calcium status was compared between Payne's and the newly derived equation with ionised calcium, respectively. The difference in classification of calcium status between Payne's with the newly derived equation was also done for all and hypoalbuminaemia patients.

MATERIALS AND METHODS

Study population

This cross-sectional study was performed at Hospital Tengku Ampuan Rahimah (HTAR), Selangor from January to July 2017 involving 1011 subjects who attended the emergency department (ED). The sample size was based on the recommendation by ACB that 1000 values are to be collected for the study with at least 30 data points for each whole integer albumin concentration (10). Recruitment of patient's data was done by universal sampling. The inclusion criteria were Malaysian subjects aged 18 years old and above who had total calcium, ionised calcium and albumin results available. They also had to have serum creatinine, urea, potassium, aspartate aminotransferase (AST) and alkaline phosphatase (ALP) results available from concurrent blood drawing for the exclusion criteria. Patients excluded were as per the ACB recommendations with the following biochemical abnormalities: i) renal impairment (creatinine >200μmol/L or urea >15mmol/L), ii) hypomagnesaemia (hypokalaemia as a surrogate marker), iii) AST and/or ALP >upper reference limit, iv) total calcium concentration <2.0mmol/L or >2.7mmol/L, v) albumin concentration <20g/L or > 50g/L and vi) patients with malignancy.

Data collection

Patients who had fulfilled the inclusion criteria were selected and their laboratory results were extracted from the hospital-web based laboratory information system. Only the first total calcium, ionised calcium and albumin results taken on the same day for each patient during the study period were recorded. Demographics and clinical data from the eligible subjects were collected and recorded into the pro-forma. Confidentiality of patient's identification was ensured. This study was approved by the Malaysian Research Ethical Committee (MREC) Ministry of Health (NMRR-16-2273-33101).

Laboratory investigations

Radiometer ABL 800 (Radiometer, Copenhagen, Denmark) was used for ionised calcium measurement. Total calcium and albumin measurements were done by NM-BAPTA and bromocresol green (BCG) methods, respectively using Cobas c702 (Roche Diagnostics International, Rotkreuz, Switzerland). During the study period, the coefficients of variation (CV) for ionised calcium, total calcium and albumin were 1.5% to 2.2%, 1.8% to 2.3% and 2.4% to 3.1%, respectively. Analytical bias based on External Quality Assurance Program was 0.025 and 0.9 for total calcium and albumin, respectively.

Data analysis

Statistical calculations were performed using the standard statistical software package, IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp. Total

calcium and albumin were compared according to age, gender and race using independent t-test and one-way ANOVA, respectively. The 1011 patients were further divided into two groups consisting of 749 subjects for the derivation population (~ 3/4 of sample size) and 262 subjects for the validation population (~ 1/4¼ of sample size) based on previous studies (11, 12). In all statistical analyses, a 'p' value of <0.05 (95% confidence interval) was considered statistically significant.

i. Derivation of the new albumin-adjusted calcium equation

Derivation sample (749 subjects) was used to determine the association between total calcium and albumin using linear regression analysis. From the analysis, the slope of the best fit line, the Y-axis intercept, which represents the probable calcium concentration at zero albumin concentration and the correlation coefficient (r) were calculated. Determination of the adjustment equation obtained from the 2015 position paper guideline on 'Albumin- adjusted calcium' by ACB (10) is as follows:

Adjusted calcium =

Total calcium – [slope (albumin)] + [mean normal total calcium – intercept calcium]

where

slope = the slope of the best fit regression line

mean total calcium = the mid-point of the normal healthy population calcium reference range

ii. Internal validation of the new albumin-adjusted calcium equation

Internal validation was undertaken using the validation sample (262 subjects). Initially, the amount of shrinkage of the newly derived equation was calculated to validate it. This was done by applying the newly derived equation to the validation sample in order to find a predicted calcium concentration for each subject. To obtain an estimate of the variance for both the derivation and validation groups, measured calcium was regressed on the predicted calcium. The adjusted r^2 for the validation sample was deducted from the derivation sample to obtain an estimate of the amount of shrinkage, which is an indicator of how much the predictive ability reduces when the equation is applied to other samples. The regression is considered internally valid if the shrinkage is small (12).

iii. Agreement between the newly derived equation and Payne's equation

To assess agreement between the new equation and Payne's equation, Bland-Altman plot with the mean difference together with the 95% limits of agreement were used. This plot shows the difference in calcium concentration between the two equations against the mean of the two values for each subject (12).

iv. Classification of calcium status comparing Payne's equation and newly derived equation with ionised calcium, respectively

To compare the agreement in the classification of calcium status between the new equation and Payne's equation with ionised calcium, the pattern of individual differences was calculated and recorded as hypo-, normo-, and hyper-calcaemia respective to each entity. McNemar test was used and exemplified as within or outside the laboratory reference range to illustrate the degree of agreement (12).

v. Classification of calcium status comparing Payne's equation with newly derived equation in all and hypoalbuminaemia patients

The same techniques in (iv) were applied to compare the agreement in classification of calcium status between Payne's equation with the newly derived equation in all and hypoalbuminaemia patients.

RESULTS

Demographics of subjects and their associations with serum total calcium and albumin

Majority of subjects were Malay (57.8%) males (53.4%) with median age of 32 years [interquartile range (IQR) = 21] (Table I). The mean ± standard deviation (SD) for total serum calcium and albumin concentrations for the overall study population were 2.22 ± 0.14 mmol/L and 41.84 ± 4.46 g/L, respectively. Statistically significant results were obtained when total calcium was compared with gender and age whereas for albumin, age and race were significantly associated (Table II).

Table I : Demographics of subjects according to age, gender and race

Demographic variables	n	%
Age:		
< 30 years old	431	42.6
≥ 30 years old	580	57.4
Gender:		
- Male	540	53.4
- Female	471	46.6
Race:		
- Malay	584	57.8
- Chinese	149	14.7
- Indian	270	26.7
- Others	8	0.8

Derivation of an albumin-adjusted calcium equation

Figure 1 illustrates the linear regression analysis between serum total calcium and serum albumin attained

Table II : Comparison of total calcium and albumin according to gender, race and age

Demo-graphic	Total calcium			Albumin		
	Mean ± SD	t or F value	p value	Mean ± SD	t or F value	p value
Gender						
- Male	2.20 ± 0.18	-0.378 [a]	0.04*	43.0 ± 5.0	3.179 [a]	0.49
- Female	2.19 ± 0.22			42.0 ± 6.0		
Age (years)						
< 30	2.20 ± 0.13	-2.270 [a]	0.02*	42.5 ± 4.1	4.245 [a]	0.00*
≥ 30	2.22 ± 0.14			41.3 ± 4.6		
Race						
- Malay	2.19 ± 0.19	0.143 [b]	0.706	42.0 ± 7.0	10.11 [b]	0.02*
- Chinese	2.20 ± 0.17			42.0 ± 6.0		
- Indian	2.21 ± 0.21			42.0 ± 5.0		
- Other	2.15 ± 0.21			41.0 ± 7.0		

*Statistically significant ($p < 0.05$)

[a] Independent t-test statistical test (t)

[b] Oneway ANOVA statistical test (F)

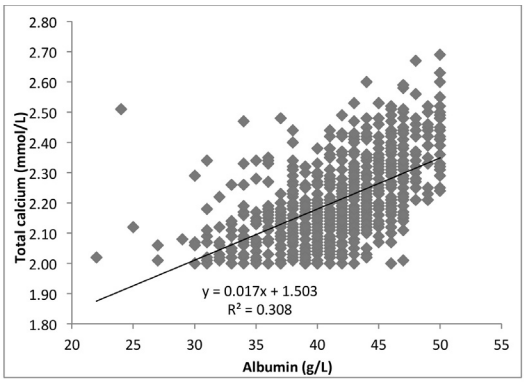

Figure 1 : Linear regression relationship of serum total calcium and albumin derived from a cohort of 749 patients with simultaneous total calcium and albumin measurements.

from the derivation cohort (749 subjects). Within the derivation cohort, the mean ± SD for serum total calcium was 2.21 ± 0.14 mmol/L. From this analysis, the regression equation = total calcium (mmol/L) - 0.017 [albumin (g/L)] + 1.503. The regression value for slope and intercept were substituted into the working equation as proposed by ACB (10):

Adjusted calcium =

Total calcium – [slope (albumin)] + [mean normal total calcium – intercept]

Total calcium – [0.017 (albumin)] + [2.206 – 1.503]

Total calcium – [0.017 (albumin)] + 0.703

Total calcium + 0.703 – [0.017 (albumin)]

Total calcium + 0.017 [(0.703/0.017) – albumin]

Total calcium + 0.017 (41.35 – albumin)

Validation of an albumin-adjusted calcium equation

The newly derived albumin-adjusted calcium equation, total calcium + 0.017 (41.35 – albumin) was applied to the derivation and validation populations to obtain the values for adjusted calcium concentration. Total calcium values were regressed on adjusted calcium value for both populations to give an adjusted r^2 value for each population. Then, the derivation cohort's adjusted r^2 value was subtracted from the validation cohort's adjusted r^2 value. This was done to calculate the amount of shrinkage of the newly derived albumin-adjusted calcium equation. The amount of shrinkage represents how reliable the newly derived albumin-adjusted equation is when it is applied to other populations apart from the population it was derived from. The amount of shrinkage was calculated as follows:

Linear regression equation for derivation sample (749 subjects):

Total calcium = 0.69 [predicted calcium (g/L)] + 0.714

Adjusted r^2 = 0.688

Linear regression equation for validation sample (262 subjects):

Total calcium = 0.731 [predicted calcium (g/L)] + 0.611

Adjusted r^2 = 0.737

Amount of shrinkage:

Derivation sample adjusted r^2 - Validation sample adjusted r^2

0.688 - 0.737 = -0.049

The amount of shrinkage of 0.049 shows good internal validity as the value obtained is small.

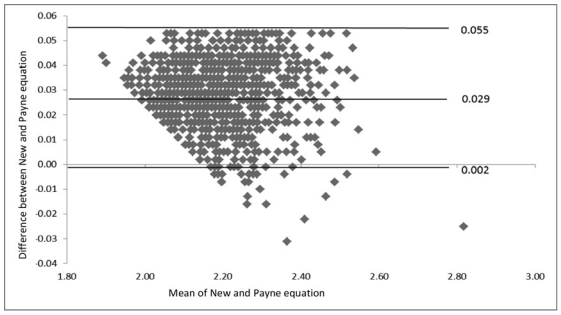

Figure 2 : Bland Altman plot including 95% limit of agreement between adjusted calcium by the Payne equation and the newly derived equation.

Agreement between the newly derived equation and Payne's equation

The mean ± SD for albumin-adjusted calcium by Payne's and the newly derived equation were 2.18 ± 0.11 and 2.21 ± 0.11 mmol/L, respectively. Bland-Altman plot shows mean difference of 0.029 mmol/L and limits of

agreement between 0.002 - 0.055 mmol/L. This shows that the newly derived equation tends to overestimate total calcium than Payne's equation (Figure 2).

Classification of calcium status comparing Payne's equation and newly derived equation with ionised calcium, respectively

Table III demonstrates the difference in classification of calcium status between the Payne's equation and newly derived equation with ionised calcium, respectively. Comparison between the Payne's equation and ionised calcium gave a different classification of calcium status in 402 patients. 379 patients were classified as normocalcaemia by the Payne's equation but hypocalcaemia by ionised calcium. A smaller proportion of patients were classified as hypocalcaemia (n=19) and hypercalcaemia (n=1) by Payne's equation but normocalcaemia by ionised calcium. Patients who were hypercalcaemia by ionised calcium measurement were hypocalcaemia (n=1) and normocalcaemia (n=2) by Payne's equation.

Table III : Classification of calcium status using Payne's equation and the newly derived equation with ionised calcium

	Ionised calcium		
	Hypocalcaemia (Ca < 1.15 mmol/L) n (%)	Normocalcaemia (Ca 1.15-1.29 mol/L) n (%)	Hypercalcaemia (Ca>1.29 mmol/L) n (%)
Payne's equation[1]			
Hypocalcaemia (Ca < 2.20 mmol/L)	568 (56.2)	19 (1.9)	1 (0.1)
Normocalcaemia (Ca 2.20 – 2.70 mmol/L)	379 (37.5)	41 (4.0)	2 (0.2)
Hypercalcaemia (Ca> 2.70 mmol/L	0 (0.0)	1 (0.1)	0 (0.0)
Newly derived equation			
Hypocalcaemia (Ca < 2.20 mmol/L)	481 (47.6)	16 (1.6)	1 (0.1)
Normocalcaemia (Ca 2.20 – 2.70 mmol/L)	466 (46.1)	44 (4.3)	2 (0.2)
Hypercalcaemia (Ca> 2.70 mmol/L	0 (0.0)	1 (0.1)	0 (0.0)

[1]Payne's equation: Corrected calcium = Total calcium (mmol/L) + 0.02 (40 - [albumin] (g/L)

[2]Newly derived equation: Adjusted calcium = Total calcium + 0.017 (41.35 – albumin)

Reference ranges used by HTAR based on ABL 800 and Roche Cobas inserts

Comparison between the newly derived equation and ionised calcium also gave discrepancy in the classification of calcium status in 486 patients (Table III). 466 patients were classified as normocalcaemia by the newly derived equation but hypocalcaemia by ionised calcium. A smaller proportion of patients were classified as hypocalcaemia (n=16) and hypercalcaemia (n=1) by the newly derived equation but normocalcaemia by ionised calcium. Similar to the comparison between Payne's equation and ionised calcium, patients who were hypercalcaemia by ionised calcium measurement were hypocalcaemia (n=1) and normocalcaemia (n=2) by the newly derived equation.

Classification of calcium status comparing newly derived equation with Payne's equation in all and hypoalbuminaemia patients

When the two equations were compared for all subjects, 90 of them were normocalcaemia with the newly derived equation but hypocalcaemia by the Payne's equation (Table IV). When the two equations were applied to 278 hypoalbuminaemia patients with serum albumin level between 20 - 39 g/L, 16 subjects were classified differently. They were normocalcaemia with the newly derived equation but hypocalcaemia by the Payne's equation (Table IV).

McNemar test for all three comparisons (Table V) showed a significant p value < 0.001 when calcium status was classified as within or outside the laboratory reference range. This indicates that the difference was significant between the two modalities for both Payne's equation and the newly derived equation with ionised calcium, respectively as well as between the newly derived equation and Payne's equation for all and hypoalbuminaemia patients.

DISCUSSION

Although the gold standard for calcium measurement is ionised calcium, most laboratories still practice measurement of total calcium as a means for evaluating calcium status. This is attributable to various limitations of ionised calcium measurement, making it unfeasible as a routine test (6). It is well known that total calcium is greatly influenced by albumin levels; hence numerous albumin-adjusted calcium equations have been developed to accommodate this effect. The chemical pathology laboratory at HTAR embarked on to derive and validate an albumin-adjusted calcium equation from our local data as recommended by the ACB (10). The equation's performance was assessed against ionised calcium to confirm the reliability of the equation.

Determination of albumin-adjusted calcium equation

Our study showed that the newly derived equation using our local data was not the same as the formerly published equation by Payne et al. and other studies (9, 11-13). Probable reasons for the difference can be divided into demographic factors (gender, age and race), types of analyser and analytical methods.

Demographic factors

i. Gender

Similar to a previous study, albumin concentrations were higher in males than females (14). In our study, mean albumin for males and females were 43 g/L and 42 g/L, respectively. However, this difference was not statistically significant in our study. The lower albumin concentrations in females can be explained by different hormonal basis in both sexes until menopause ensues

Table IV : Classification of calcium status comparing Payne's equation with the newly derived equation in all and hypoalbuminaemia patients

	Newly derived equation[2]					
	All patients n = 1011			Hypoalbuminaemia n = 278		
Payne's equation[1]	Hypocalcaemia (Ca < 2.20 mmol/L) n (%)	Normocalcaemia (Ca 2.20 – 2.70 mmol/L) n (%)	Hypercalcaemia (Ca > 2.70 mmol/L) n (%)	Hypocalcaemia (Ca < 2.20 mmol/L) n (%)	Normocalcaemia (Ca 2.20 – 2.70 mmol/L) n (%)	Hypercalcaemia (Ca > 2.70 mmol/L) n (%)
Hypocalcaemia (Ca < 2.20 mmol/L) n (%)	498 (49.3)	90 (8.9)	0 (0.0)	126 (45.3)	16 (5.8)	0 (0.0)
Normocalcaemia (Ca 2.20 – 2.70 mmol/L)n (%)	0 (0.0)	422 (41.7)	0 (0.0)	0 (0.0)	135 (48.5)	0 (0.0)
Hypercalcaemia (Ca > 2.70 mmol/L) n (%)	0 (0.0)	0 (0.0)	1 (0.1)	0 (0.0)	0 (0.0)	1 (0.4)

[1]Payne's equation: Adjusted calcium= Total calcium + 0.02 (40 – albumin)
[2]Newly derived equation: Adjusted calcium= Total calcium + 0.017 (41.35 – albumin)

Table V : Classification of calcium status using 'within or outside range' between Payne, newly derived equation and ionised calcium. McNemar Test

	Ionised calcium n (%)			Newly derived equation[2] n (%)					
				All patients n = 1011			Hypoalbuminaemia n = 278		
	Within range (Ca 1.15 - 1.29 mmol/L)	Outside range (Ca < 1.15 or Ca > 1.29 mmol/L)	p value	Within range (Ca 2.20 – 2.70 mmol/L)	Outside range (Ca < 2.20 or Ca > 2.70 mmol/L)	p value	Within range (Ca 2.20 – 2.70 mmol/L)	Outside range (Ca < 2.20 or Ca > 2.70 mmol/L)	p value
Payne equation[1]									
Within range (Ca 2.20 – 2.70 mmol/L)	20 (2.0)	569 (56.3)	0.001	422 (41.7)	0 (0.0)	0.001	135 (48.6)	0 (0.0)	0.001
Outside range (Ca < 2.20 or Ca > 2.70 mmol/L)	41 (4.0)	381 (37.7)		90 (8.9)	499 (49.4)		16 (5.7)	127 (45.7)	
Newly derived equation[2]									
Within range (Ca 2.20 – 2.70 mmol/L)	44 (4.4)	468 (46.3)	0.001						
Outside range (Ca < 2.20 or Ca > 2.70 mmol/L)	17 (1.7)	482 (47.6)							

[1]Payne equation: Adjusted calcium= Total calcium + 0.02 (40 – albumin)
[2]Newly derived equation: Adjusted calcium= Total calcium + 0.017 (41.35 – albumin)

(14). In this study, total calcium was slightly higher in males (2.20 mmol/L) than females (2.19 mmol/L). This is similar to Wallace et al. (15) who found lower total calcium in menopausal women. Low oestrogen level indirectly affects serum calcium by decreasing intestinal calcium absorption. Oestrogen stimulates conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D thus enhancing intestinal calcium absorption (15). Another reason is that men have higher dairy intake than women (15). Although our study only found a minute difference in total calcium values, it was statistically significant supporting that gender variation affects total calcium concentration.

ii. Age

We found that mean albumin was significantly lower in subjects ≥ 30 years old than < 30 years old (41.3 g versus 42.5 g). Another study also found that albumin level peaks around the age 20 and starts to decline with advancing age in both males and females (16). This can be explained by limited dietary intake that contributes to malnutrition especially in the older population (16). A study done by Ko et al. in Hong Kong found no significant difference in total calcium concentrations when stratified by 10-year age groups in men but otherwise in women. However, our study showed higher total calcium levels in subjects ≥ 30 years old and males. This finding may be due to aging where there is relative increase in bone resorption but low bone formation (17).

iii. Ethnicity

Albumin concentrations showed statistically significant difference between ethnicities. Thus, the difference in our newly derived equation maybe due to the multiethnic population studied. However, the difference was not clinically significant at medical decision limits.

At medical decision limits of 1.75 mmol/L, 2.69 mmol/L and 3.24 mmol/L (18), our equation did not show a significant difference in 'adjusted' calcium values since the difference was 0.03 mmol/L, which was smaller than the medically allowable error (0.09, 0.12 and 0.14 mmol/l) at each level by The Clinical Laboratory Improvement Amendments of 1988 (CLIA 88). This was similar to the equation by Davies et al., which was developed in the British population (11). Therefore, although the equations differed, the total calcium results still agreed with each other at medical decision levels. Unfortunately, studies comparing albumin-adjusted calcium equations among Asians are limited. Nevertheless, this finding may be explained by non-similar type of diet consumed by each ethnicity (19). Redmond and colleagues encountered that calcium and phosphate metabolisms differed in White American and African American populations and attributed these findings to non-identical dietary styles as well as different vitamin D and parathyroid hormone milieu. However, our data did not show a significant difference in calcium levels between ethnicities in this study population.

Type of analyser

Various studies had utilised different combination of assay methods and analysers (9, 11-13). These different combinations affect the final albumin-adjusted calcium equation obtained. We used NM-BAPTA method for total calcium and BCG method for albumin measurement on Roche Diagnostics Ltd analyser, similar to Davies et al. However, the equations were different, which may be explained by the different genetic make-up of populations studied (Asian versus British) (12).

A study by Ashby et al. (20) found a change in regression coefficient after a change of analytical platforms within the same laboratory (Vickers M-300 and Technicon SMA 11) with similar analytical methods for both platforms (O-CPC and BCG). However, they noticed that albumin performance on those two analysers were different (Vickers M-300, 2.15 g/L; Technicon SMA 11, 0.7 g/L). This was evident by a small change in the population mode (42 g/L to 43 g/L) as albumin values became less distributed. Hence, the authors postulated that the improvement in precision and accuracy of albumin method upon change in analytical platforms may have contributed to the difference in regression coefficient observed. For that reason, the equation is believed to be dependent on the performance of the analytical platform used.

Analytical method

i. Albumin method

Study has shown that albumin methods give greater impact on the equation since dye-binding methods have distinct sensitivity towards albumin (21). A similar finding was also observed by Barth et al. (22), who found differences in regression coefficient among three different laboratories, which utilised similar analytical methods (O-CPC and BCG). In our study, the BCG method was used but it is less specific than bromocresol purple (BCP) since it also reacts with globulin, especially α_2-globulin (23). α_2-macroglobulin causes a significant high bias for BCG method in severe hypoalbuminaemia due to nephrotic syndrome. Similarly, haptoglobin induced by inflammatory cytokines, also interferes with BCG method (24). Although we had applied certain exclusion and inclusion criteria for our study data, there is still a possibility that we may have included patients with subclinical inflammatory conditions. This may explain the higher mean value of albumin measured for the study population contributing to the dissimilarity in the regression line.

Barth et al. concluded that difference in BCG reagent formulation contributed to the dissimilarity in the equations. Although the laboratories were using the similar BCG method, the reagents were supplied by different manufacturers. Different types of BCG formulation would react differently with globulins (22). This could affect the albumin measurement and eventually translate into the derived equations. During our study period, the same albumin reagent lot was utilised from the same manufacturer to ensure consistency in results.

When we compared our equation with the equation from James et al. (12) that used different methods than us for both albumin (BCP) and total calcium (Arsenazo III), the differences were also not clinically significant at medical decision limits (data not shown). However, our study did not compare between BCG and BCP. So, we cannot conclude that BCP would give a better regression line than BCG.

ii. Calcium method

A change in analytical methods for calcium (O-CPC to NM-BAPTA) is recognised to contribute only a small change to the overall final equation as noted by Davies et al. NM-BAPTA is the latest calcium assay developed by Roche Diagnostics. A method comparison study between O-CPC and NM-BAPTA by Willaert et al. revealed that NM-BAPTA had picked up more samples with hypocalcaemia. This shows that NM-BAPTA is more sensitive in identifying low calcium values compared to O-CPC (NM-BAPTA: sensitivity 90%, specificity 100%, O-CPC: sensitivity 52%, specificity 97%) (25).

NM-BAPTA method showed good analytical CV (1.5%) (26), which was further supported by our finding (CV: 1.5% to 1.8%). This new assay has additional advantages as it is more stable with longer on-board stability, does

not react with magnesium ions, has wider analytical measuring range as well as high sample output (25). With these evidences, NM-BAPTA assay might not contribute so much to the dissimilarity of the equations.

Agreement between the newly derived equation with Payne equation

Based on Bland-Altman plot, the mean difference between our newly derived equation with Payne's equation still complied with CLIA 88. According to CLIA 88 and its regulations, acceptable test performance and allowable error criteria for calcium performance is ± 0.25 mmol/L. This value applies to all medical decision levels for calcium. Thus, if the Payne's equation is to be replaced by the new equation, the newly derived equation is still acceptable in its performance even with slight positive bias since the mean difference (0.029 mmol/L) is lower than 0.25 mmol/L.

Difference in classification of calcium status between Payne's equation and the newly derived equation with ionised calcium, respectively

Majority of studies, which derived albumin-adjusted calcium equation did not compare their new equations with ionised calcium. It is therefore difficult to conclude whether their equations performed more or less similarly with the gold standard. We compared Payne's equation and our newly derived equation with ionised calcium, respectively to investigate which equation performed better in comparison with the gold standard. Surprisingly, both equations showed a statistically significant difference in calcium status classification when compared with ionised calcium. Thus, this finding proves that Payne's equation is not the best equation to be generalised for all laboratories. It also shows that our new equation has its own restrictions.

In this study, 48.1% of the patients fell under different categories when the two modalities were compared. The new equation has a propensity to overestimate the incidence of normocalcaemia in a group of patients deemed hypocalcaemia by ionised calcium. One possible reason for this may be due to the type of syringe used for drawing blood for ionised calcium. During this study period, the hospital was using a 1 ml syringe manually prepared with heparin. As a known fact, heparin chelates significant amount of calcium in the specimen if the concentration is above the recommended level, depending upon the type of heparin utilised (27). This will lead to falsely low ionised calcium values, which could have contributed to this finding. Ionised calcium measurement requires strict preanalytical requirements, which are extremely difficult to control in clinical practice (28). Ideally, the specimen should be drawn using anaerobic technique, kept closed and only be opened at the time of analysis to avoid loss of carbon dioxide (CO_2). This is because low CO_2 will result in a raise in pH causing ionised calcium levels to decrease (29). On top of that, taking meals may temporarily decrease ionised calcium values due to higher blood protein concentration and blood pH. These factors may escalate the binding of calcium to albumin and other ions (28). Unquestionably, these uncontrollable factors could also have contributed to discrepancies in our findings, which showed almost half of the patients being reclassified differently between these two modalities.

Use of different kinds of samples may contribute to the differences observed. It has been shown that the best sample type for measurement of ionised calcium is whole blood as it has fewer propensities for preanalytical errors (30). In our study, we had tried to lessen this effect by using whole blood specimen for ionised calcium measurement; hence it is unlikely this factor contributed to the discrepancy.

Another contributing factor to the inadequacy of the new albumin-adjusted equation is the issue with albumin standardisation (31). Reference material for albumin, BCR-470 or ERM-DA470 was released in 1993. Later, the secondary reference material called ERM-DA470k/IFCC was distributed in 2008 by the Institute for Reference Materials and Measurements (IRMM) to replace the ERM-DA470 that was out of stock at that time (32). Nowadays, most manufacturers claim their albumin assay is traceable to the BCR-470/ERM-DA470 but do not explain whether this was done directly or through the secondary reference material (ERM-DA470k/IFCC) since the former has been sold out for years. Similarly, our albumin assay is stated as traceable to BCR-470 in the manufacturer's insert without further clarification of the standardisation procedure. Thus, there is uncertainty as to the traceability of the calibrator. Effort to harmonise serum albumin assay using ERM-DA470k/IFCC has been undertaken but results have been unpromising (31). ERM-DA470k/IFCC needs to be reconstituted by diluting the material with deionised water prior to use. Unexpectedly, the value acquired on the ERM-DA470k/IFCC by BCG method was lower than the certified value provided by the IRMM. So, diluted ERM-DA470k/IFCC is unsuitable to calibrate a BCG method (32). If it is still being used to align commercial BCG assays, it could give imprecise results that would further contribute to errors when total calcium is regressed on albumin. This error would be evident by an incorrect slope and/or intersect value during derivation of the equation, which was not apparent in our study.

Difference in classification of calcium status between Payne's equation with the newly derived equation

Any disorder that causes low albumin concentration will affect total calcium value but not ionised calcium. Accordingly, it is recommended that total calcium value should be corrected for hypoalbuminaemia patients. In the present study, implementation of the new equation in all and hypoalbuminaemia patients (albumin < 40 g/L) demonstrates a statistically significant difference

in calcium status classification. In general, the new equation categorised more patients as normocalcaemia as compared to hypocalcaemia by the traditional Payne's equation. Both Payne's and the new equation deviated from ionised calcium measurement presumably due to non-adherence to stringent preanalytical requirements of ionised calcium. However, Payne's equation gave greater discrepancy in terms of the actual number of patients who were classified differently compared to ours. So, it could be inferred that this difference was contributed more by Payne's equation. Nevertheless, at medical decision limits (1.75, 2.69 and 3.24 mmol/L), total calcium results did not show clinically significant difference with our equation when total calcium results were reported to two decimal places (difference was 0.02 mmol/L).

Our equation by some means was better than Payne's equation since it was able to correct several hypoalbuminaemic patients from hypocalcaemia to normocalcaemia group. The clinical significance of these findings is reliant on the prevalence of abnormal albumin level within the community studied. Thus, in populations with a higher prevalence of hypoalbuminaemia, the application of this new equation would have a more substantial effect on calcium status classification. In our study, 27.5% (278 of 1011) of the patients were hypoalbuminaemic. When this equation was implemented by our laboratory, lesser number of hypoalbuminaemia patients were reported as hypocalcaemia, which was statistically significant. Thus, if this equation is going to be used, clinicians will see lesser hypoalbuminaemia patients with hypocalcaemia. Indirectly this will reduce unnecessary investigations and treatment. However, comparison with the gold standard under strict, controlled preanalytical conditions is needed to confirm this finding.

CONCLUSION

In conclusion, the new equation tends to overestimate adjusted calcium values, based on ionised calcium as well as Payne's equation, resulting in fewer patients being classified as hypocalcaemia. Its implementation appears statistically significant in terms of reclassification of calcium status especially in hypoalbuminaemia patients. However, to confirm this significance, results need to be compared with the gold standard ionised calcium under strict, controlled preanalytical conditions. In terms of clinical significance, there was no difference in classification of calcium status between the old and new equations at medical decision limits.

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