

**The clinical and analytical aspects of albumin adjusted calcium and
an assessment of the validity of harmonisation between
laboratories**

A thesis submitted to the University of Manchester for the degree of Doctor of
Clinical Sciences in the Faculty of Biology, Medicine and Health

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Abbreviations

AACB	Australian Association of Clinical Biochemists
ACB	Association of Clinical Biochemistry and Laboratory Medicine
AFP	Alpha Fetoprotein
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AMM	All Methods Mean
AST	Aspartate Aminotransferase
BCG	Bromocresol Green
BGP	Bromocresol Purple
CaR	Calcium Sensing Receptors
CLSI	Clinical And Laboratory Standards Institute
CKD	Chronic Kidney Disease
CNS	Central Nervous System
C-RIDL	Committee On Reference Intervals And Decision Limits
EDTA	Ethylene Diamine Tetraacetic Acid
EFLM	European Federation Of Laboratory Medicine
eGFR	Estimated Glomerular Filtration Rate
ENH	Early Onset Hypocalcaemia
EQAS	External Quality Assurance Scheme
FHH	Familial Hypocalciuric Hypercalcaemia
iCa	Ionised calcium
HRT	Hormone Replacement Therapy
IFCC	International Federation Of Clinical Chemistry
ISE	Ion Selective Electrode
LIMS	Laboratory Information System
LNH	Late Onset Hypocalcaemia
LRL	Lower Reference Limit
K/DOQI	Kidney Diseases Outcome Quality Initiative
NEQAS	National External Quality Assessment Scheme
NIST	National Institute Of Standards And Technology
NORIP	Nordic Reference Interval Project
OPG	Osteoprotegerin
PPI	Proton Pump inhibitors

PTH	Parathyroid Hormone
PTHrP	Parathyroid Hormone Related Peptide
RANKL	Receptor Activator of Nuclear factor kappa B Ligand
URL	Upper Reference Limit
VDR	Vitamin D Receptor
WEQAS	Welsh External Quality Assessment Scheme

Abstract

The routine measurement of the biologically active fraction of calcium “ionised calcium” is fraught with difficulties. Therefore, an assessment of this fraction is estimated through a mathematical equation using the regression of albumin on calcium. In clinical practice, the most widely used equations for calcium estimation were derived by Payne et al (1973). To date this equation has been derived using hospitalised patients’ data after excluding patients with diseases that may disturb calcium metabolism. Since the inception of their use, reports have described many inherent problems with applying a calcium equation to adjust total calcium measurements across different patient groups. However, no robust alternative approach or method for calcium measurement has been proposed by these critics. This study aims to elucidate the reasons behind suboptimal performance of the free calcium estimation (hereafter called adjusted calcium), causes for variability in adjusted calcium reporting as well as exploring new concepts to improve adjusted calcium performance such as the derivation and validation of a population specific equation for ambulant patients. For this purpose, three data sets were collected; first, a retrospective data set from 14 hospitals and communities representing various analytical platforms. These data were collected from all the methods that are commercially available for calcium measurement and were used to derive calcium equations, for hospitalised patients and ambulant patients. The second was a prospectively collected data set. This set was used to validate the newly proposed population specific equation for ambulant patients for a single analytical platform (Roche Cobas). The third data set includes an external quality data set that was collected to assess analytical and post analytical factors that impact on adjusted calcium classifications such as analytical performance goal of calcium and albumin and their reference intervals. Results from this study showed that in comparison to “ionised calcium”, the population specific equation performed better in ambulant patients than an equation derived from hospitalised patients. Replacing creatinine $\leq 200 \mu\text{mol/L}$ with estimated glomerular filtration rate (eGFR) $> 60 \text{ mL/min/1.73}^2$ in the inclusion criteria prior to an equation derivation was without effect whereas replacing the calcium population mean with a mean of 2.4 mmol/L in the equation worsened the equation performance, leading to misclassification of calcium status. Reference interval studies for calcium and albumin showed a wide variation between various analytical platforms and methods. This finding was supported by the analytical performance studies. The Association of Clinical Biochemistry (ACB) proposed approach for data gathering (which includes total calcium values between 2.0 and 2.7 mmol/L) for equation calculation, appears promising and may lead to reduced adjusted calcium variability between laboratories. However, it has been shown that one of the major problems adding to increased adjusted calcium variability is related to analytical variability of the calcium and albumin methods. Albumin methods (Bromocresol green and Bromocresol purple) accounted for significant differences in the classification of adjusted calcium results which was found to be related to a difference in albumin concentrations measured by these assays. The mean albumin concentration of these assays was consistently higher for BCG than BCP methods irrespective of platform. Calcium methods showed a closer agreement with the exception of Siemens calcium assays, for which re-calibration should be seriously considered. This study showed a population specific equation concept can be a solution for improving the adjusted calcium equation in ambulant patients. The same concept opens the door for more sub-division and consideration of an age and gender related adjusted calcium equation. It has been found that standardising adjusted calcium equations and reducing variability in adjusted calcium results is a remote possibility without eliminating albumin and calcium calibration issues. Until these issues are resolved, laboratories should continue deriving their own equations according to Payne’s described method. However, the introduction of a population specific equation in combination with the ACB recommended data collection approach is a step forward towards reducing variability in adjusted calcium reporting.

Declaration

The work undertaken in this thesis is a collection of a number of studies to resolve a single research question.

In the first scientific paper (chapter 2), laboratory staff assisted me in loading the samples on to the analysers. The selection of subjects, administration of protocols and all pre-analytical and analytical work of ionised calcium, written work and analysis of results has been performed by the author. The retrospective calcium data was collected from various trusts via a pre-devised protocol. Protocols, data analysis and written work have been performed by the author.

A small portion (reference interval raw data, chapter 4) of the work undertaken in this thesis has been performed in collaboration with the Reference Interval in Laboratory Medicine in Yorkshire project (A. Luvai FRCPath project, 2015). The selection of subjects, administration of the study protocols and most pre-analytical work was performed jointly. Data analysis and written work has been performed by the author.

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Dedication

To my mother for her immense support

To Dr Julian Barth for providing me with advice, support and friendship throughout my career

To Dame Professor Sue Hill for offering me this opportunity

To my children for being the biggest source of joy in my life

The author

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The author's main research interest is harmonisation within clinical networks. Current research interests include establishing a new approach for developing analytical performance specifications based on both technical capability and clinical need and standardisation in clinical biochemistry. Professionally, the author has chaired a number of working groups and committees regionally, nationally and at European level and currently is the chair of the European Federation of Laboratory Medicine working group on the Register.

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1. Introduction

1.1 Background to study

In clinical practice, measurement of calcium in serum is critical for the assessment of bone diseases. The adult human skeleton contains approximately 1 kg of calcium (Baird, 2011) with only 1% of total body calcium in soluble form. Soluble calcium is distributed equally in blood and intracellular fluids. Approximately 45% of blood calcium is protein bound, principally to albumin, 10% is bound to small anions such as citrate, phosphate and carbonate and proteins such as globulins and 45% is unbound. The unbound fraction is referred to as ionised or free calcium and considered as the physiologically active form. The sum of all fractions, bound and unbound is known as total calcium (Fraser et al., 1987).

At physiological pH, the calcium ion (Ca) binds to the negatively charged sites on the albumin molecule. McLean and Hastings (1935) identified the correlation between total calcium and total serum protein (albumin and globulins) and suggested that ionised calcium concentration can be predicted from the two. In health, the measurement of total calcium is a good reflection of calcium status. However, in disease, this assumption does not hold true. Low albumin concentration is common in diseases, a situation which results in an alteration in the binding dynamics and leads to an increase in unbound calcium fraction relative to total calcium; therefore serum total calcium measurements may underestimate the free calcium concentration (Payne et al., 1979). In an ideal world, direct measurement of ionised calcium would resolve this problem. However, the direct measurement of ionised calcium is technically difficult, expensive and thus not widely routinely available. Therefore, to correctly estimate calcium status and to overcome the fluctuations in serum albumin concentrations, an equation that predicts the physiologically active calcium fraction has been proposed and widely used around the globe (Labriola et al., 2009), although there are contrasting views (Toffaletti, 2011).

The terms corrected calcium or adjusted calcium are usually used interchangeably. In a recent national guidelines release from the Association for Clinical Biochemistry, it was recommended that the most preferred term is “adjusted calcium” (O’Kane et al., 2015). Therefore, in this thesis the term adjustment and adjusting calcium equation will be used.

1.2 The clinical significance of calcium

Calcium plays two vital roles in the human body. Calcium compounds provide the structural integrity of the bones, which makes the bones an inexhaustible reservoir of calcium to extracellular and intracellular pools. Extracellularly, calcium concentration is important to the maintenance of a number of metabolic processes such as muscle contraction, cardiac output, arterial pressure regulation, enzymes secretion, neurological stability and other physiologic processes (Ramasamy, 2006). In health, calcium concentration in serum is tightly regulated and maintained within a narrow reference range of 2.2-2.60 mmol/L, figure 1.1.

1.3 Calcium homeostasis

A stable extracellular ionised calcium concentration is vital for physiological functions at cellular level. Extracellular calcium concentration is therefore tightly regulated. The system regulating calcium homeostasis includes organs and hormones. Organs involved are the kidneys, skeleton, intestine and parathyroid glands. Amongst others, hormones which regulate calcium include parathyroid hormone (PTH), vitamin D and calcitonin.

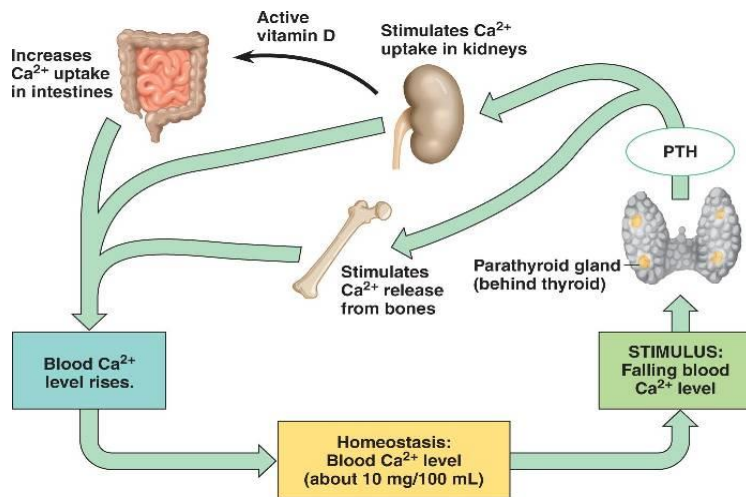


Figure 1.1: Calcium metabolism. Taken from: Human Medical Physiology.

1.3.1. Vitamin D

1,25 dihydroxy vitamin D is the active form of vitamin D and it plays a pivotal role in the control of calcium homeostasis and calcium transport. The rate of vitamin D conversion from 25 hydroxy vitamin D to 1, 25 dihydroxy vitamin D is dependent on the calcium requirements of an individual. The process for the hydroxylation of vitamin D switches to the formation of 24 hydroxy vitamin D rather than 1,25 dihydroxy vitamin D when there is an abundance of vitamin D (Van Leeuwen et al., 2001). This mechanism is considered the first step of the inactivation process that aims to control extracellular calcium concentration.

Approximately 90% of dietary calcium absorption takes place in the small intestines via saturable and non-saturable vitamin D dependent pathways (Bronner et al., 1986). The intestinal active transport of calcium is controlled by 1, 25 dihydroxy vitamin D via vitamin D receptors (VDRs). In a healthy individual, the intestinal calcium absorption is balanced by urine calcium excretion. The glomeruli excrete up to 270 mmol of calcium daily. But the net intestinal absorption is 4 mmol/day only; therefore, this massive filtration process is coupled by almost complete reabsorption process. 98% of filtered calcium is reabsorbed along the renal tubule via passive and active mechanisms. Renal calcium reabsorption

largely takes place in the proximal tubules through a passive mechanism driven by an electrochemical gradient that is generated by sodium and water reabsorption (Hoenderop et al., 2002). Calcium passive transport that takes place in the proximal tubules and the intestine is mediated by a vitamin D dependent calcium binding protein called Calbindin (Ramasamy, 2006). The active reabsorption of renal calcium occurs at the distal convoluted tubules under the effect of vitamin D and other hormones such as PTH and calcitonin.

A small fraction of total bone calcium is exchanged regularly with circulatory calcium through the bone formation and resorption dynamic process that involves bone cells; the osteoblasts, osteoclasts and osteocytes. The resorption process is regulated by PTH and vitamin D hormones. 1, 25 dihydroxy vitamin D is a potent stimulator of osteoclast resorption activity. 1, 25 dihydroxy vitamin D has been shown to exert its effect on the bone, directly through VDRs in the osteoblasts (Ramasamy, 2006), or indirectly through the stimulation of alkaline phosphatase activity and the synthesis of other bone matrix proteins. The stimulation of the osteoclast activity by 1,25 dihydroxy vitamin D is an indirect one through a mechanism involving PTH and a membrane associated ligand called receptor activator of nuclear factor kappa B ligand (RANKL). The main role of RANKL is facilitating osteoblast cell and osteoclast cell interaction (Kenkre and Bassett, 2018) (Wheater et al., 2013). RANKL is constitutively expressed on osteoblast cells. Interaction of RANKL with its receptor RANK that's expressed on osteoclast precursor promotes osteoclast differentiation and its interaction with RANK receptors on mature osteoclast cells results in its activation and prolonging its survival. Therefore, it has been postulated that a physiological dose of 1,25 dihydroxy vitamin D is capable of suppressing bone resorption through a mechanism that involves suppression of the PTH induced proteins called RANK/ RANKL/ osteoprotegerin (OPG). OPG competes with RANK (mature and precursor osteoclast cell) by blocking its interaction with RANKL, hence it acts as a physiological suppressor to osteoclast activity and survival (Kenkre and Bassett, 2018).

1.3.2 Parathyroid Hormone

The second organ that plays a pivotal role in calcium haemostasis is the parathyroid gland. Parathyroid hormone is a polypeptide of 84 amino-acids secreted by the parathyroid gland. PTH secretion is under the control of 1,25 dihydroxy vitamin D, oestrogen, phosphate and calcium. Physiological levels of 1,25 dihydroxy vitamin D decrease the transcription of the PTH gene, while hypocalcaemia, hyperphosphataemia and oestrogen stimulate the mRNA expression of the PTH gene. PTH acts to increase extracellular calcium concentration via its action on kidney and bones. Endogenous PTH acts to maintain extracellular calcium concentration by stimulating osteoclastic bone resorption via a mechanism involving OPG/RANK/RANKL signalling system (Kenkre and Bassett, 2018). PTH was found to directly stimulate RANKL expression and inhibit OPG production by osteoblasts, and thus promote osteoclastogenesis (Hofbauer et al., 2004). Hence chronic PTH elevation, as primary or secondary hyperparathyroidism, stimulates bone resorption and bone loss. On the contrary, intermittent pharmacological administration of PTH exerts anabolic effects and stimulates bone formation through an unclear mechanism that involves preventing mature osteoblast cells apoptosis (Rodan & Martin, 2000) (Kenkre and Bassett, 2018).

In the kidney, PTH functions by increasing the re-absorption of calcium and increasing the excretion of phosphate and stimulating the hydroxylation of 25 vitamin D to 1,25 dihydroxy vitamin D. In the intestine, there is evidence that PTH has a direct effect on increasing dietary calcium and phosphate absorption. PTH shares considerable amino acid homology with another peptide called PTH-related peptide (PTH-rP). PTH-rP is usually expressed in the same tissues that express PTH receptors and has the same biphasic effects to those of PTH on bone (Murry, 2003). In pregnancy, PTH-rP plays a diverse role in the regulation of placental calcium transfer and protecting maternal skeleton by inhibition of bone resorption (Simmonds and Kovacs, 2010). Other than its role in pregnancy, in health, there is no role

for PTH-rP in controlling calcium haemostasis. However, in disease (eg lymphoma, squamous tumour of the head, neck and lung), ectopic secretion of PTH-rP is a cause of hypercalcaemia.

1.3.3 Calcium sensing receptors

Another factor involved in calcium regulation is calcium sensing receptors (CaR). The effect of extracellular calcium is mediated through CaR. CaR is a G protein-coupled cell surface receptor that senses change in calcium concentration. CaR is expressed on the parathyroid gland tissue and many other organs such as the kidney, gut, thyroid, skin, brain, heart and pancreas (Kos et al., 2003). The role of CaR in bone tissue as a promoter of osteoblast differentiation revealed new insights into the regulation of calcium homeostasis and showed another level of coordination between the parathyroid gland and bone that is mediated by CaR (Brown and Lian 2008). The expression of CaR is controlled by calcium and 1,25 dihydroxy vitamin D. The degree of CaR expression determines the degree of responsiveness of different organs to calcium.

In the parathyroid gland the CaR modulates PTH secretion via its action as a detector of extracellular calcium concentration. There is evidence to suggest that CaR has a pivotal role as a mediator of calcium inhibition of PTH secretion. For example, individuals with an inactivation mutation in CaR show a shift in the set point for PTH secretion. Those with a homozygous inactivation of CaR usually present with severe hypercalcaemia and elevated PTH. On the other hand, activating mutations lead to increases in the sensitivity of the PTH gland to calcium, therefore, individuals with activating mutations present with hypocalcaemia and inappropriately low PTH (Brown and McLeod, 2001). In the kidney, the activation of CaR in the thick ascending tubule leads to the generation of an electrical gradient and results in a lumen positive voltage that drives a calcium, magnesium and sodium passive re-absorption mechanism. CaRs have been detected at the basal side of the epithelial cells of duodenal villi. The presence of CaR in this tissue suggests a possible role in the intestinal

calcium absorptive process. However, this mechanism is not yet fully explored (Gama et al., 1997) (Brown and Lian, 2008).

1.3.4 Calcitonin

Thyroid gland C-cells secrete another hormone, “calcitonin” which reportedly opposes the actions of PTH. The effect of calcitonin is exerted through calcitonin receptors in bone. The main effect of calcitonin is inhibition of bone resorption and increase in the renal excretion of calcium. Hence, calcitonin prevents abnormal increase in both serum calcium and serum phosphate. Several studies suggested that calcitonin inhibits bone resorption in the face of acute hypercalcaemia challenge (D'Souza et al, 1986) (Ramasamy, 2006).

In pregnancy, like PTH-rP, calcitonin plays a major role in regulating extracellular calcium concentration in maternal and foetal blood. Calcitonin increases during pregnancy and is evidently coming from placenta and breasts in addition to the C-cells of the thyroid. Furthermore, higher oestrogen levels during pregnancy may also stimulate calcitonin synthesis (Kovacs, 2015). Maternal calcitonin secretion promotes renal calcium excretion (Murry, 2003). Foetal bone mineralisation is dependent on a process of active transplacental calcium transfer in the final trimester of pregnancy. In pregnancy, calcitonin serves to protect the maternal skeleton, whilst allowing the foetus to accumulate calcium. In the third trimester, maternal PTH levels rise to accommodate the increased demand for calcium. The foetus therefore sustains low PTH levels because maternal PTH hormone does not cross the placenta, while calcitonin hormone does. After birth, calcium levels drop in healthy neonates due to the cessation of maternal calcium transport and the peak in calcitonin (Aggarwal et al., 2001) (Kelner & Butler, 2008). Normal calcium concentration is achieved due to a gradual decline of calcitonin accompanied by an increase of PTH to normal levels (Reynolds et al., 1975).

1.3.5 Other calcitropic hormones

Oestrogen is another hormone that has an effect on calcium homeostasis. Oestrogen inhibits bone resorption by directly inducing apoptosis of the bone-resorbing osteoclasts (Takashi et al., 1997). Oestrogen effects on calcium homeostasis are demonstrated by the fact that oestrogen deficiency in postmenopausal women results in negative calcium balance and bone loss (Murry, 2003). Research supports the concept that the reduction in calcium absorption in the bowel and reabsorption in the kidney in postmenopausal women is oestrogen-dependent. Furthermore, oestrogen status can affect the requirement for vitamin D in the same group (Prince and Dick, 1997). Thus, the role of oestrogen in regulating calcium transport in the bowel and kidney appears to be an important part of calcium homeostasis, particularly in the pathogenesis of both postmenopausal and age-related osteoporosis.

Diseases affecting any of the organs regulating calcium would lead to disruption of calcium metabolism. Therefore, diseases of abnormal calcium metabolism cover a wide array of specialties ranging from nephrology, endocrinology, haematology, gastroenterology, respiratory medicine and oncology.

1.4 Albumin

Albumin is the most abundant human serum protein and has various physiological functions. Albumin is the main determinant of plasma oncotic pressure and it plays a significant role in modulating the distribution of fluids between compartments. Albumin acts as a non-specific binding protein and a transporter for several hormones and substances, e. g. certain drugs toxins and ions like calcium. It exists in serum in either reduced or oxidised forms. It is synthesised in the liver at a rate of ~12 g a day with a half-life of 3 weeks. Albumin synthesis is increased by a fall in plasma oncotic pressure and decreased by pro-

inflammatory substances, including interleukin-6 and tumour necrosis factor- α 1,2 (Caraceni et al., 2013). The measurement of albumin for the prognostic assessment of several diseases is well-established clinically (Greipp et al., 2005). Albumin is measured in dialysis patients as a marker of therapy adequacy, in myeloma patients to support disease staging, in nutritional assessment as a core nutritional marker, in patients undergoing replacement therapy for human albumin, in liver disease and finally for the estimation of calcium status.

Serum albumin concentration is measured routinely by dye binding methods using reagents such as the bromocresol green (BCG) and bromocresol purple (BCP) with the nephelometric immunochemical method accepted as the reference method. Albumin BCG method is known for its non-specificity. BCG reacts not only with albumin, but also with α -globulins, haptoglobin and immunoglobulins, resulting in overestimation of albumin concentrations in disease processes that elevate these globulin concentrations in serum (Ueno et al., 2016). By contrast, in similar pathological conditions, the albumin BCP method showed a closer alignment with the nephelometric methods (Ueno et al., 2013). The difference in albumin concentrations resulting from the use of BCG and BCP has been reported to impact on the clinical classification of hypoalbuminaemia, subsequently influencing an increase in albumin prescription (Coley-Grant et al., 2016).

Clinically, albumin is of pivotal importance for the estimation of a patient's calcium status. Thus, physiological and pathological factors affecting albumin concentrations would have a direct impact on calcium measurements. For example, albumin concentrations are known to be affected by a number of pre-analytical factors. Venous stasis from tourniquet placement during venepuncture is known to overestimate albumin and subsequently total calcium concentration (Lippi et al., 2005). This effect is considerably minimised if tourniquet application is limited to less than one minute (McMullan et al., 1990). Total calcium intra-individual variability was attributed to the impact of posture on serum albumin (Pedersen, 1972). Pathologically, hypoalbuminaemia is the commonest cause of hypocalcaemia. A

correlation between decreased albumin concentration and hypocalcaemia was observed several decades ago which led the way to the introduction of the adjustment of calcium to albumin concept (Imrie et al., 1976).

1.5 Disruptions of calcium homeostasis

Calcium disorders are characterised by either a lowered level of blood calcium (hypocalcaemia) or an increased level of blood calcium (hypercalcaemia). Hypo and hypercalcaemia are indicative of disruption in calcium metabolism. Hypo/hypercalcaemia conditions are not always symptomatic. Signs and symptoms are often related to both calcium level and the extent of calcium decrease/increase as well as its chronicity. Whether calcium is measured directly or estimated by adjusting equations, accurate prediction of calcium status is of vital importance for diagnosis and management of calcium disorders.

1.5.1 Hypercalcaemia

Hypercalcaemia is defined as an ionised calcium concentration of greater than 1.33 mmol/L, adjusted calcium > 2.6 mmol/L in adults or adjusted calcium > 2.7 mmol/L in paediatrics. Hypercalcaemia is associated with symptoms relating to various body systems such as the central nervous system (CNS), musculoskeletal, cardiovascular and gastrointestinal systems and genitourinary tract. In patients with severe hypercalcaemia, symptoms can progress rapidly from lethargy, to stupor to coma. In contrast, chronic and mild hypercalcaemia tend to be asymptomatic or present with non-specific symptoms such as fatigue, weakness, mood swings, confusion, and nausea and vomiting. Hypercalcaemia decreases the ability of the kidney to concentrate urine; hence polyuria and polydipsia may develop in patients with chronic hypercalcaemia. Hypercalcaemia may be detected in patients presenting with bone pain and muscle weakness and/or pathological fracture. The cardiovascular presentation of

hypercalcaemia can be life threatening and includes uncontrolled hypertension, arrhythmias, atherosclerosis and progressive cardiac dysfunction (Karthikeyan et al., 2006).

There are numerous causes of hypercalcaemia, but the pathogenesis in approximately 90% of patients is hyperparathyroidism or malignancy (Murry, 2003). Hypercalcaemia of malignancy can be associated with osteoclast resorption stimulating factors such as cytokines, growth factors or PTH-rP. PTH-rP mimics the effect of PTH causing hypercalcaemia, phosphateuria and stimulates the synthesis of 1,25 dihydroxy vitamin D. Hypercalcaemia also occurs in patients with T-cell or B-cell lymphomas and granulomatous disease (e.g. sarcoidosis and tuberculosis) due to an increase in the production of 1,25 dihydroxy vitamin D secondary to granuloma. In primary hyperparathyroidism, the prominent biochemical picture is an increase of both calcium and PTH concentrations, low/normal phosphate and hypercalciuria. Familial hypocalciuric hypercalcaemia (FHH), an asymptomatic genetically inherited mutation affecting CaR, is a condition also characterised by a similar biochemical picture of hyperparathyroidism. In contrast to primary hyperparathyroidism, FHH patients are characterised by hypocalciuria. Pseudo-hypercalcaemia occurs in myeloma patients due to a high total protein concentration in this group of patients. Calcium binds albumin and immunoglobulin and gives rise to elevated total calcium (Annesley et al., 1982). The direct measurement of ionised calcium plays an important role in the investigation of pseudo-hypercalcaemia (Glendenning, 2013).

In neonates and paediatric patients, chronic hypercalcaemia manifests as irritability, anorexia and failure to thrive (Payne et al., 2001). Hypercalcaemia due to primary hyperparathyroidism is rare in paediatric populations although it is potentially fatal. Idiopathic hypercalcaemia of infancy manifests itself as hypercalcaemia and gives rise to mental retardation, heart defects and unusual facial features (Martin et al., 1984) (Kelnar & Butler 2008). It is a condition associated with hypersensitivity to vitamin D and a delay in its diagnosis has been found to have a damaging effect on cognitive development (Udwin et al.,

1986). Other causes of hypercalcaemia in paediatrics include FHH, a rare autosomal dominant disorder known as Williams's syndrome or conditions due to mutations in CaR. Hypercalcaemia has the potential to cause kidney stones and consequent renal damage and as such prompt diagnosis and treatment is advocated.

1.5.2 Hypocalcaemia

Hypocalcaemia defined as serum adjusted calcium < 2.2 mmol/L. It is a frequently encountered and potentially life threatening condition in adults and paediatrics. Prevalence of hypocalcaemia varies based on the population and the clinical setting; hypocalcaemia has a prevalence of 18% in all in-patient and 85% in intensive care units (Cooper and Gittoes, 2008).

The pathogenesis of hypocalcaemia can be divided into two main groups according to PTH concentration; hypocalcaemia with reduced PTH concentration (hypoparathyroidism) or with high PTH concentration (secondary hyperparathyroidism). Common causes of hypocalcaemia include hypomagnesemia, hyperphosphatemia, vitamin D deficiency, malabsorption and renal failure. The presence of citrated blood products, or PTH antagonists, such as glucocorticoids, also commonly results in hypocalcaemia. Severe hypocalcaemia can be encountered in osteoporotic patients on bisphosphonate with untreated vitamin D deficiency. Chelation of calcium due to acute pancreatitis, early rhabdomyolysis or massive tumour lysis is not an uncommon cause of hypocalcaemia in hospitalised patients (Hannan and May, 2013). Hypocalcaemia has also been associated with many drugs, including bisphosphonates, cisplatin, antiepileptics, aminoglycosides, diuretics, and proton pump inhibitors (Fong and Khan, 2012).

Pseudo-hypocalcaemia is commonly encountered in clinical practice due to hypoalbuminaemia, thus total calcium is adjusted to reference albumin concentration. The

clinical presentation of hypocalcaemia ranges from asymptomatic to a severe life-threatening condition. Patients may be asymptomatic with mild hypocalcaemia, although more severe or long term hypocalcaemia may result in acute symptoms such as neuromuscular irritability which can cause seizures, tetany and paraesthesia. Chronic hypocalcaemia is a risk factor for chronic disorders including osteoporosis, cardiovascular and neurodegenerative conditions.

The frequent drop in calcium concentration is a well-known phenomenon that makes hypocalcaemia in neonates a relatively common occurrence (Early Onset Neonatal Hypocalcaemia (ENH)). ENH is likely to be asymptomatic, however, symptoms may include irritability, lethargy, apnoea and seizures. Screening for ENH is recommended in those predisposed patients in order that calcium supplements can be administered if necessary (Aggarwal et al., 2001). Hypocalcaemia in paediatrics is associated with several conditions including prematurity, respiratory distress syndrome, nephrotic syndrome, hepatic disease and malnutrition but the level of the free fraction of calcium is not necessarily affected. On the other hand, Late Onset Hypocalcaemia (LNH) in paediatrics is an uncommon finding. LNH can be idiopathic or due to congenital disorders such as Di George Syndrome or vitamin D deficiency as a result of a maternal deficiency or due to intestinal malabsorption in the neonate (Camadoo et al., 2007).

Hypocalcaemia is a common finding in critically ill patients (Baines et al., 2000) (Singhi et al., 2003). It has been suggested that calcium is regulated to a lower level in the critically ill and that hypocalcaemia may be beneficial to this group of patients (Baird, 2011). Broner and colleagues, however, noted that hypocalcaemia was associated with a poorer outcome in critically ill patients (Broner et al., 1990) a finding which contradicts this postulated beneficial effect.

1.6 Laboratory measurement of calcium

1.6.1 Calcium adjusted equations

The concept of adjusting calcium to albumin was based on the assumption that there is a linear relationship between total calcium and albumin and the slope of the regression of calcium on albumin that does not change amongst individuals at a defined pH (Payne et al., 1996). This concept has led to the application of a common adjustment equation.

In the early 1970s, at least three well known equations for the 'adjustment' of total calcium for serum albumin were published. (Payne et al., 1973) (Berry et al., 1973) (Orrell,1971).

The difference between calcium adjusted results from Payne's and Berry's equations was significant and resulted in a clinical uncertainty (Sanderson, 1974). Currently, Payne's formula is one of the most widely used formulae although another popular formula is the one derived by Orrel (see table 1.1).

Name	Equation	Reference
Orrell	Adjusted Calcium= TCa+ 0.0176 (34-albumin)	Orrell, 1971
Berry	Adjusted Calcium=TCa+0.0227 (46-Albumin)	Berry, 1973
Payne	Adjusted Calcium= TCa+0.0246(40.4-Albumin)	Payne 1973
In-house derived equation based on Payne's formula	Adjusted Calcium=TCa+ 0.0213 (42-Albumin)	The author's in-house formula

Table 1.1: the most widely used equation in clinical practice. TCa; total calcium. Total and adjusted calcium (mmol/L), albumin (g/L).

1.6.2. Derivation of calcium adjustment equation

The linear correlation between total calcium and albumin is mathematically described by the following equation:

$$\text{Adjusted calcium} = \text{total calcium} - (\text{slope} \times \text{albumin}) + (\text{mean calcium} - \text{intercept})$$

The adjustment equation was first derived by calculating the regression coefficient of calcium on albumin and using the intercept from the regression at zero albumin concentration as the non-protein bound calcium. The statistical methods of calcium equation derivation were all based on linear regression. The least square regression and linear regression were used to calculate the regression coefficients. Payne, however, argued that the use of Deming regression is a more statistically valid approach (Payne et al., 1984).

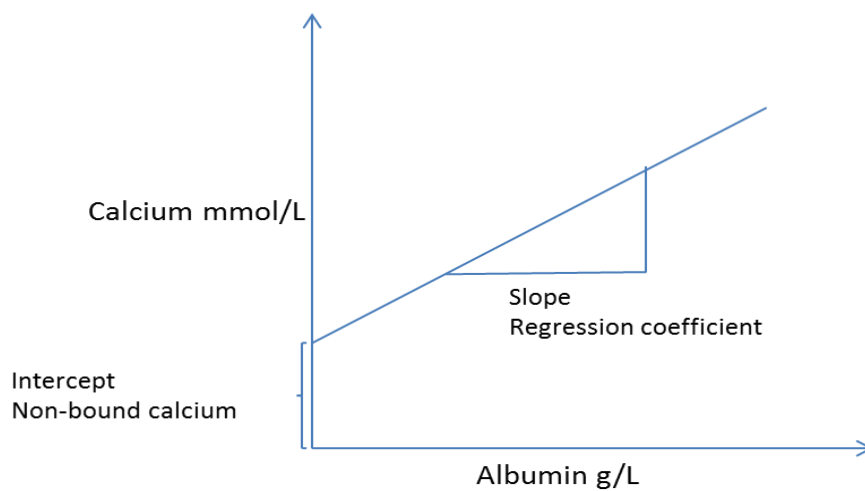


Figure 1.2: the regression of calcium against albumin

The values for regression coefficients; the slope and intercept are obtained from the linear regression of calcium against albumin (figure 1.2). The equation is re-arranged to give the widely known format as:

$$\text{Adjusted calcium} = \text{total calcium} - 0.02 (\text{Albumin} - 40) \dots\dots\dots \text{Payne's equation}$$

The slope value of 0.02 and the value of 40 g/L are variables, their values are influenced by the population from which the equation is derived (various patient groups) (Ladenson et al., 1978), age (paediatric, adult, elderly) (Jassam et al., 2011) (Lucy and Jassam, 2012), gender or methodology used to measure calcium and albumin and analytical platform (Barth et al., 1996).

1.6.3 Data collection

To derive a calcium adjusting equation, researchers retrieved albumin and total calcium data from laboratory biochemical records. The number of data points used varied significantly from a hundred to several hundreds of data points (Orrell, 1971) (James et al., 2008). The number of the data points included may have an effect on the values of the slope and intercepts which consequently affect the values of adjusted calcium derived from these equations. Therefore, to harmonise the derivation of calcium equations, recent recommendations demanded the inclusion of a minimum of 1000 pairs of albumin and calcium results, with the use of a single pair per patient (O’Kane et al., 2015).

Another factor that may affect the accuracy of adjusted calcium values is the inclusion/exclusion criteria of the patient populations used in the equation derivation process. The method of equation derivation and the patient inclusion criteria were established by Payne in 1973. The same criteria were later used by several researchers in this field (Ashby et al., 1986). (James et al., 2008). Payne excluded patients from endocrinology, oncology, haematology, nephrology departments and those under 18 years of age. Biochemically, data were excluded if urea concentration was > 15 mmol/L, creatinine > 200 μ mol/L, potassium < 3.5 mmol/L, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) $>$ the reference range (Payne et al., 1973). These criteria are still in use today, and are meant to exclude patients in whom there are other conditions that might affect calcium homeostasis. However, patients with creatinine of 100-200 μ mol/L may have chronic kidney disease, with eGFR < 60 mmol/min/1.73m². It is evident that patients with eGFR < 60 mmol/min/1.73m² have disrupted calcium metabolism (NICE, 2008). Patients with creatinine close to 200 μ mol/L may present with acid-base disturbance hence pH may vary among these patients which invalidates the equation assumption.

It is known that protein concentration in the blood, including albumin, is affected by venous stasis and the prolonged use of tourniquets may alter total calcium concentration due to the rapid increase of protein in the blood sample. Orrel's, but not Payne's, equation excluded data from patients where blood samples were collected with tourniquets. Including patient samples with possible venous stasis may affect adjusted calcium accuracy and contribute to the difference between Payne's and Orrel's equations (Orrel, 1971).

1.6.4 Validation methods

The literature describes several statistical methods to validate the newly derived adjustment equation. Validation by applying the new equation to a different cohort or population and comparing the outcome statistically was a method used widely (Ferrari et al., 2009). Determining the Z value of the regression factor for the equations or the intercept values was used as a method of validation for new equations or comparing new to old equations (Jassam et al., 2011). In order to ensure the stability of regression equations, one approach was to compare the outcome of established equations to a newly derived equation using *t*-test (Jassam et al., 2011). However, comparing the adjusted calcium to ionised calcium measurement remains the gold standard method of validating any newly derived equations that deviate from Payne's equation (Clase et al., 2000) (Byrnes et al., 2005) (Smith et al., 2018). From a statistical point of view, Clase and colleagues argued that the use of interclass correlation coefficient as a measure of agreement between the predicated calcium versus ionised calcium is more suited than Pearson correlation coefficient (Clase et al., 2000).

1.7 Over what albumin concentration range are equations valid?

Ashby et al (1986) and Besarb et al (1981) have established that the relationship between calcium and albumin is only linear when albumin concentrations are between 32 and 50g/L

and 30-50 g/L, respectively. Outside of these ranges calcium cannot be reliably adjusted. Barth et al in 1996 confirmed and extended the linearity range that Ashby et al reported by looking at albumin ranges from 16-35 and 36 to 51g/L. The products of the regression of calcium on albumin were two slopes. Although statistically significant, Barth et al concluded the difference in the two slopes is not clinically significant, thus the range can be extended to 16-50 g/L. It is worth mentioning that the Ashby et al and Barth et al studies examined the linearity relationship using the BCG albumin method and it is unknown whether the reported non-linearity was related to the non-specificity of the BCG albumin method. Smith et al, however, studied the relationship between adjusted calcium and ionised calcium using the BCP albumin method in a large cohort of hospitalised patients (Smith et al., 2018). Smith and colleagues disagreed with the Barth study and reported the unreliability of adjusting calcium to albumin lower than 30 g/L. Critics disagree with this study's conclusion because; firstly this study did not define the population for the adjusting equation used and secondly it was thought that limiting the linearity of the adjustment equation at 30 g/L of albumin risked missing results of clinical importance (Payne, 2019). Smith et al is the first study that reported albumin calcium linearity using BCP method. Thus, studies to confirm the albumin calcium linearity relationship using the BCP method and adjusted calcium equation derived according to Payne's criteria are needed to affirm or refute the findings and conclusions of Smith et al.

1.8 The analytical imprecision of adjusted calcium measurement

Diagnostically, correct classification of calcium status using the adjusted calcium also relies on precise determination of all components of the equation. Reported regression factors of albumin against calcium vary considerably (Payne et al., 1973) (Orrell, 1971) (Ashby et al., 1986), whether this reflects variation in analytical performance or differences in populations and patients or both is uncertain.

The adjusted calcium is a value derived using a regression factor; thus the adjustment is not valid if there is a wide biological and analytical variability in the coefficient regression value (Ryan, 1979). The regression coefficient variability originates from analytical variability of albumin and calcium methods and from the biological variability of the population used. The error in the coefficient of regression is small if the analytical error of calcium and albumin is low. Barth et al (1996) and Ashby et al (1986) established that differences in regressions occur when data are collected from different analytical platforms, even when the same analytical principles have been applied, indicating that variability between quality indices of analytical platforms may partly contribute to the variability of regression coefficient between sites. Previous calcium methods may have contributed to the regression coefficient variability due to poor precision, but current calcium methods have analytical standard deviation (SD) a tenth of that previously reported (Weykamp et al., 2017). Whilst this is true for calcium methods, the same is not true for albumin methods. Albumin analytical performance, despite technological advancements, varies between different albumin methods and reports have shown that the albumin BCG method in particular failed to meet the minimal analytical performance goal (Koerbin et al., 2019). Therefore, the recommendations that discourage laboratories from adjusting calcium using a regression factor derived from other laboratories are still valid (Barth et al., 1996) (O’Kane et al., 2015).

1.9 Limitations of the concept of adjusting calcium to albumin

Several inherent problems with using equations to adjust total calcium measurements have been described. The notion of adjusting calcium to albumin equations assumes a constant coefficient of calcium binding to albumin. It has been realised that the binding constant K_a varies markedly with albumin concentrations and this concept is only true in the physiological range (Besarab et al., 1981). Besarab studied *in vitro* calcium albumin binding over a range of concentrations covering hypo/hyperalbuminemic states and hypo/hypercalcaemic states.

The percentage of calcium bound to albumin increased at the concentration below 20g/L, and decreased over the range of 30 to 90 g/L. However, at the range from 30-50 g/L the amount of calcium bound per gram of albumin did not change significantly. This finding suggests that changes in binding characteristics occur with alteration in albumin concentrations. *In vivo* studies produced similar results with a more significant effect at low albumin concentrations leading to a number of hypoalbuminaemic subjects having normal adjusted calcium levels in spite of low ionised calcium (Besarab and Carr, 1981). Therefore, it has been unanimously agreed that an adjustment factor is not accepted to adjust calcium at albumin concentration below 20 g/L (Byrnes et al., 2005).

Following their study, Besarab and colleagues proposed at physiological conditions, that there was one predominant class of calcium-albumin binding sites (Besarab et al., 1981). This is in agreement with earlier postulations in that one particular class of calcium-albumin binding sites was responsible for the majority of the binding at a physiological pH and albumin concentration (Fogh-Anderson, 1977). However, ultrafiltration studies showed there to be numerous low affinity binding sites (up to 32 sites) with slightly differing binding constants (Martin, 1953) (Kragh-Hansen, 1993). The disparity amongst these studies stems partially from the use of albumin contaminated with other proteins, such as globulins (Martin, 1953).

In vitro studies showed that the binding of albumin to calcium was not saturable at physiological concentrations of both molecules (Carr, 1953) (Kragh-Hansen and Vorum, 1993). This finding is consistent with the belief that albumin acts as a short term buffer for calcium, to sustain steady concentrations of ionised calcium.

The albumin adjusted calcium concept was deemed suitable for clinical practice based on the assumption of no intra-individual variability in the binding affinity of albumin for calcium (Payne and Walker, 1979). This assumption was earlier proved untrue, as a considerable

intra-individual variation was found (Phillips et al., 1977) (Ryan and Masarei, 1979). In fact, individual correction factors have been reported to vary in health between 0.013 and 0.044 and this variation is assumed to be more pronounced in disease (Phillips et al., 1977). These findings illustrate the influence of population differences on the adjustment equation and make the use of a common regression factor for hospitalised and ambulant patients questionable.

1.10 To what extent do equations differ for different albumin and calcium methods?

The adjusting equations are sensitive to the effect of different analytical methods of calcium, albumin, protein and any other test used for the derivation of a regression coefficient. It is known that the method used for the measurement of albumin has a significant impact on the regression values of total calcium on albumin. In clinical laboratories albumin is measured routinely by automated dye-binding methods such as BCG and BCP methods. These two methods yield somewhat different results in a normal and in a chronic kidney disease population (Speicher et al., 1978). (Joseph et al., 1996). Although another research group showed that the different behaviour of BCP is specific to haemodialysis patients rather than renal failure patients in general (Beyer et al., 1994). A recent study showed that the difference between these methods is not limited to renal disease; it in fact extends to many conditions that induce the acute phase response or α_2 -globulins (Ueno et al., 2013). Reported difficulties with the use of BCG include falsely elevated albumin levels in serum containing heparin or fibrinogen and globulin fractions (Webster et al., 1974) (Gustafsson et al., 1976) (Halibach et al., 1991) (Ueno et al., 2016). Labriola and colleagues (2009) made this point clear when they reported that the difference in BCG and BCP methods accounted for significant differences in adjusted calcium results. James and colleagues (2008) established a clinically significant difference in calcium status classification when they compared BCP in house derived equations to BCG based published equations. It has been

reported that the BCG method overestimates serum albumin by as much as 10 g/L compared with BCP (Wells et al.,1985) (Carfray et al.,2000) (Clase et al.,2001). Barth et al (1996) elaborated that the increase in globulins associated with hypoalbuminaemia was the cause of the steeper regression slope obtained at lower albumin concentrations and this was due to globulin interference with the BCG albumin assay.

Albumin exists in reduced and oxidised forms. The reactivity of BCP methods with albumin is affected by the redox status of thiol groups attached to the albumin molecules (Mabuchi et al., 1987). For example, uremic toxin inhibits the binding of BCP to albumin in sera, thereby possibly leading to the underestimation of serum albumin concentration in uremic patients. While previous studies have shown that BCP can be superior to BCG, some believe with the development of a shorter reaction time BCG methods, the superiority of BCP to BCG is probably of historical interest only and the new versions of the BCG assay are more widely used than BCP (Lolekha and Charoenpol, 1974) (NEQAS report, 2018).

The majority of serum albumin is in a reduced form, but a significant amount of albumin is oxidised during storage. Current BCP assays react more with the oxidised than the reduced form. An updated version of BCP assay, however, reacts equally with both forms (Muramoto et al., 1999). Recent BCP method agree more with the nephelometric method and as such are increasingly now being favoured over BCG methods (Ueno et al., 2013) (Ueno et al., 2016) (Koerbin et al., 2014). Most of the published equations have been derived using BCG albumin methods. According to the UK NEQAS report, approximately 36% of participants are using the more specific BCP methods. This renders the use of published equations inappropriate for use in clinical laboratories today.

Indeed Jain and colleagues partly attributed the superior performance of their derived equation over conventional equations to the fact that Payne's and Orrell's equations were derived from albumin concentrations measured by BCG reagents while his equation used

BCP albumin method (Jain et al., 2008). Despite the enhancement of the BCP and BCG methods, different albumin values are still yielded from different dye-binding albumin assays. This is evident from the albumin results distribution for various analytical platforms in the UK National External Quality Scheme (NEQAS). This wide albumin value distribution may contribute to variation in the product of calcium adjusting equations (figure 1.3 UK NEQAS). This difference influences adjusted calcium equations and results in a discrepancy in the classification of calcium status (Kato, 2011). Therefore, it has been recommended as best practice that every laboratory derives their own equation (Barth et al., 1996) (Jane et al., 2008). This recommendation was strongly supported by the evidence from accuracy studies that demonstrated significant differences between albumin methods (Bachmann et al., 1984) (Infusino and Panteghini, 2013). In conclusion, the extent that difference in calcium and albumin methods impact on the interpretation of adjusted calcium results and clinical management is a question that has no clear answer thus albumin method choice should be considered carefully until harmonisation of albumin methods has been achieved.

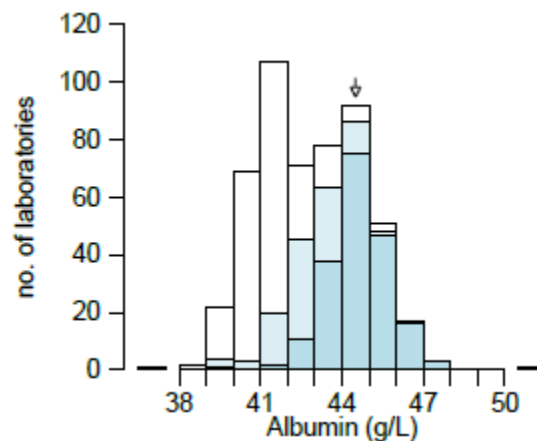


Figure 1.3: NEQAS report shows that a target value of albumin of 44.4 g/L varied between 37 and 47 g/L using different analytical platforms using BCG and BCP. NEQAS report, (Distribution No. 1013, reproduced with permission of NEQAS). The arrow shows the author's lab result. The dark blue columns represent BCG method users. The light blue columns represent BCG users using Roche analytical platform. The white columns represent BCP method users.

1.11 Limitations of using calcium adjusting equations - Clinical implications.

Since 1953, there have been reports that calcium binding capacity of serum albumin is affected by disease (Martin, 1953). Several reports followed this finding. The use of an adjusted calcium value has been questioned in critically ill patients due to altered calcium – albumin binding properties (Zaloga et al., 1987) (Broner et al., 1990) (Dickerson et al., 2004) (Ward et al., 2004). It has been suggested that calcium binds albumin with a greater affinity in critically ill patients therefore lowering ionised calcium concentration independent of albumin concentration. Other factors such as low albumin concentrations (< 20 g/L), acid-base disturbances, excessive production of fatty acid, and the presence of lactate and drugs such as heparin are common in critically ill patients. These factors alter the equilibrium between free and bound calcium thus invalidating the application of an adjusted calcium equation to this group of patients. Slomp and co-workers (2003) found that the use of an albumin adjusted calcium equation in intensive care units has led to an increase in the number of false positive hypercalcaemia and false negative hypocalcaemia cases and thus it is not suitable for use in the critically ill. Further support came from Dickerson et al (2004), this group of researchers reported low sensitivity for prediction of the correct calcium status of twenty two previously published calcium equations applied to critically ill patients and they concluded the measurement of ionised calcium is recommended in such patient groups.

Following the same trend, several equations for the estimation of adjusted calcium or ionised calcium in these patient groups have been proposed, but so far none has been found to replace satisfactorily direct ionised calcium measurement in end stage renal failure or in those on dialysis (Ring et al., 1995) (Morton, 2010). Lian and Asberg (2008) compared 6 equations, including Payne et al, Orrel, James et al and Berry's et al equations. The finding from this study was in agreement with previous studies and also recommended that the use of albumin adjusted calcium should be abandoned in renal failure patients. Clase and colleagues (2000) and more recent research groups reported that the adjusted calcium

equation led to more misclassification than total calcium did in haemodialysis patients (Goransson et al., 2005) (Obi et al., 2018). They elaborated that the lack of concordance between total and adjusted calcium may result in misclassification of calcium status and impact on the clinical decision of prescribing vitamin D or phosphate binders in patients on dialysis (Goransson et al., 2005) (Gauci et al., 2008). The disturbed acid-base haemostasis in this group of patients may provide a partial explanation for the discrepancy between the measured ionised calcium and predicated calcium (Jain et al., 2008). Another group of researchers reported that the regression of albumin binding to calcium is not effected by renal disease. This group, however, looked at a very small population with albumin in the normal interval (Leme and Silva, 1977). In conclusion, almost all studies in this field agreed that that the use of albumin adjusted calcium does not predict ionised calcium better than total calcium and the use of adjustment equations should be abandoned in favour of the use of total calcium or ionised calcium. Nevertheless, Kidney Disease Outcome and Quality Improvement (K/DOQI) guidelines advocate the use of ionised calcium measurement only when exact values are required (K/DOQI, 2003).

Jain et al (2008) argued that Payne's equation excluded end stage kidney disease and haemodialysis, and proved that an equation derived from haemodialysis patients applied to the same patient group outperformed Payne's formula. Albumin and globulin are not the only molecules that interact with calcium in patients with end stage renal failure on dialysis. Phosphate binds to calcium as well. Ferrari et al, (2009) and more recently Obi et al, (2018) proved that a calcium equation containing factors correcting for both albumin and phosphate in dialysis patients improved the comparison of adjusted calcium with ionised calcium.

A study by Payne suggested that the regression of total calcium on albumin may also differ in the pregnant population (Payne et al., 1990) and in menopause (Sokoll, 1989) (Nordln et al., 1989). They found an increase in the level of albumin adjusted calcium not seen with the direct measurement of ionised calcium. Protein bound calcium increases owing to the mild

compensated respiratory alkalosis associated with pregnancy and slightly decreased albumin in pregnancy which is perhaps the main reason for this finding. Research findings consistently showed that published equations performed inadequately in identifying calcium status in malignancy, in hospitalised elderly and in patients with calcium metabolic diseases when adjusted calcium was compared to ionised calcium measurements (Sorva et al., 1988) (Thode et al., 1989) (Ijaz et al., 2006).

None of the published equations allow for variation in non-protein binding e.g. citrate and phosphate. Although in health the error from these sources is likely to be small, studies in conditions such as post plasma exchange (high citrate), dialysis patients (high phosphate) or myeloma (high globulin) proved that different calcium binding properties exist. Accounting for some of these ligands in the calcium equation may improve the equation performance within this specific population (Ferrari et al., 2009).

In another study, comparing 13 of the published equations to ionised calcium in different populations, Ladenson and colleagues (1978) found that none of the equations consistently performed better than a measurement of total calcium. However, Ladenson and colleagues established that deriving an equation from a population and applying it to the same population improved the performance of the equation. This hypothesis was tested later and it has been proved that the new population specific equations out-performed common Payne's equations (Pfitzenmeyer et al., 2007) (Jane et al., 2008). In support of the population specific equation, Ryan reported that differences in albumin on the calcium regression coefficient among individuals are significant and it is due to true biological differences (Ryan, 1979). Critics of adjusted calcium concept argued that binding albumin to calcium has considerable intra-individual variation which makes the use of an average correction factor questionable (Thod et al., 1989) (Larsson, 2003). Finally, healthy mobile patients have slightly wider biological variation than those in recumbent posture (Humphrey et al., 1977) which again suggests the use of single adjusted regression may be not valid.

In summary, numerous researchers have questioned the ability of adjusted calcium to correctly assign patient calcium status in different patient groups. The difference in regression of total calcium on albumin in the above patient groups can be attributed to number of factors. These are summarised in figure 1.4.

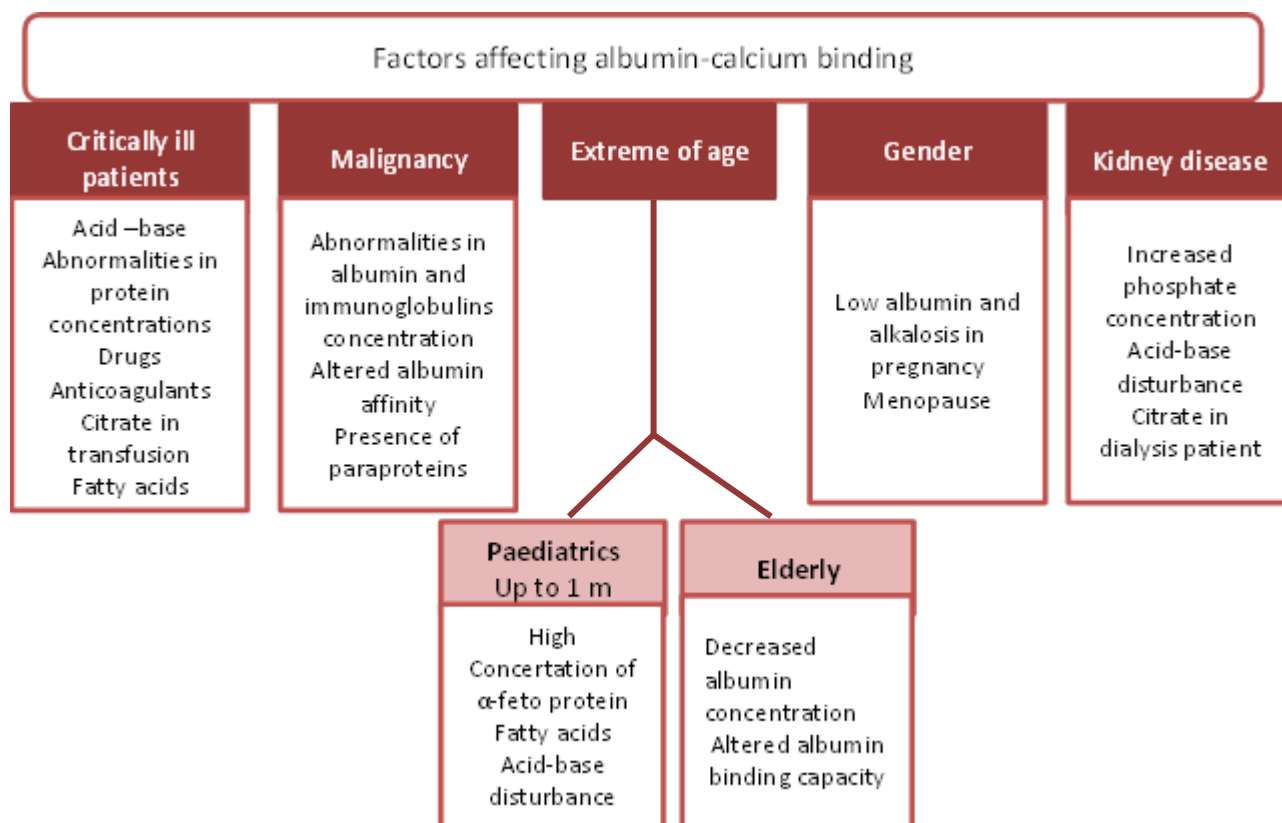


Figure 1.4: Factors affecting albumin calcium binding in different patient groups and invalid application of adjusted calcium equation.

1.12 What is the effect of age and gender on albumin and calcium adjusting equations?

Marshall et al (1982) established an increase in plasma total calcium concentration in women at the menopause and found that the increase in calcium concentration with age was independent of protein concentration. This change was attributed to an increase in ionised calcium which occurs as a result of reduced oestrogen level (Marshall et al., 1982).

A study by Whitehead et al of 55 000 healthy adults showed serum total calcium and albumin concentrations in men fell in parallel with increasing age, while there was a small increase in total calcium in women over 54 years despite a fall in albumin concentration (Whitehead et al., 1994).

In agreement with the previous studies, Payne and Barth (1996) showed that females, but not males, older than 50 years old had a mean adjusted calcium concentration significantly higher than those of a younger age. In this study, ionised calcium concentration was found to be similar irrespective of gender or age implying that older women have higher concentrations of bound calcium. Insensitivity of adjusted calcium values calculated using Payne's equation in the detection of hypocalcaemia was, however, observed when compared to the ionised calcium measurements. All subjects were hypoalbuminaemic, therefore the overestimation of calcium is likely to be due to the hypoalbuminaemia, rather than age. In support of this conclusion, Pfitzenmeyer and colleagues also observed that the use of Payne's equation in frail elderly patients resulted in the production of falsely elevated adjusted calcium results, but these phenomena disappeared when an equation derived from a population of frail elderly was applied, thus resulting in the conclusion that an age specific equation was superior to Payne's equation (Pfitzenmeyer et al., 2007)

In agreement with the reports of Payne and Barth, Sorva and colleagues also established in their study of a geriatric population that ionised calcium levels remained stable with age (Sorva et al., 1988) (Sorva et al., 1992). Despite these observations, the least squares regression coefficients of total calcium on albumin were not found to differ significantly between genders or between adults and children thus implying that one calcium adjusting equation fits all (Sokoll and Dawson, 1989). It is worth mentioning, however, that the age groups used in this study covered a wide range from paediatrics to adult and no age separation was included in the study design, which weakens the validity of their conclusion.

At the other end of age spectrum, it was realised that in newborn infants, serum calcium values obtained from the Maclean-Hastings nomogram (equivalent of adjusted calcium) did not correlate with plasma ionised calcium (Brown et al, 1972), thus implying the nomogram was unsuitable for use in neonates. The population of patients in this study had a high morbidity incidence owing to the nature of the intake of patients to this hospital, and thus may not have been representative of a normal neonatal population. The finding from the Maclean-Hastings study was recently confirmed; Jassam et al (2011) demonstrated that adjusted calcium did not correlate with ionised calcium in neonates and children of less than 1 year old implying that the use of an adult derived equation in an infant population is an invalid approach. It was also observed that mean albumin for the infant population was significantly lower than the adult population thus it is possible that the lack of correlation in this study was associated with the hypoalbuminaemia rather than the age of the subjects alone. Furthermore, in neonates, other proteins such as alpha-feto protein (AFP) exist in high concentrations until the age of one year. This high concentration may alter albumin calcium binding characteristics. However, unlike in adults, deriving an adjusted calcium equation from this group did not improve the sensitivity of the adjusted calcium equation. Therefore, the use of total calcium, supported by ionised calcium was advocated in this age group (Jassam et al., 2011).

In a more recent study, Weaving et al (2016) reported variation in serum albumin with age and gender. The mean albumin increased with age at around 20 years and then decreased with increasing age thereafter. The findings of Weaving et al strongly challenge the concept of the use of a single equation that adjusts calcium to a standard albumin concentration (Weaving et al., 2016). Whilst some research has been conducted to examine the age effect on the regression equations, age specific equations are not yet widely used in clinical practice.

1.13 Evidence against the use of a single adjusted calcium equation.

As described above, a number of studies have indicated that the performance of an albumin adjusted equation may be enhanced successfully by deriving a population specific equation.

The reasons discussed above are summarised in Table 1.2.

Albumin concentrations vary with age	Weaving et al., 2016 Jassam et al., 2011 Sokoll, 1989
Albumin concentration is lower in in-patient than out-patient populations	Orrell, 1971
Albumin binding affinity varies in health and disease	Martin, 1953 Zaloga et al., 1987
Mobile patients have wider biological variation than recumbent hospitalised patients	Humphrey et al., 1977
Wide inter-individual regression variation	Ryan, 1979 Larsson, 2003 Philips, 1977
Presence of drugs and metabolic factors affects albumin-calcium binding	Slomp et al., 2003 Ferrari et al., 2009

Table 1.2: Factors supports the derivation of population specific equation

Nationally and internationally, there is inclination within the laboratory medicine professionals to harmonise reference intervals for tests with established traceability to a higher metrological standard. In the UK, the Pathology Harmony (UK) working group has recently recommended using adjustment equations normalised to a mean calcium concentration of 2.4 mmol/L, with a reference interval of 2.2 to 2.6 mmol/L as an attempt to harmonise adjusted calcium reporting (Berg, 2012). This practice was supported by the Association for Clinical Biochemistry (ACB) working group on adjusted calcium (O’Kane et al., 2015). This move contradicts previous UK based studies which concluded that an adjusted calcium equation is not transferable between laboratories due to difference in methods used to measure albumin and calcium and differences in patient populations (Bachmann et al.,

1986) (Barth et al., 1996). Figure 1.5 summarise the factors presented in this review that may contribute to the variability of adjusted calcium.

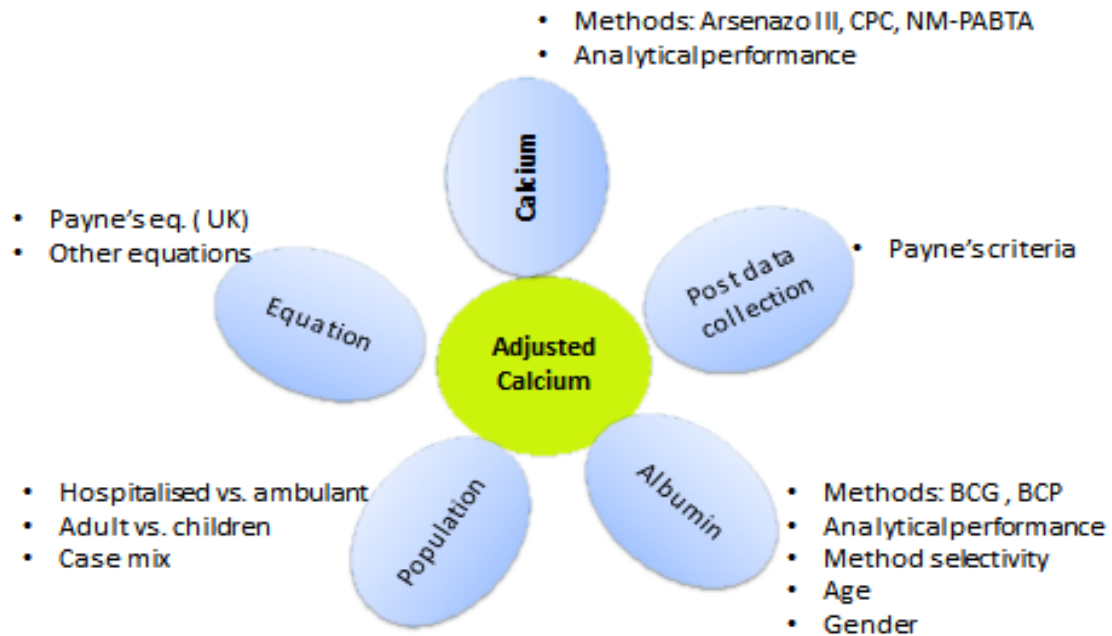


Figure 1.5: illustrating all the reported factors affecting adjusted calcium variability

1.14 Summary

With the exception of a few clinical settings, the current practice is comprised of application of a common regression coefficient to all patients irrespective of patient group. This practice has its own limitations; it may not be a valid practice, may result in misclassification of a patient's calcium status and may lead to unnecessary investigations or delay necessary investigations. Despite these observations, no further research was published and there have been no investigations into the validity of adjusted calcium values from more recently derived equations that adjust for albumin in specific patient groups. Furthermore, the adjusted calcium equation is a product of calcium and albumin measurements, therefore analytical, pre-analytical or clinical factors influencing the performance of calcium and albumin would also affect the adjusted calcium values.

It is therefore necessary to conduct research to address the derivation of population specific equations and examine all factors that may affect the variability of adjusted calcium with the aim of optimising the performance of current calcium adjusted equations. The albumin adjusted calcium practice was established in 1973 and has not changed since then. There are no studies that have assessed the Payne inclusion criteria for creatinine following advances in renal medicine practice associate with the introduction of estimated GFR measurement into clinical practice.

1.15 Research hypothesis and objectives

The weight of evidence indicates that equations used to adjust total serum calcium levels based on linear regression analysis of total calcium concentration against albumin concentration, derived from hospitalised patient data, are not suitable for the adjustment of total calcium concentrations from community and out-patient settings. The current inclusion criteria as described by Payne in 1973 include patients with creatinine up to 200 $\mu\text{mol/L}$. A

creatinine of $\leq 200 \mu\text{mol/L}$ includes patients with severe renal failure and eGFR of as low as $10 \text{ mL/min/1.73m}^2$. Patients with advanced CKD stage 3-5 are known to have metabolic bone disease and therefore disturbed calcium metabolism. A population including all those with creatinine $\leq 200 \mu\text{mol/L}$ would include such patients.

Objectives

The hypothesis to be tested is that the calcium estimation could be improved by deriving population specific calcium adjusting equations.

This research study aims to derive and validate new equations for primary care populations and to examine modified inclusion criteria consistent with the change in the diagnosing criteria of kidney disease

Study outlines: Various types of data will be used to address the research questions in this study. A small size population of healthy volunteers will be used to address questions require definitive answers (e.g. ionised calcium and reference interval populations). The second set of data will comprise of a large volume of retrospective data collected from a small number of laboratories comprised of a two populations, one with mild illness of minor severity (primary care patients) and the second population comprised of severely ill patients (hospitalised patients). The third type of data that will be used in this study is from an external quality assurance (EQA) scheme which examines the variation between analytical methods by circulating a small number of samples to a large number of laboratories. In this study, primary care setting/patients were also referred to as community setting or ambulant patients.

Primary Objectives

To validate a population specific calcium equation

To validate new inclusion/exclusion criteria for the derivation of a calcium adjustment equation

Secondary Objectives

To explore the possibility of standardising adjusted calcium by examining the causes which are leading to variability in adjusted calcium:

- To explore the analytical accuracy of calcium and albumin methods.
- To review reference intervals for albumin and calcium.
- To explore a post data collection sifting algorithm

Chapter 2: Prospective study comparing the outcome of a population-specific adjusted calcium equation to ionised calcium.

2.1 Background

In clinical practice, the most widely used equations were derived by Payne et al and Orrell et al (Payne et al., 1973) (Orrell, 1971). To date these equations have only been derived using hospitalised patients' data after excluding patients with diseases that may affect calcium albumin binding such as patients from endocrinology, oncology, haematology, nephrology departments and those under 18 years. In the absence of a valid equation to report calcium for the community and out-patient clinics, laboratories report adjusted calcium values using a regression equation obtained from in-patient data. It is postulated that this may not be a valid practice because the factors that influence calcium binding to albumin are less marked in ambulant community patients than acutely sick patients. There is evidence that albumin concentrations vary between genders, age and populations (Weaving et al., 2016) (Jassam et al., 2011). Reports have described many inherent problems with applying equations to adjust total calcium measurements across different patient groups. For example, the equation derived by Payne et al in 1973, has been found to be most effective when used on patients with low to normal albumin and total protein concentrations, which is a similar population to the group of patients from which the equation was derived (Ladenson et al., 1978). This finding suggests that the effectiveness of the adjusted calcium equation would be enhanced by deriving an equation for each population. In support of this conclusion, Payne's original study excluded patients from the renal medicine department. However, Jain et al proved that an equation to adjust calcium derived from haemodialysis patients and applied to them outperformed most of the published equations including Payne's equation (Jain et al., 2008).

The concept of adjusting calcium to albumin equations assumes a constant coefficient of calcium binding to albumin. Besarab and colleagues demonstrated that this is rarely true outside the physiological range of albumin in health. They found that the binding constant K_A varies significantly with albumin concentrations over the range from 10-90 g/L. (Besarab et al., 1981). *In vitro* studies confirmed that albumin-bound calcium concentrations vary inversely with albumin concentrations. Moreover, calcium binding is not constant even in the range 30-70 g/L where it too shows a trend to decline. But this trend of decreased calcium to albumin binding where albumin is 30-70g/L was considered insignificant and was therefore assumed to be constant. The increased rate of calcium binding at low albumin was later confirmed by *in vivo* studies that showed a more marked effect at low albumin concentrations leading to a number of hypoalbuminaemic subjects having normal adjusted calcium level despite low ionised calcium (Besarab & Caro, 1981). Further support for Besarab's study came from *in vitro* studies that have also demonstrated that the binding of calcium to albumin was not saturable at physiological concentrations of either molecule (Carr, 1951). The important conclusion from these studies is that the binding characteristics of proteins change with its concentration.

Comparable findings were obtained in several further studies implying that variations in albumin concentration alter protein binding characteristics and therefore are not supportive of the use of a single regression value in different clinical settings and populations (Sorva et al., 1988). Other disagreements with the fixed albumin corrected calcium factor concept include the fact that the binding affinity of albumin for calcium has a considerable intra-individual variation (Larsson and Magnussen, 2003) (Philips et al., 1977). It's believed that the use of a single equation to adjust total calcium results may result in misclassification of a patient's calcium status and may lead to unnecessary investigations or delay necessary investigations. Therefore, we postulate that different populations e.g. hospitalised vs ambulant patients may also generate different regression equations. In this study, we aim to

generate an adjusted calcium equation specific for ambulant population. This equation will be validated against the gold standard calcium measurement, which is ionised calcium.

2.2 Materials and methods

2.2.1 Harrogate adjusted calcium equations: prospective population

This phase of the study was approved by the National Research Ethics Committee of Northern Ireland (Ref 17/NI/0010), Appendix Ia.

Healthy volunteers were recruited from among members of staff and visitors to the Harrogate Hospital. The inclusion criteria described a reference individual as:

1. Subjectively well.
2. Over 18 years. There is no upper age limit.
3. Not having been a hospital in-patient nor been subjectively seriously ill during the previous 4 weeks.
4. Ideally are not taking any medication but if they are taking medications, these should be recorded (medication, dose and frequency).
5. Not have had any alcohol in the previous 24 hours.
6. Not smoked in the hour prior to blood sampling.

Ineligible candidates included, pregnant or lactating women, known cancer or renal, bone, liver disease patients or artificially fed patients. Participants with unusual or strenuous exercise during the previous days were excluded from participation.

2.2.2 Informed consent and health questionnaire

Poster advertisements were displayed in the clinical areas and electronic invitations were sent to staff within Harrogate Hospital. Written and verbal information was presented to the participants explaining the nature of the study and any risks involved in taking part. It was

stated that participants can withdraw from the study at any time with no obligation to give the reason for withdrawal (Appendix Ib).

The participant personally signed and dated the approved version of the informed consent form before blood was collected. Participants were asked to complete a short health questionnaire about their general health (Appendix Ic). The health questionnaire included demographics data such as date of birth, gender, race and habitual alcohol and tobacco consumption. Details of medical history of disease in any systems and prescribed and over the counter medications were recorded. The blood collections occurred over a 3 month period to ensure the provision of flexible appointments for participants based on their availability. A table was constructed with the date and time of blood collection, ionised calcium measurement and participant's name and ID number.

2.2.3 Sample collection

Subjects were rested in a seated position for 10 mins prior to venepuncture. Blood was taken without the use of a tourniquet when possible, to avoid venous stasis. On some occasions, blood was collected with the use of a tourniquet for a very short time (McMullan et al., 1990). If a tourniquet was used, it was applied 7-10 cm above the venepuncture site and released within 30 seconds to 1 minute to minimise the effects of venous stasis. Repeated fist pumping was not allowed. A minimum blood volume of 2.5 mL in heparin tube and 2 x 5 mL of serum and plasma was collected from each participant. The following blood samples were collected.

1. Heparin tube 1 x 2.5 mL for ionised calcium
2. Plain tube sufficient to collect 5 mL serum, for total calcium, albumin and vitamin D
3. EDTA tube sufficient to collect 5 mL plasma for PTH measurement

The heparinised blood was measured immediately for ionised calcium. The serum sample was allowed to clot at room temperature, and then separated by centrifugation within a maximum of 4 hours from venepuncture and the serum stored for a maximum of 24 hours at 4°C until analysis. The following analyses were undertaken; total calcium, albumin, vitamin D and PTH.

2.2.4 Analytical methods

Blood tubes were allowed to clot at room temperature for 30 mins and analysed within 24 hours for total calcium, albumin and vitamin D. EDTA samples were analysed for PTH on the day of collection. Total serum calcium, albumin, vitamin D and PTH concentrations were measured on one of two Roche Cobas 802 analysers (Roche Diagnostics UK Ltd, West Sussex, UK) in the Blood Sciences Laboratory at Harrogate Hospital. Calcium was measured using a spectrophotometric method “NM-BAPTA”, while albumin was measured using the bromocresol green photometric method (BCG). Vitamin D and PTH were measured by immunoassay based methods.

The analytical performance in terms of precision (coefficients of variation) was within acceptable limits defined by this laboratory on either analyser on all of the four analytes and on three levels of internal quality control levels over the period of study (Appendix II). There was no significant bias on External Quality Assurance Scheme during the period of study and bias did not exceed -0.3 % (performance limit $\pm 3.5\%$) for calcium, 2% (performance limit $\pm 5\%$) for albumin, 10% (performance limit $\pm 25\%$) for Vitamin D and -6.4% (performance limit $\pm 15\%$) for PTH.

2.2.5 Ionised calcium analysis

Ionised calcium was measured by an Ion Selective Electrode (ISE) based method on the 9180 Electrolyte Analyser (Roche Diagnostic Ltd, West Sussex, UK). All plasma samples were measured within 30 mins from blood collection. Analysis was performed according to

the manufacturer's protocol. The ionised calcium assay performed within acceptable limits defined by the manufacturer in terms of Internal Quality Control (IQC) for the duration of the study. The EQA assessment showed agreement with the overall national mean (appendix III).

2.2.6 Equation derivation

Retrospective biochemical data for calcium equation derivation was extracted from two settings; in-patient and primary care. Primary care extracted data included the following parameters; age (>18 years), gender, calcium, albumin, ALP, ALT, potassium, creatinine, urea and eGFR. The same above criteria were used for in-patient data extraction with the exception of eGFR. Data were collected using Laboratory Management Information system "LabCentre, Clinisys".

Data collected excluded patients attending the departments of Endocrinology, Haematology, Nephrology, Oncology or artificially fed patients. Data were collected for a defined period time (2-3 months) to allow the availability of at least 1000 data points per equation with the use of a single set of albumin and calcium per patient. Biochemically, data were further filtered to exclude patients with ALT > 40 iu/L, ALP > URL, Creatinine > 200 $\mu\text{mol/L}$, urea > 15 mmol/L and potassium outside the reference range (Barth et al., 1996). Primary care setting data were also biochemically filtered according to Payne's criteria (Barth et al., 1996).

2.2.7 Mathematical derivation of calcium adjustment equations

Adjustment equations were derived for each population according to Payne's described method (Barth et al., 1996). The slope and intercept were obtained from the linear regression plot of total serum calcium against albumin.

The mean total calcium concentration in the population was also calculated using a normal plot histogram. The linearity of albumin regression against calcium was assessed by plotting

albumin against calcium using the linear regression. This analysis produced the slope and intercept (non-bound calcium) values. The values for the intercept, slope and mean total calcium were entered into equation 1 for each laboratory and each population.

Equation 1

$$\text{Adjusted calcium} = \text{Total Calcium} - (\text{slope} \times \text{albumin}) + (\text{mean total calcium of the population} - \text{intercept}) \dots\dots\dots 1$$

Equation 1 is mathematically rearranged to give the final format represented in equation 2 (for full details of equation derivation and re-arrangement see appendix IV). Adjusted calcium and total calcium are measured in mmol/L, albumin measured in g/L.

Equation 2

$$\text{Adjusted Calcium} = \text{Total Calcium} - \text{Slope} \times (\text{Albumin} - \text{Constant}) \dots\dots\dots 3$$

This method results in two equations:

- In-patient equation using hospitalised patients data set
- Community equation, using primary care patients data set

2.2.8 Validation of equations: comparison to ionised calcium

The Harrogate Hospital in-patient and primary care derived equations were validated by comparing adjusted calcium values from those equations to ionised calcium measurements. For each data set, the respective adjusted calcium equation was applied to total calcium and albumin pairs, to provide adjusted calcium values. To validate the newly derived equations (namely in-patient and primary care equations); the adjusted calcium equations were applied to the reference populations. Adjusted calcium values obtained from these equations were compared to ionised calcium concentrations that were obtained from the reference population (123 subjects).

2.2.9 Statistical analyses

All data were analysed by a statistical package, Analyse it (Microsoft Excel 2010) (version 2.10). A normal probability plot was used to find the constant factor value (Intercept) which represents the non-protein-bound calcium and to calculate the mean calcium in each population. Linear regression was constructed to derive the regression factor (slope) for each population. Linear regression model was also used to compare the regression factor R^2 for the in-patient and community equations. The comparison of adjusted calcium results to ionised calcium was presented using Altman Bland plot and scatter plot. The t-test was used to test the statistical significance between adjusted calcium mean of the newly derived equation and the routine equation. The Z test was used to test the statistical significance between the albumin and calcium mean from in-patient and primary care populations.

2.3 Results

2.3.1 Harrogate equations: perspective population

125 healthy subjects were recruited for the Ionised calcium study. The age and gender distribution is shown in figure 2.1. The ethnic distribution of the studied population was 92% White, 4% Asian and 4% of various other ethnic groups. 53% of the studied population took no medications. Only 9% and 15% were on vitamin D or multivitamins, respectively. The rest of the group, which accounts for a total of 23%, took one or more of antidepressants, HRT, thyroxine, PPI, Ventolin inhaler, Simvastatin and diuretics.

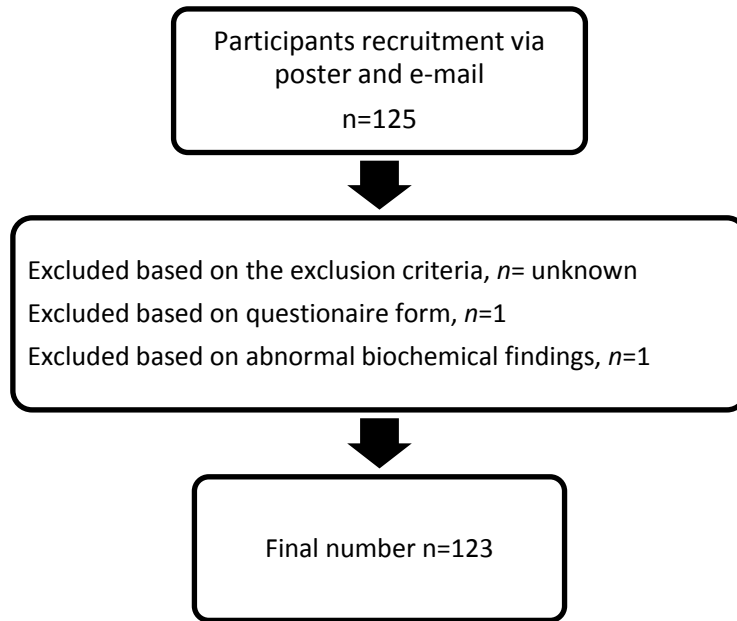


Figure 2.1: Flow chart of exclusion process.

Gender	27 (22%) male and 96 (78%) female
Age range	18-69 years old
Exercise in the last 24 hour	91% no exercise, 8% mild level, 1% strenuous exercise.
Ethnicity	92% white, 4% Asian and 4% various ethnic groups
Vitamin D status	
Sufficient > 60 nmol/L	Sufficient = 66.4%
Insufficient 20-59 nmol/	Insufficient =33%
Deficient < 20 nmol/L	Deficient = 0.8%
PTH (1.6-7.0 pmol/L, Roche Cobas)	86% within reference interval and 14% within (7.3-14.0) Median 8 pmol/L.
Medication	9% on Vitamin D, 15% on multivitamins

Table 2.1: Population characteristics, $n=123$.

2.3.2 Excluded subjects

All participants considered themselves to be healthy at recruitment. No participant was known to have chronic kidney, liver disease, history of cancer or metabolic bone disease.

No records had missing data, insufficient sample volume or inaccuracy sufficient to warrant

exclusion. One subject was excluded due to a heavy strenuous exercise in the last 24 hours before bleeding session. A single subject was regarded as unhealthy and excluded for the following biochemical results: raised PTH, iCa 1.33 mmol/L (1.18-1.33) (in-house derived reference interval, appendix III) and total calcium of 2.58mmol/L (2.20-2.60). A referral to endocrinology for possible primary hyperparathyroidism has been arranged.

2.3.3 Community and in-patient equations

Retrospective data from Harrogate Hospital have been divided into two main sets; hospitalised patients and primary care patients. Two calcium equations have been derived, one derived for each data set. Table 2.2 presents these equations and Table 2.3 presents the mean albumin and calcium of the hospitalised and ambulant populations. The outcome of these equations has been compared to ionised calcium in figure 2.3 and figure 2.4.

Equation Name	Calcium Equations	<i>n</i> value
In-Patient Equation	Adjusted Ca=T.Ca- 0.018 (Alb. - 38.3)	1141
Community Equation	Adjusted Ca=T.Ca- 0.014 (Alb. – 44.9)	6062

Table 2.2: shows the derived adjusted calcium equations for Harrogate hospitalised patients and community population. Platform: Roche Cobas 702, Calcium method: NM-BAPTA, Albumin method: BCG. Adjusted and total calcium (mmol/L) and albumin (g/L).

Equation Name	Regression Coefficient	Intercept	Mean Calcium mmol/L	Mean Albumin g/L
In-Patient Equation	0.018	1.579	2.279, <i>CI</i> (2.269-2.289)	38.7, <i>CI</i> (38.3-39.0)
Community Equation	0.014	1.77	2.378, <i>CI</i> (2.376-2.381)	44.9, <i>CI</i> (44.7-45.1)

Table 2.3: shows the characteristics of hospitalised versus ambulant patients in terms of albumin and calcium. The calcium and albumin means were found significantly different at $p < 0.0001$.

2.3.4 Validation of the community regression equation against the routine equation

In order to validate the newly derived adjusted calcium for the community population, the routine in-patient equation and the community specific equation were applied to the data set obtained from 123 healthy subjects. The significance of the difference in the mean of adjusted calcium produced by these equations was compared by *t* test. A statistically significant difference between the two populations means was observed with a mean difference of - 0.156 mmol/L, 95% *CI* of (-0.1586 to -0.1539, $p < 0.0001$). The adjusted calcium community equation mean of 2.33 mmol/L was higher than the mean from the routine in-patient equation (mean of 2.177 mmol/L). This difference of - 0.156 mmol or (7.6%) is both statistically and clinically significant different as it exceeds the allowable difference of 5% or 0.1 mmol/L for this laboratory or the minimal allowable error of 4.6% as set by the biological variation model (Fraser et al., 1997). Similar to *t*-test finding, the Deming Fit analysis for the adjusted calcium values of the two equations showed that routine in-patient equations underestimated calcium status by 0.160 mmol/L in comparison to the community specific equation. This finding agrees well with the calcium mean analysis and the difference in adjusted calcium values obtained from these equations exceeds the allowable limits for calcium, which suggests that these equations are distinctively different equations.

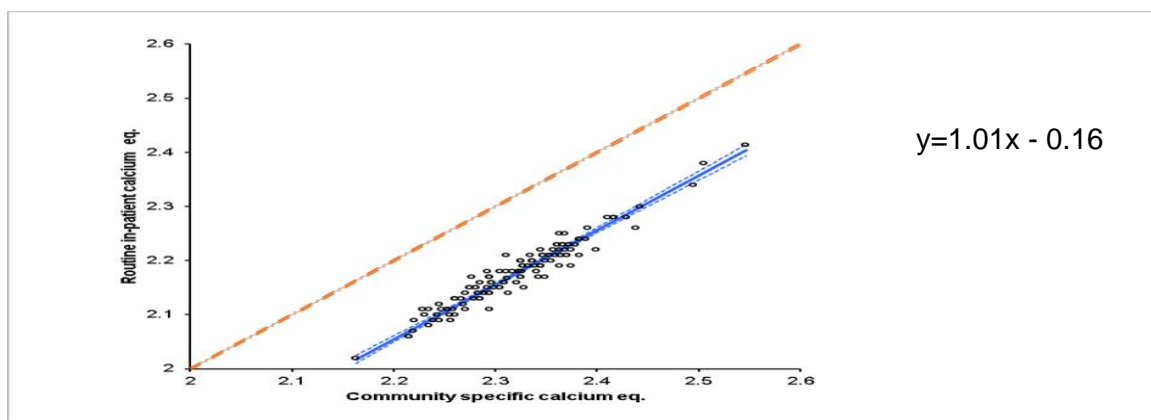


Figure 2.2: Deming Fit shows a comparison between the adjusted calcium values for 123 healthy subjects (○) obtained from in-patient equation and the community equation. Red dashed line denotes the line of best fit. Blue dashed lines represent 95% *CI*.

2.3.5 Validation of the community regression equation with ionised calcium

Paired samples for ionised calcium and total calcium were analysed for 123 healthy subjects. The routine in-patient equation and the community specific equation have been applied to the data set obtained from 123 healthy individuals. The comparison between adjusted calcium that was calculated using the routine in-patient equation and ionised calcium is as follows:

$$\text{Adjusted calcium} = 1.292 + 0.7181 \times \text{Ionised Calcium}, R^2 = 0.20$$

$$\text{Intercept} = 1.292 \quad 95\%CI (0.983 \text{ to } 1.601, p < 0.0001)$$

$$\text{Slope} = 0.7181 \quad 95\%CI (0.4679 \text{ to } 0.9683, p < 0.0001)$$

The comparison between the newly derived adjusted calcium equation for the community population and ionised calcium is as follow:

$$\text{Adjusted calcium} = 1.361 + 0.7887 \times \text{Ionised Calcium}, R^2 = 0.26$$

$$\text{Intercept} = 1.361 \quad 95\%CI (1.065 \text{ to } 1.658, p < 0.0001)$$

$$\text{Slope} = 0.7887 \quad 95\%CI (0.5483 \text{ to } 1.0291, p < 0.0001)$$

Calcium status was classified as hypo/hypercalcaemia according to the reference intervals of 1.18-1.33 mmol/L for ionised calcium (in-house derived, appendix III) and 2.2-2.6 mmol/L (Berg, 2012) for adjusted calcium and total calcium. Using ionised calcium as a gold standard, the number of patients in whom calcium status was correctly predicted using the routine in-patient adjusted equation was 56/123 (46%), by the community specific equation was 113/123 (92%) and by total calcium was 112/123 (91%). However, our data shows that adjusted calcium by the routine in-patient equation underestimated calcium in healthy individuals. Our data shows that the use of the in-patient adjusted calcium equation which was derived using Payne's exclusion criteria significantly misclassifies calcium in healthy

subjects. On the contrary, the newly derived community equation, which was also derived using Payne's exclusion criteria compares well with ionised calcium.

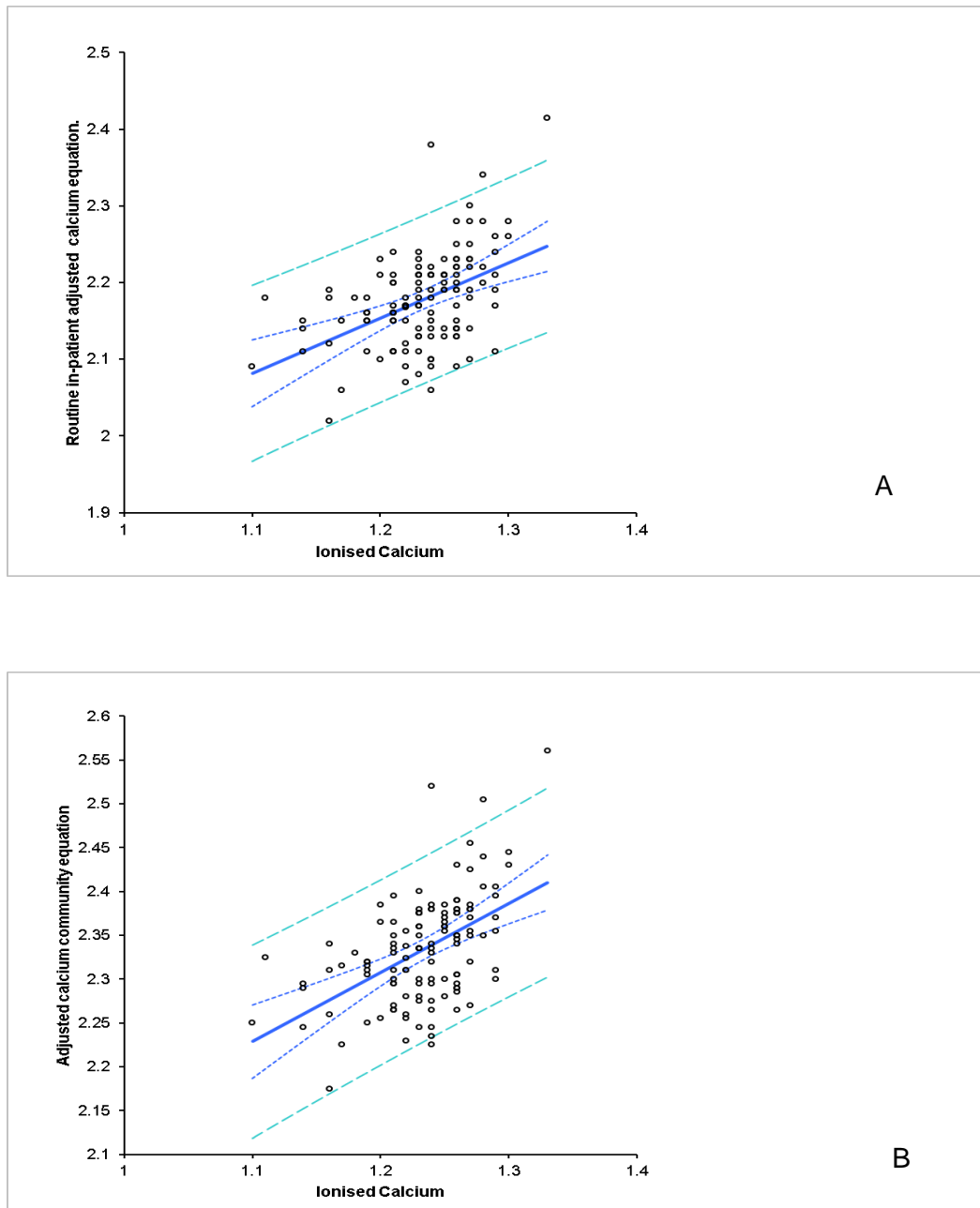


Figure 2.3: shows comparison of measured ionised calcium and adjusted calcium equation in 123 healthy subjects. (A) Using the routine in-patient adjusted calcium equation. (B) Using the newly derived community equation. Dark blue dashed lines represent 95% CI. Light blue dashed lines represent 95% prediction interval.

2.4 Discussion

At present, adjusted calcium measurement reported on all patients whether they are hospitalised patients, out patients or in primary care settings using equations that have been derived from hospitalised patient's data. It was postulated that this practice may cause misclassification of calcium status in non-hospitalised patients. In this study, the use of in-patient adjusted calcium equation in a primary care setting has been evaluated. A locally derived adjusted calcium equation specific to the community population was calculated. The newly derived equation was validated against ionised calcium results obtained from 123 healthy subjects that participated in this study. To our knowledge there are no previous studies that have validated an equation specific for community population.

The adjusted calcium community specific equation correlated well with ionised calcium and predicted the correct calcium status in 92% of the 123 healthy individuals. On the other hand the routine in-patient equation only correctly predicted the calcium status of less than 50% of the healthy participants. In support of the above finding, *t*-test showed that the routine in-patient equation produced lower adjusted calcium results (with an average of - 0.156 mmol/L) compared to the community specific equation. These findings support the suggestion that the application of a single regression equation to different clinical settings may result in misclassifying calcium status. In this study, I present an argument against the general application of a single regression equation in different clinical settings and populations. Albumin regression on calcium is a mathematically derived factor which depends on a number of variables related to albumin, calcium and the environment where the binding occurs. Affinity studies showed that albumin-calcium binding is known to vary widely between individuals and this variation is wider in severely diseased populations than in healthy populations (Zaloga et al., 1987) (Martin, 1953) (Ryan, 1979). This variation in albumin binding affinity is mathematically translated to a wide regression variation (Ryan, 1979). One can conclude that regression variation would only be larger within diseased

individuals due to the presence of disturbed metabolic processes which may alter albumin affinity and presence of drugs that may compete with calcium on albumin binding sites (Martin, 1953). (Slomp et al., 2003) (Dickerson et al., 2004).

Binding capacity is another factor that affects the albumin calcium binding relationship. In fact Pedersen (1971) showed that the binding constant depends on the concentration of both albumin and calcium. There is also evidence that albumin concentrations in recumbent patients are lower than that seen in supine patients (Humphrey et al., 1977). In agreement with Orrell (1971), the data herein showed that mean albumin and calcium concentrations in hospitalised patients were significantly lower ($p < 0.0001$) than in primary care populations (table 2.3). Indeed, albumin as an acute phase protein is lower in acutely ill patients than in ambulant patients. A recent study showed that mean albumin concentration on ambulant patients reaches a peak at the age of 20 years and this declines with old age (Weaving et al., 2016). In this study, Payne's data collection criteria excluded all those under 18 years old in both clinical settings; therefore, the proportion of patients with old age would certainly be higher in the hospital data set and thus contribute to a lower albumin mean in this population. Whilst calcium disorders are prevalent in both hospital and primary care settings; hypocalcaemia of acute severe illness is well documented and thus contributes, among many other causes to a lower calcium mean in hospitalised patients (Hannan and Thakker, 2013).

The argument presented above renders the use of a single equation derived from hospitalised patients and applied to ambulant patients as questionable. The differences in mean calcium and albumin also suggest that hospitalised patients and primary care patients are two distinct populations. In support of this point, a previous study that evaluated the use of adult adjusted equation in neonates and children also concluded that the significant difference in mean albumin between these age groups invalidated the use of an adult adjusted calcium equation in neonates and children (Jassam et al., 2013).

To highlight the different characteristics of these two populations, these populations can be described as follows; the hospitalised population consists of a group of supine, severely ill patients with a higher mean of age than the primary care population. The primary care population on the other hand consists of ambulant, chronically ill patients, those who are attending for health screening and a young population with short episodes of minor illnesses. One can conclude that these are two populations with different calcium albumin binding characteristics and concentrations, therefore different regression equations would arise from those distinctively different populations.

The literature presents compelling evidence supporting the concept of a population specific equation. Ladenson et al (1978) compared 13 published equations versus ionised calcium, and he noted some improvement in calcium classification when an algorithm was derived from a given population's own data. More recently Jain et al (2008) also produced a population specific equation from end stage renal patients on haemodialysis that outperformed Payne's calcium equation. Ferrari and colleagues proved that the inclusion of phosphate in the regression equation improved the diagnostic accuracy of Payne's equation. It is not known however, if the main reason for equation performance improvement was the addition of phosphate to the regression or the application of an equation to the population from which it was derived. On the contrary, another research group presented a similar concept but could not confirm that the addition of phosphate improved the adjusted calcium equation in renal failure patients (Lian and Åsberg, 2017).

In the healthy individuals it was found that total calcium was superior to the routine in-patient adjusted equation and predicted the correct calcium status in 91% of healthy subjects. This finding is not surprising, because the concept of albumin adjusted calcium was introduced to counteract the hypoalbuminaemia effect in the diseased population and this population consisted of healthy subjects. However, in disagreement with the critics of adjusted calcium

practice, total calcium completely failed to pick out those with hypocalcaemia or hypercalcaemia in the studied healthy participants.

Calcium is one of the most frequently requested biochemical tests. In primary care, calcium requests by primary care physicians ranged from 46.2 - 526.3 per 1000 practice population (The NHS Atlas of Variation, 2013). An American study demonstrated that a change in calcium concentration as small as 0.025 to 0.125 mmol in either direction, would increase the cost of health care \$8-89 per patient due to the increase in number of biochemical and non-biochemical follow up tests requested and drug prescription (NIST, 2004). In this study it was found that the mean calcium difference between the in-patient equation and community specific equation was 0.156 mmol/L. Therefore, our findings are of clinical and financial importance because enhancing the diagnostic accuracy of the adjusted calcium equation by using a population specific calcium equation has the potential impact of saving health care resources.

In this study, regression analysis of adjusted calcium equations gave low R^2 regression factors of 0.2 and 0.26 for in-patient and community specific adjusted calcium equations, respectively. It is worth mentioning that low R^2 values, but with a good residual plot, can still be an indicative of a good regression model (Frost, 2019). In support of this, the small change in R^2 value from 0.2 to 0.26 with the introduction of the population specific equation resulted in improving the prediction of the correct calcium status from 46% (in-patient equation) to 92% (community equation).

This is the first study that has derived and validated an equation specific for ambulant patients. The strength of our findings stems from comparing the newly derived community equation outcome to ionised calcium, which is considered the gold standard for calcium measurement. However, some limitations of this study should be discussed.

It has been shown previously that different analytical platforms produce different regression equations (Barth et al., 1996). This implies that the validated community adjusted calcium equation could only be relevant to a Roche Cobas analytical platform, NM-BAPTA calcium method and BCG albumin method, as used in the current study. The process of replicating this work on all commercially available analytical platforms and to account for different methodologies of albumin and calcium would require the participation of at least 12-18 laboratories to cover all the combinations of commercially available calcium and albumin methods. Therefore, from a resource point of view, such an exercise is outside of the scope of this study. Therefore, future studies comparing the impact of community specific equations to routine in-patient equations for different analytical platforms are needed.

In conclusion: the use of in-patient equation in a primary care setting has been evaluated. The mean albumin and calcium concentrations are significantly different between these two populations which is supportive of the use of a population specific equation. The literature reports a plethora of attempts to improve the diagnostic accuracy of calcium. This work is an addition to all previous efforts in this field. The diagnostic accuracy of the adjusted calcium equation was improved by the derivation of a population specific equation. This new practice is likely to lead to saving health care resources.

Chapter 3: The effect of different analytical platforms and methods on the performance of a population-specific adjusted calcium equation

3.1 Background

Approximately 45% of total serum calcium is protein bound, principally to albumin, 10% is bound to small anions and 45% is ionised. Ionised calcium is the biologically active form of calcium. Routine measurement procedures measure total calcium, which includes the bound fractions. Calcium measurement is critical for the assessment of bone diseases, but it is widely accepted that total serum calcium concentrations are unreliable markers of the physiologically important ionised calcium fraction in serum. Calcium-albumin binding is affected by abnormalities in albumin concentration, acid-base status and by the presence of molecules such as drugs or fatty acids that shift the balance between the bound and the free fraction of calcium (Landson and Shyong, 1977) (Mimouni et al., 1991). In order to compensate for these effects, calcium concentrations are adjusted using regression equations. In the mid-seventies of the last century the first few widely used calcium adjustment equations emerged (Orrell, 1971) (Berry et al., 1973) (Payne, et al., 1973). For the construction of a calcium adjustment equation, in-patient data were used to calculate the regression of total calcium on albumin. The most frequently used equation was; Adjusted calcium = Total calcium - 0.02 (Albumin-40) (adjusted and total calcium (mmol/L), albumin 40g/L). The regression coefficient of albumin of 0.02 is used to calculate the expected change in calcium for each gram change in albumin concentration away from the baseline of 40 g/L.

The diagnostic value of calcium adjusting equations has always been a subject for criticism (Clase et al., 2000). Whilst calcium equations continue to be used in clinical practice, researchers continued to explore reasons for the poor performance and attempted to improve the diagnostic accuracy of these equations (James et al., 2008) (Ferrari et al.,

2009). Yet all these attempts proved to be less than optimal and adjusted calcium based on albumin regression was considered, by a very recent study, less accurate than total calcium in predicting patient calcium status in renal failure patients (Lian and Asberg, 2018).

A recent attempt to improve the diagnostic value addressed a population specific adjusting calcium concept (chapter 2). The move from the use of a general regression equation to a specific regression equation is not necessarily a new practice. Due to the variation in regressions obtained from various albumin, calcium methods and platforms, adjusted calcium equation is deemed analytical platform and method specific (Barth et al, 1996). For example, an adjusted calcium equation derived for over 18 year olds proved to be invalid for application in newborn and paediatric populations (Jassam et al, 2011). There is evidence that there is a scope for improving calcium reporting in hospitalised patients by using an age specific equation for those over 60 years old (Welsh and Jassam, 2012). In the UK, best practice guidelines recommend that laboratories have to derive an equation specific for their analytical platform and methods of calcium and albumin (O' Kane et al., 2015).

The recently derived ambulant patient specific equation was only validated for a single analytical platform, BCG albumin method and NM-PABTA calcium method (chapter 2). This study aims to derive and validate community specific equations for most of the commercially available analytical platforms and albumin and calcium methods.

Historically, validation of a newly derived calcium equation was best confirmed against ionised calcium (Smith et al., 2018). However, statistical methods for calcium equation validation were also used previously (Clase et al., 2000) (Jassam et al., 2011). In this study, for the first time the impact on the calcium classification status will be used as indirect validation method.

3.2 Materials and methods

Retrospective and anonymised biochemical data for calcium equation derivations were collected from other hospitals' laboratories to cover the four most commonly used analytical platforms in the UK. These are; Roche Cobas (Roche Diagnostics Ltd, West Sussex, UK), Siemens Advia (Siemens Healthcare Diagnostics, Surry, UK), Abbott Architect (Abbott Diagnostics, Kent, UK) and Beckman Olympus or DXI (Beckman Coulter Ltd, High Wycombe, UK). An invitation for participation was sent to the UK laboratories. Participant laboratories were asked to collect data from their laboratory information system (LIMS) according to a circulated protocol. The protocol specified the following:

- 1- Collecting data from primary and secondary care settings
- 2- Retrospective data collected for a period of 2-3 months to allow the availability of at least 1000 data points per equation.
- 3- Excluding conditions which could affect calcium haemostasis as defined by Payne's criteria (section 2.2.6).
- 4- Primary care extracted data included the following parameters age (>18 years), gender, hospital number, calcium, albumin, ALP, ALT, potassium, creatinine, urea and eGFR.
- 5- The same above criteria were used for secondary care data extraction.
- 6- Providing the name of the analytical platform, albumin and calcium methods and the reference range for the collected tests.

All data were then filtered to include over 18 year olds, one set per patient and then further filtration was undergone according to Payne's biochemical criteria (Payne, 1973). Two adjusted calcium equations were derived per laboratory; one equation for hospitalised patients and another for primary care patients. The equation calculation was the same as that described in section (2.2.7).

The participating laboratories were grouped by analytical platform and referred to by the name of the analytical platform followed by a number as presented in the following table (3.1):

Lab. No.	Location	Manufacturer
Roche 1	Harrogate	Roche Cobas 702
Roche 2	Bolton	Roche Cobas 702
Roche 3	North Cumbria	Roche Cobas 702
Roche 4	Peterborough	Roche Cobas 702
Roche 5	Sheffield	Roche Cobas 702
Roche 6	Manchester	Roche Cobas 702
Roche 7	Bristol	Roche Cobas 702
Beckman 1	Hull	Beckman Coulter AU 5800/680
Beckman 2	York	Beckman Olympus
Abbott 1	Dublin	Abbott Architect
Abbott 2	Cardiff	Abbott Architect
Abbott 3	Plymouth	Abbott Architect
Abbott 4	Gwent	Abbott Architect
Siemens 1	Leeds	Siemens Advia XP

Table 3.1: shows the list of participating laboratories.

Derived equations from hospitalised patients and from primary care patients were then applied to their populations and the performance of an equation was defined by its impact on the prevalence of hypocalcaemia and hypercalcaemia in the studied population. The outcome of these equations was compared across laboratories, analytical platforms and methods.

3.2.1 Statistical analyses

The Z test was used to test the statistical significance of differences between the albumin and calcium means from different analytical platforms. Adjusted calcium equations obtained from different analytical platforms were compared by calculating the percentage of hypo/hypercalcaemia for each equation. The statistical analysis was undertaken using Analyse-it Statistical Package (Microsoft Excel 2010) (version 2.10).

3.3 Results

Fourteen laboratories from various locations in the UK have participated in this study. Retrospective data from in-patient and community were collected by each participating laboratory. Hospitalised patient data were collected according to Payne et al (Payne et al., 1973). To reduce/eliminate source of variations, a protocol was circulated to all participating laboratories describing the required data and preferable format. All participating laboratories fulfilled the requirements for the inclusion in this study with the exception of a single laboratory. Abbott 4 laboratory provided A&E data for the hospitalised patients set rather than in-patient data. However, community data were collected according to the requested criteria, thus only community data were presented for this laboratory.

In-patient and community data were filtered according to Payne's exclusion criteria. Therefore, two equations were produced for each participating laboratory, one for the in-patient setting and another one for the primary care setting. The participating laboratories, analytical platform used and methods for albumin and calcium are given in Table 3.2.

Lab. No.	Manufacturer	Calcium Method	Albumin Method
Roche 1	Roche Cobas 702	NM-BAPTA	BCG
Roche 2	Roche Cobas 702	NM-BAPTA	BCG
Roche 3	Roche Cobas 702	NM-BAPTA	BCG
Roche 4	Roche Cobas 702	NM-BAPTA	BCG
Roche 5	Roche Cobas 702	NM-BAPTA	BCG
Roche 6	Roche Cobas 702	NM-BAPTA	BCP
Roche 7	Roche Cobas 702	NM-BAPTA	BCP
Beckman 1	Beckman Unicel DXI	Arsenazo III	BCP
Beckman 2	Beckman Olympus AU5800/680	O-Cresolphthalein	BCG
Abbott 1	Abbott Architect	Arsenazo III	BCG
Abbott 2	Abbott Architect	Arsenazo III	BCP
Abbott 3	Abbott Architect	Arsenazo III	BCG
Abbott 4	Abbott Architect	Arsenazo III	BCP
Siemens 1	Siemens Advia XP	Arsenazo III	BCP

Table 3.2: gives a list of platforms, calcium and albumin methods for all participating laboratories. BCG is bromocresol green. BCP is bromocresol purple.

3.3.1 Calcium adjusted equations

The adjusted calcium equation format for hospitalised and ambulant patients is:

$$\text{Adjusted calcium} = \text{Total calcium} - \text{Regression Coefficient} \times (\text{Albumin} - \text{Constant})$$

Table 3.3 and Table 3.4 present the Regression Coefficients and the Constant values for both hospitalised and ambulant patient equations categorised by platform.

Hospital	Regression Coefficient In-patient	Constant In-patient	<i>n</i> In-patient	Regression Coefficient Community	Constant Community	<i>n</i> Community	Albumin method
Roche 1	0.018	38.3	1141	0.014	44.9	7779	BCG
Roche 2	0.016	36.0	2021	0.016	45.0	2938	BCG
Roche 3	0.018	37.5	7135	0.013	45.5	5134	BCG
Roche 4	0.019	36.6	1347	0.015	44.0	1991	BCG
Roche 5	0.019	36.9	1848	0.013	45.5	5226	BCG
Roche 6	0.018	35.0	6994	0.015	39.3	5934	BCP
Roche 7	0.019	34.0	1123	0.012	39.9	2103	BCP
Beckman 1	0.019	36.0	1959	0.014	39.4	5272	BCP
Beckman 2	0.020	39.2	5102	0.016	41.5	13077	BCP
Abbott 1	0.015	36.0	6290	0.014	41.0	4562	BCG
Abbott 2	0.012	36.1	1360	0.014	39.0	7647	BCP
Abbott 3	0.021	36.0	1504	0.017	41.2	6642	BCG
Abbott 4*				0.014	38.0	9581	BCP
Siemens	0.016	36.8	3358	0.015	38.8	9428	BCP

Table 3.3: shows in-patient equations and community equations for all participating laboratories, grouped by analytical platforms. *In-patient data were excluded.

In this study, it was found that mean calcium and mean albumin were higher for the ambulant patients than hospitalised patients irrespective of albumin method (table 3.4). The baseline for albumin in the community equation is higher than the baseline albumin in in-patient equations and higher than the mean albumin for in-patients.

Laboratory	In-Patient Albumin g/L	Community Albumin g/L	Z-test	In-Patient Calcium mmol/L	Community Calcium mmol/L	Z-test
Roche 1	38.7	44.9	$p < 0.0001$	2.279	2.378	$p < 0.0001$
Roche 2	35.4	44.6	$p < 0.0001$	2.200	2.359	$p < 0.0001$
Roche 3	37.5	45.8	$p < 0.0001$	2.269	2.391	$p < 0.0001$
Roche 4	34.6	43.9	$p < 0.0001$	2.192	2.339	$p < 0.0001$
Roche 5	34.4	40.9	$p < 0.0001$	2.170	2.343	$p < 0.0001$
Roche 6*	33.1	39.0	$p < 0.0001$	2.266	2.356	$p < 0.0001$
Roche 7*	29.9	39.1	$p < 0.0001$	2.142	2.339	$p < 0.0001$
Beckman 1*	38.7	41.4	$p < 0.0001$	2.300	2.353	$p < 0.0001$
Beckman 2*	32.2	38.7	$p < 0.0001$	2.227	2.344	$p < 0.0001$
Abbott 1	35.4	41.4	$p < 0.0001$	2.302	2.352	$p < 0.0001$
Abbott 2*	35.8	36.6	$p < 0.0001$	2.357	2.363	$p = 0.0007$
Abbott 3	35.2	40.9	$p < 0.0001$	2.225	2.328	$p < 0.0001$
Siemens 1*	35.1	38.7	$p < 0.0001$	2.278	2.361	$p < 0.0001$

Table 3.4: Mean albumin and mean calcium for hospitalised and ambulant patients. Laboratories used BCP methods are marked with *, the rest of the participating laboratories have used BCG albumin method.

3.3.2 The impact of calcium equations on the prevalence of hypo/hypercalcaemia in hospitalised patients

The Pathology Harmony reference interval of (2.2-2.6) was used to define the limits for hypo/hypercalcaemia. Figure 3.1 and figure 3.2 give the impact of the derived equations on the prevalence of hypocalcaemia and hypercalcaemia in hospitalised patients, respectively.

The prevalence of low calcium according to total calcium among participating hospitals ranged from 22.5-55.0%. Post adjusting total calcium to albumin, the prevalence of hypocalcaemia in participating laboratories reduced to 6.6 - 44.0%. It was observed that following post adjustment to albumin, hypocalcaemia prevalence could be divided into two groups based on albumin method, with a higher prevalence among BCG method users (figure 3.1).

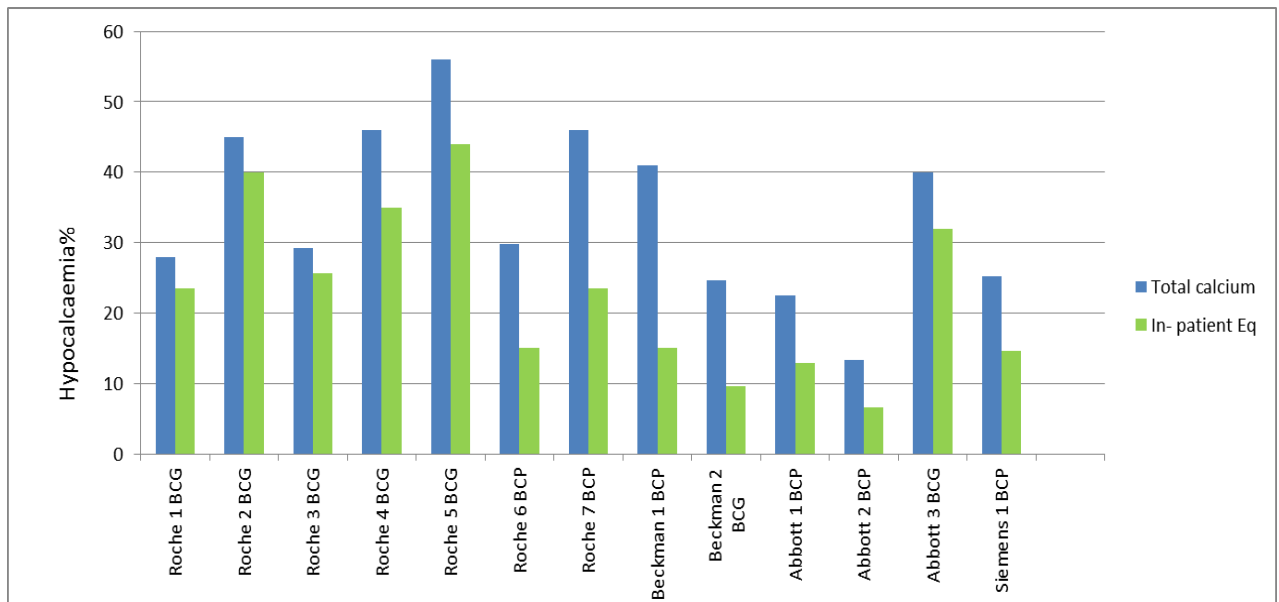


Figure 3.1: shows the impact of various analytical platforms and albumin methods on the prevalence of hypocalcaemia in hospitalised patients

In comparison to total calcium, the percentage reduction of hypocalcaemia post the adjustment of total calcium to albumin using equations derived for different albumin methods is presented in table 3.5. Table 3.5 shows that the adjusting power of equations derived from BCG methods, with the exception of Beckman platform, is weaker than equations derived from BCP methods. Equations derived from Roche, Abbott BCG albumin method adjusted on average 19% of total calcium (range 8.1-21.8%). Whilst equations derived using BCP albumin methods adjusted total calcium and lowered hypocalcaemia by an average of 50%, irrespective of the analytical platform.

	Albumin method	% of Adjusted calcium
Roche 1	BCG	15.9
Roche 2	BCG	8.1
Roche 3	BCG	15.1
Roche 4	BCG	11.9
Roche 5	BCG	21.8
Roche 6	BCP	49.5
Roche 7	BCP	47.8
Beckman 1	BCP	63.7
Beckman 2	BCG	61.2
Abbott 1	BCP	43
Abbott 2	BCP	50.3
Abbott 3	BCG	19.4
Siemens 1	BCP	41.3

Table 3.5: gives calcium equation adjusting power, measured as a reduction in the percentage of hypocalcaemia in comparison to total calcium.

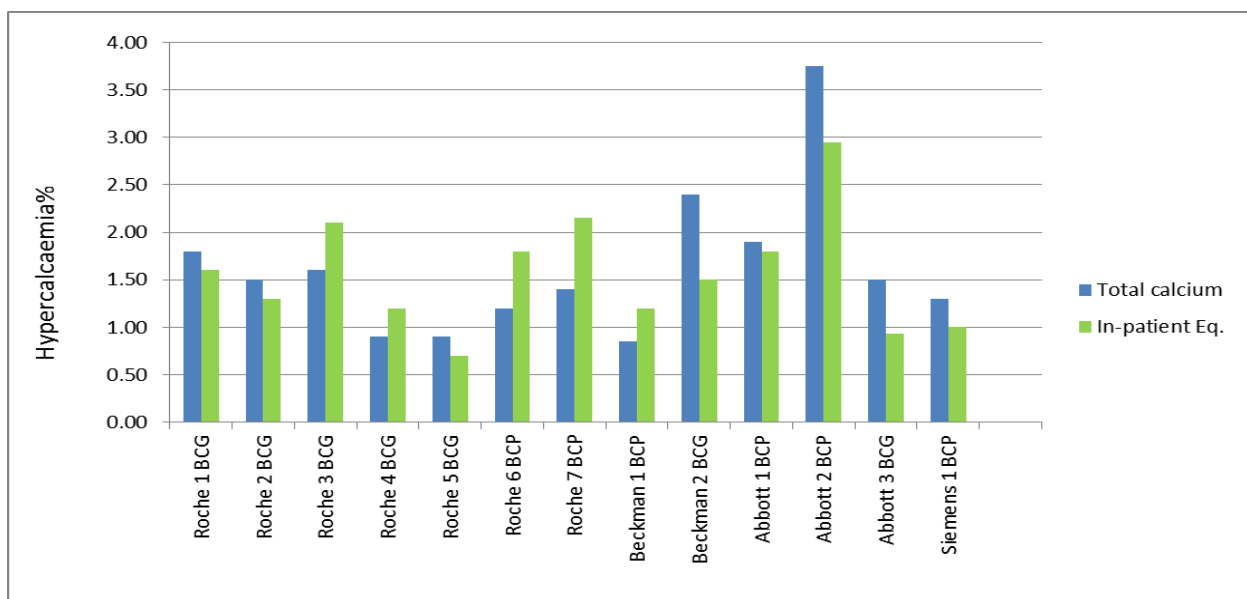


Figure 3.2: shows the impact of various analytical platforms and albumin methods on the prevalence of hypercalcaemia (%) in hospitalised patients from 14 participating laboratories.

The prevalence of hypercalcaemia among the participating hospitals populations appeared to be less variable than hypocalcaemia. The in-patient equations, irrespective of analytical platform or albumin method used yielded average prevalence of 1.3% (range from 0.9-2.9%).

3.3.3 The impact of calcium equations on the prevalence of hypo/hypercalcaemia in community setting

The application of adjusted calcium equation to total calcium is expected to reduce the prevalence of hypocalcaemia. In the primary care population, however, the application of in-patient equations overestimated hypocalcaemia with the exception of Siemens Advia analytical platform and a single user of Abbott Architect (figure 3.3). According to total calcium, the prevalence of hypocalcaemia in the community populations ranges from 2-9.6% across all participated laboratories. All equations, with the exception of 2 laboratories (Siemens1 BCP & Abbott 2 BCP) yielded an increase in the prevalence of hypocalcaemia when total calcium from primary care was adjusted to albumin using in-patient equations (figure 3.3).

The performance of various equations also varied significantly between participating laboratories; therefore, hypocalcaemia prevalence ranged from 1.8% to 53%. This large variation is albumin method dependent. For instance, the prevalence of hypocalcaemia for BCG method users, irrespective of analytical platform, ranges from (5.4 - 53%), while Roche Cobas BCG users alone had a prevalence range from 22% to 53%. BCP method users yielded a lower prevalence which ranged from 1.3-9.0% across all analytical platforms. However, the application of a community specific equation to primary care data showed greatly reduced hypocalcaemia percentage with an average of 1.7% and a range of (0.8%-3.7%). The community equations also greatly reduced hypocalcaemia prevalence variation across all participating laboratories.

In-patient equations underestimated hypercalcaemia in the community patients in comparison to the community equations (figure 3.4).

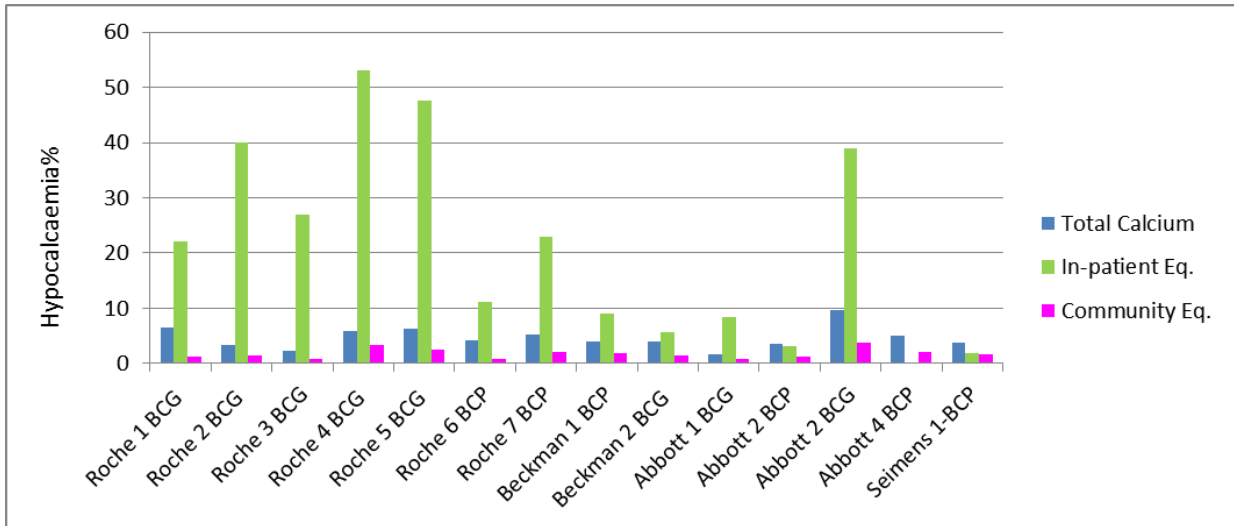


Figure 3.3: Shows a comparison of the impact of in-patient equation and community equation on the prevalence of hypocalcaemia in the primary care population.

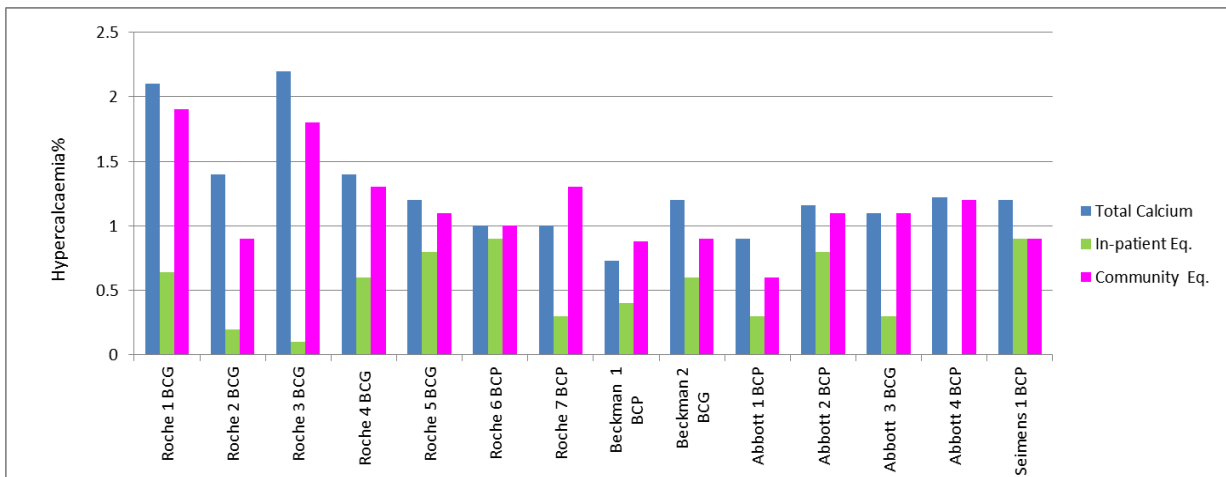


Figure 3.4: Shows a comparison of in-patient equations and community equations on the prevalence of hypercalcaemia in the primary care population.

3.4 Discussion

It is a common practice that the in-patients derived adjusted equation is applied to ambulant patients from outpatient clinics and the primary care setting. In the previous chapter, a community specific equation for Roche Cobas, albumin BCG method was derived and validated for a single laboratory. In the current study we attempted to derive community specific equations for the other analytical platforms and methods using retrospective data from various laboratories in the UK. The impact of in-patient and community equations on

the prevalence of hypo/hypercalcaemia for all participating laboratories was compared. The performance of the newly derived community equations to the Roche community adjusted equation that was previously validated with ionised calcium was also compared.

Calcium disorders are frequently encountered in primary care as well as in secondary care, however, limited data are available on the epidemiology of hypo/hypercalcaemia in these two settings. Common causes of hypocalcaemia in hospitalised patients are many and can include hypomagnesemia, vitamin D insufficiency, renal failure, parathyroid loss and chelation of calcium such as in pancreatitis, rhabdomyolysis, tumour lysis or blood transfusion. Hypocalcaemia can be associated with many drugs that inhibit bone resorption eg. cinacalcet, calcitonin, denosumab bisphosphonates, or chemotherapy. Hypocalcaemia can also be precipitated by drugs that exert effect on factors (vitamin D or magnesium) included in calcium metabolic pathway eg. antiepileptics, aminoglycosides, diuretics, and proton pump inhibitors. Hypocalcaemia of acute severe illness is a well-documented common cause of hypocalcaemia in hospital (Hannan and Thakker, 2013). Prevalence of hypocalcaemia was reported as 3%, 18% and 27.7% in all hospitalised patients and up to 85% in intensive care patients (Aishah, 1995) (Catalano et al., 2018) and (Hästbacka and Pettilä, 2003).

In this study, the prevalence of hypocalcaemia in hospitalised patients showed a bimodal pattern based on the albumin method used. Prevalence of hypocalcaemia among BCG users varied from 23.5-44%, while BCP method users showed a lower prevalence of 4.6-15%. This suggests this prevalence variation could be attributed to variation in albumin concentrations measured by these assays. Indeed, it was found that mean albumin of these assays was consistently higher for BCG methods irrespective of platform (table 3.3). Systematic differences between albumin methods (BCG versus BCP) has long been recognised (Wells and Addison, 1985) as has the fact that the BCG method provides higher albumin results because it is subject to non-specific interference by immunoglobulins

(Webster et al., 1974). This difference was confirmed even in a newer version of these assays (Clase et al., 2000). Clase and colleagues (2000) compared the performance of BCG and BCP methods and demonstrated method differences, with BCG significantly higher than BCP in renal and non-renal patients. It has been reported that a newer version of BCP methods agrees more with the reference method and as such is increasingly now being favoured over BCG methods (Koerbin et al., 2019). BCP method can also be criticised as it underestimates albumin in haemodialysis but not in renal failure patients (Beyer et al., 1994). Another study made this point clearer when it reported that the difference in BCG and BCP methods accounted for significant differences in the classification of adjusted calcium results in renal failure patients on haemodialysis (Labriola et al., 2009). Labriola and colleagues assessed the impact of albumin assays on the achievement of K/DOQI target for calcium; in agreement with the results reported herein, they established that a larger number of hypocalcaemia and fewer patients with hypercalcaemia were classified by BCG compared with BCP. In conclusion, despite the reports of enhancements of the BCP albumin method, the current study confirms that different albumin values are still yielded from different dye-binding albumin assays and the choice of BCG or BCP continues to have a major impact on albumin adjusted calcium equation performance and thus impacts on calcium classification.

In the in-patients setting a large variation in hypocalcaemia prevalence (4.6-44%) was observed. All participants are NHS laboratories from acute hospitals in the UK and the same protocol from data collection to equation derivation was applied. Therefore, this large difference is unlikely to be due to variation in equation derivation. In-hospitalised patients, patient mix and variation in demographics are still a possible cause, as variation even within the same method/analytical platform users was reported. There is evidence that the prevalence in calcium status changes even over time in the same hospital (Catalano et al., 2018).

In primary care, calcium disorders are frequently encountered too. Hypocalcaemia from general practice is most commonly a consequence of vitamin D insufficiency, which is

dependent upon population demographics, may have a prevalence of 50% (Horlilick, 2007). Few studies have reported the prevalence of hypocalcaemia in ambulant patients and even within these studies, the prevalence varied significantly based on the population demographics. For example, a study that assessed the prevalence of hypocalcaemia in patients attending various clinics in a Nigerian university hospital, reported a prevalence of 11.53% (Ogunkolo et al., 2006), whereas hypocalcaemia in children attending outpatient clinics in Yemen was reported to be 58% (Bin Mohanna et al., 2005). Another study reported a disease specific hypocalcaemia prevalence of 29% in patients receiving anticonvulsant therapy (Schmitt et al., 1984). In a healthy population however, Philipson et al (1978) reported a much lower prevalence of 0.6%. The reports above suggest that the prevalence of hypocalcaemia in ambulant patients varies significantly due to population demographics and cause of hypocalcaemia. In agreement with the above studies, our data showed a high prevalence of hypocalcaemia and large variation between participating laboratories. Unlike other studies, however, the variation in hypocalcaemia prevalence cannot be explained by population differences alone, firstly because the participating laboratories are all UK based with insignificant differences in demographics. Secondly, because hypocalcaemia prevalence in primary care populations based on total calcium was much lower (1.3-9%) than that obtained from adjusted calcium. Almost all BCG and BCP (with the exception of two BCP users) yielded equations which significantly overestimated the prevalence of hypocalcaemia above that of total calcium. These results suggest that the main reason for the increase in the prevalence and the large variation in hypocalcaemia was the application of the conventional Payne's in-patient equations to primary care populations. The variation in prevalence was also similar to that seen in hospitalised patients and found to be platform independent but albumin method dependent. Like in hospitalised patients our data also showed bimodal distribution of hypocalcaemia. Whilst this finding explains the high percentage of hypocalcaemia in the community, it contradicts the principle of the calcium adjusted equation. In the absence of ionised calcium it is impossible to be certain what true prevalence of hypo-and hypercalcaemia is. It is worth mentioning that this finding

was identical to that seen in the previous study (chapter 2), as the use of an in-patient equation overestimated hypocalcaemia in both healthy volunteers and the primary care patients. Indeed, the application of community specific equations to the primary care population significantly reduced the magnitude and variation in the prevalence of hypocalcaemia from 1.3 - 53% to 0.8 - 3.7%. This suggests that community specific equations reduced the prevalence and the magnitude of variation between laboratories and therefore there is potential to harmonise reporting of adjusted calcium in the primary care setting. This finding supports the hypothesis, in which the application of a population specific equation enhances the performance of an adjusted calcium equation. It also implies that equations from different analytical platforms behave in the same way as the Roche Cobas/BCG method validated equation.

The reason behind the increased prevalence of hypocalcaemia in the primary care population upon the application of a conventional in-patient equation is attributed to two facts, both of which relate to the function of the adjusting equation. The regression coefficient of albumin is used to calculate the expected change in calcium for each gram change in albumin concentration away from the baseline albumin in the equation. For example;

Adjusted calcium = Total calcium - 0.02 (Albumin - 40) equation has albumin baseline of 40. If the patient's albumin is lower than 40 g/L, adjusting total calcium to albumin would result in an increase in the adjusted calcium concentration. However, if the patient's albumin is higher than 40 g/L, we would see a lower adjusted calcium concentration. In this work, with no exceptions, it was found that mean albumin for primary care was higher than the baseline albumin in in-patient equations (table 3.3 and table 3.4); hence the significant increase of hypocalcaemia with the use of the conventional in-patient equation in the primary care setting.

In agreement with the previous study (chapter 2), it was found that calcium and albumin means are higher for ambulant patients than for hospitalised patients, which implies that these are different populations and would benefit from derivation of an equation for each population.

Other than adjusted calcium equations and demographics, the variation in prevalence of hypocalcaemia can also be attributed to the use of various reference intervals, different platforms and methods (WEQAS, 2012), or/and analytical variation (Walker and Payne, 1979) (Petersen and Klee, 2014). In this study, the variation that stems from derivation of adjusted calcium equations was removed by the use of a local standardised protocol. But variation that stems from the reference interval or analytical performance has not been evaluated here. Nevertheless, the observed variation in the prevalence of hypocalcaemia is rather large to be explained by the reference interval alone. The majority of BCG method users in this study were Roche Cobas users. A BCG method from Abbott Architect, but not from Beckman Olympus, showed a similar performance to BCG from the Roche Cobas platform. One of the limitations of this study was that the number of other non-Roche BCG users is very small (2 other users); therefore a conclusion cannot be reached about the BCG performance using other analytical platforms.

In conclusion, this study confirms the finding from chapter 2 and shows that the impact of the application of population specific equations to primary care patients followed the same pattern that was seen for the ionised calcium validated Roche Cobas community equation. The community specific equations reduced both the percentage and the magnitude of variation of hypocalcaemia between different laboratories. The community equation appears to harmonise calcium reporting from different laboratories using different analytical platforms.

Chapter 4: Prospective study of healthy individuals: calcium and albumin reference intervals.

4.1 Background

Disturbances in calcium homeostasis are common in primary and secondary care settings but the prevalence of hypo- and hypercalcaemia in a clinical setting varies widely in the literature (Fong and Khan, 2012) (Minisola et al., 2015). Amongst many other factors, the reference interval used for calcium status classification is an important factor affecting the prevalence of hypo/hypercalcaemia. There is a growing assumption among physicians and patients that test results from different laboratories are equivalent and hence can be interpreted by the same evidence based reference interval. This concept was behind several professional efforts to harmonise reference intervals at least for assays with an established standardised calibration system. The UK Pathology Harmony working group which was formed under the umbrella of the Association for Clinical Biochemistry and Laboratory Medicine worked towards harmonisation of reference ranges for a number of biochemical and haematological tests, including calcium and albumin (Berg, 2014). The Harmony initiative used a mixture of pragmatic and scientific approaches to dealing with existing variations in reference intervals. They recommended reference ranges of 2.2-2.6 mmol/L and 35-50 g/L for calcium and albumin respectively, across all commercially available methods.

According to the Directive on *in Vitro* Diagnostic Medical Devices of the EU, manufacturers have a responsibility to provide medical laboratories with reference intervals specific for their methods and analytical platforms. The International Organisation for Standardisation ISO 15189 mandated that medical laboratories are responsible for the periodic re-evaluation of their reference intervals (ISO, 2012). Alternatively, medical laboratories may establish their

own reference intervals as recommended by the International Federation of Clinical Chemistry (IFCC) (Solberg, 1991).

The IFCC has renewed its interest in the reference interval topic and is currently coordinating worldwide multi-country reference interval studies using a standardised protocol which covers all aspects of reference interval derivation via the Committee for Reference Interval and Decision Limits (C-RIDL) (Ozarda et al., 2013). This study aims to derive reference intervals for four analytical platforms that are commonly used in the UK medical laboratories. The local reference interval data for albumin and calcium in the current study was based on the data collected in West Yorkshire using the C-RIDL protocol. In the previous section, the Pathology Harmony reference range was used to assess the diagnostic accuracy of the newly derived calcium equations. Therefore the secondary aim is to assess the impact of the newly established reference intervals on the diagnostic accuracy of calcium classification in comparison with the Pathology Harmony reference intervals.

4.2 Materials and methods

4.2.1 Reference interval studies

This is a collaborative project which was undertaken under the auspices of the Reference Interval in Laboratory Medicine in Yorkshire (A.Luvai FRCPath project, 2015). This study was approved by the National Research Ethics Committee (Ref 11/H/1302/5).

4.2.2 Reference population

A prospective reference population of healthy individuals was recruited according to the IFCC C-RIDL Criteria (Ozarda et al., 2013). Subjects were recruited using advertisements at three hospitals in the region; the Leeds Teaching University Hospitals, Hull Royal Infirmary and Harrogate Hospital. Exclusion criteria for reference individuals were:

- 1) Regular drug therapy for chronic diseases (diabetes, hypertension, hyperlipidemia, gout, depression),
- 2) Within 2 weeks after recovery from any acute diseases requiring hospitalisation or surgery,
- 3) Pregnant, lactating or within 1 year after delivery.

Samples were excluded after analysis if there were missing or incomplete data, insufficient sample volume.

4.2.3 Informed consent and health questionnaire

Poster advertisements were displayed in the clinical and non-clinical areas and electronic invitations were sent to staff within participating hospitals. Written and verbal information was presented to the participants explaining the nature of the reference interval study and any health hazard involved in taking part. It was stated that participants could withdraw from the study at any time with no obligation to give the reason for pulling out.

The participants personally signed and dated the approved version of the informed consent form before blood was collected. Participants were asked to complete a short health questionnaire about their general health. The health questionnaire included demographic data such as date of birth, gender, last menstrual period, race and habitual alcohol and tobacco consumption. Details of medical history of disease and prescribed and over the counter medication were recorded.

4.2.4 Blood collection

Blood was collected by a single phlebotomist. A tourniquet was avoided, however, when necessary a tourniquet was applied and released within less than 1 minute to minimise the effects of venous stasis. To avoid variation due to postural influence, the participants were requested to sit for 10 minutes prior to blood collection. Repeated fist pumping was not allowed. Each tube was inverted at least 5 times.

4.2.5 Analysis

Blood tubes were allowed to clot at room temperature for 30 minutes and within 6 hours of venepuncture samples were centrifuged at 3000g for 10 minutes at room temperature. Serum samples were aliquoted into storage tubes. The storage tubes were well sealed and stored at -80°C. On the day of analysis one set of samples was thawed slowly at room temperature for one hour. Homogenisation was achieved by leaving the tubes on a rotator for 10 minutes and analysis undertaken within 4 hours from the start of thawing.

Samples were analysed on the four most widely used analytical platforms, for total calcium and albumin. Analysis was undertaken on a Roche Cobas (Roche Diagnostics Ltd, West Sussex, UK), (Harrogate), Beckman Unicel Dxl (Beckman Coulter Ltd, High Wycombe, UK) (Hull), Abbott Architect i2000SR Plus (Abbott Diagnostics, Kent, UK) (Doncaster) and Siemens Advia XP (Siemens Healthcare Diagnostics, Surry, UK) (Leeds). This included two calcium methods 1) - NM-BAPTA (Roche), 2) - ArsenazoIII (Beckman, Siemens Advia, and Abbott Architect) and two albumin methods 1) BCP method (Beckman and Abbott Architect), 2) BCG (Roche Cobas and Siemens Advia). All four laboratories which participated in this study are accredited laboratories at the time of undertaking this study. All analysers were maintained as per the manufacturer's standards, and manufacturer's reagents were used as

per their protocols. External quality was assured through UKNEQAS and quality markers were acceptable throughout the duration of the study.

4.2.6 Reference range statistical analysis

Statistical analysis was performed using Analyse-it Statistical Package (Microsoft Excel 2010) (version 2.10). Dixon test was used to detect outliers. Reference intervals were calculated using the Quantile function method. After establishing a normal distribution, reference interval was based on taking the central 95% range in accordance with the IFFC recommendations (CLSI, 2008). Distributions were assessed for normality using Anderson Darling test. The test rejects the hypothesis of normality when the p-value is ≤ 0.05 . Normally distributed values were suitable for parametric reference interval derivation whereas a log transformation method was applied for non-normally distributed values.

4.3 Results

The characteristics of the studied reference interval population are presented in table 4.1.

Our data shows predominance of white Caucasian, female and young aged subjects.

Gender	186 (62%) female, 113 (37.7%) male
Age	Median 41 year old (18-65). 173 (58%) <50 years old and 44 (23%) post-menopausal.
Exercise	208 (69%) subjects engaged in moderate intensity regular exercise (at least twice weekly)
Ethnicity*	284 (82%) Caucasian
Smoking	27 (11%)

Table 4.1: Reference interval population characteristics

*Non-Caucasian ethnic groups mainly composed of black (African and Caribbean), Asian (Indian, Pakistani and Bangladeshi), Arabs and mixed race participants.

Platform	Albumin Method	Calcium Method	<i>n</i> =
Abbott	BCP	Arsenazo III	106*
Beckman	BCP	Arsenazo III	298
Roche Cobas	BCG	NM-BAPTA	278*
Siemens Advia XP	BCG	Arsenazo III	299

Table 4.2: States the analytical platform, method for calcium and albumin on each platform and the total number of healthy individuals included in the derivation of reference interval values. Due to the exclusion of insufficient volume samples (e.g. samples leaked in transit), the total number of analysed samples on Abbott and Roche analytical platform were < 299.

4.3.1 Albumin and calcium reference intervals

The 2.5 and 97.5 percentiles for calcium and albumin are presented in Tables 4.3 and 4.4. This study showed a close agreement between the calcium derived reference intervals and the nationally recommended reference intervals described by the Pathology Harmony initiative (2.2-2.6 mmol/L), with the exception of Siemens Advia XP, whereas local reference interval is approximately 0.1 mmol/L (<5%) lower than both reference intervals limits. The Siemens Reference interval obtained from this study is lower than the manufacturer's quoted one (table 4.5). The Pathology Harmony recommended albumin reference interval is 35-50 g/L. The derived reference intervals are in a good agreement with the Pathology Harmony reference interval with a difference of a maximum of 1-2 g/L or < 5% at the upper reference limit (URL). However, the lower reference limit (LRL) difference varies by 3-6 g/L or (9-17%). The Roche BCG method reference interval is higher than other methods' reference intervals and significantly higher than the Pathology Harmony reference interval at both lower and upper limits. BCP reference intervals (for Beckman DXI and Abbott Architect) are lower than BCG reference intervals (Siemens Advia and Roche Cobas) at both the upper and lower limits. There is a close agreement between calcium reference intervals derived

from this study and the manufacturers' reported ones, with the exception of Siemens, with a marginal difference < 5%.

Platform	95% Interval				Normality
	LRL	90% CI	URL	90%CI	Anderson Darling
Abbott Architect	37.8	37.1- 38.6	48.5	47.7 - 49.2	0.95 p = 0.051
Beckman DXI	37.9	37.49-38.32	49.2	48.69- 49.77	1.9 p< 0.0001
Roche Cobas	40.4	36.50-41.30	51.7	51.30-53.0	1.26 p = 0.0027
Siemens Advia XP	40.0	39.7-40.5	50.0	49.6-50.6	2.95 p <0.0001

Table 4.3: Reference intervals for albumin on various analytical platforms; Albumin was suitable for parametric analysis when data were normally distributed, whereas a log transformation method was applied for non-normally distributed values

Platform	95% Interval				Normality
	LRL	90% CI	URL	90%CI	Anderson Darling
Abbott Architect	2.16	2.14-2.19	2.56	2.53-2.58	0.52 p=0.178
Beckman DXI	2.20	2.19-2.22	2.58	2.57-2.60	1.18 p=0.0045
Roche Cobas	2.20	2.20-2.30	2.60	2.60 - 2.60	0.65 p=0.0587
Siemens Advia XP	2.13	2.07-2.16	2.51	2.50 - 2.54	1.05 p=0.092

Table 4.4: Reference intervals for total calcium on various analytical platforms; Calcium was suitable for parametric analysis when data were normally distributed, whereas a log transformation method was applied for non-normally distributed data.

Platform	Calcium mmol/L	Reference material
Abbott Architect	2.1-2.55	NIST SRM 956
Beckman DXI	2.15-2.57	NIST SRM 909b
Roche Cobas	2.2-2.6	NIST SRM 956
Siemens Advia XP	2.18-2.6	NIST SRM 915

Platform	Albumin g/L	Reference material
Abbott Architect	35-50	ERM-DA470/IFCC
Beckman DXI	34-48	CRM470/IFCC
Roche Cobas	35-50	BCR470/CRM470
Siemens Advia XP	32-48	ERM-DA470/IFCC

Table 4.5: Reference interval for albumin and calcium as provided by the manufacturers and traceability of methods used to calculate the reference interval.

4.4 Discussion

Reference intervals are an important tool for improving the diagnostic accuracy of biochemical tests and are a critical factor for interpretation of test results. In the present study, method specific reference intervals for calcium and albumin were derived using a local healthy population with the view of harmonisation of reference intervals for calcium and albumin in mind.

The observed calcium reference intervals for Abbott Architect, Beckman DXI and Roche were all in good concordance with the Pathology Harmony recommended reference interval and with those reported by the manufacturers with the exception of Siemens Advia. Siemens reference interval for calcium was lower than the Pathology Harmony reference interval for the lower and upper limits. All participating laboratories performed within the acceptable limits in internal quality assessments and in the UK National External Quality Assessment scheme for calcium and albumin. As for Siemens Advia, the laboratory has never exceeded the allowable bias and consistently performed at bias < 2% during the period of study. The analytical producer in each laboratory followed the manufacturer's recommendations and the pre-analytical procedures were applied consistently for all

samples, therefore, the difference in Siemens reference interval is likely to be due to reagent, calibration differences or the use of non-commutable calibrator (Jansen et al., 2014) (Perich et al., 2014). Indeed, recent data from the Welsh External Quality Assurance scheme (WEQAS) showed evidence of calibration error in Siemens Advia XP calcium assay (chapter 6).

The LRL for albumin reference intervals derived in this study differed widely from the Pathology Harmony and the LRL for BCG albumin reference intervals was higher than that for BCP methods. Accumulative evidence demonstrated that BCG methods have high bias when compared to BCP methods and in fact, the BCG methods hardly met the minimum analytical performance limits based on biological variations (Koerbin et al., 2019). Among many harmonisation initiatives, the Australian Association of Clinical Biochemistry (AACB) developed an evidence-based approach for harmonising reference intervals for tests with a sound, standardised calibration system. The AACB proposed a harmonised calcium reference interval wider than that of the UK Pathology Harmony (table 4.6). Nevertheless, AACB have not proposed one for albumin because of the wide variation between results produced by BCG and BCP methods. Harmonisation of reference intervals initiatives aim to improve reporting to patients and physicians. In the presence of a strong evidence of bias, method specific reference intervals became a safer practice. In the present study, albumin assays total error range obtained from WEQAS was larger than even the minimal performance limits (chapter 6). In agreement with the AACB, our finding also supports a method specific reference interval case (Koerbin et al., 2019).

The Nordic Reference Interval Project (NORIP), however, also employed a scientifically valid approach that ensured harmonisation of methods through the use of commutable calibrator materials before the measurement of samples from healthy volunteers. The reported reference interval for calcium in serum/plasma and for albumin was significantly different

from the other initiatives (Rustad et al., 2004) and lower than the calcium and albumin reference intervals obtained from this study (table 4.6).

One can suggest that this difference could be explained by population differences. There is evidence that there are a number of biochemical tests affected by population demographics (Ozarda et al., 2014). The characteristics of the reference population from this study which consisted of a mainly white Caucasian population are similar to the NORIP study population. The NORIP study circulated reference samples to participating laboratories to correct for calibration difference (Rustad et al., 2004). Unlike the NORIP study, the design of this study lacked the measurement of reference material across the studied analytical platforms. Therefore, differences in calcium and albumin reference intervals are more likely to be attributed to calibration differences rather than population differences. The most recent multicentre study that aimed to standardise reference intervals looked at the impact of population differences across Japan on reference intervals for up to 25 biochemical tests including calcium and albumin. This study recommended a single reference range for calcium and albumin regardless of age or gender differences (Yamamoto et al., 2013). In contrast, Carlsson et al (2010) reported that the calcium and albumin reference interval in a population of 70 year olds without CVD was higher at the upper limit for calcium and lower for albumin than this study. It was also observed that differences between genders exist, but the 90% *CI* for the reference value was not separated, suggesting that gender differences are so small, that the same reference interval could be used for both genders. It is worth mentioning that Carlsson and colleagues ruled out that the higher upper limit for calcium could be due to unknown cases of hyperparathyroidism in this age group.

Calcium is a standardised method; the difference between various reported reference intervals including this study did not exceed 5%. Some of the studies e.g. the NORIP study reported a reference interval similar to the one obtained for the Siemens platform in the present study. The difference, however, is that the NORIP study multiplied by a factor

obtained from reference samples to eliminate routine method bias, whilst in the present study this measure was lacking. However, a recent study reported that calibrators traceable to NIST SRM 915 (in this study used by Siemens) had lower calcium values compared to NIST SRM 909 or 956 (Perich et al., 2014). Therefore, from a harmonisation point of view, Siemens users should not be using the Pathology Harmony until the calibration issue has been resolved. Our finding is in keeping with the Hughes et al (2016) study in which harmonisation for calcium reference interval was recommended for all calcium methods with the exception of Siemens Dimension RxL. This case proves the need for laboratories to validate manufacturers' reference intervals as recommended by ISO 15189 (ISO, 2012).

On the other hand, reports on albumin methods standardisation reflect a problematic standardisation process that is unfit for clinical need (Infusino et al., 2011) (Infusino and Panteghini, 2013) (Koerbin et al., 2014). Therefore the likely reason for the different reference intervals obtained from different studies and this study is related to the standardisation of albumin method.

This current study had a number of limitations; age partition for albumin was not possible because the studied population consisted of healthy healthcare professionals and relatively young subjects with a median age of 41 years old. Only a few in the present study were older than 65 year old of age. Due to the small size of the population and the relatively low percentage of male participants, it was not possible to explore gender reference interval partitioning. Whilst this study covers almost all UK-based commercially available calcium methods, this statement is not true for albumin. Each manufacturer provides both BCP and BCG methods. This study measured reference interval samples on only 4 out of 8 possible combinations of analytical platform/methods; hence any conclusion on albumin reference interval harmonisation is incomplete without the inclusion of all available albumin assays in use. Despite the utilisation of a strict protocol for analytical quality control, the design of this study lacked the measurement of reference material across the studied analytical platforms;

therefore a harmonisation conclusion from this study is of limited value when there is an obvious difference in reference intervals.

In conclusion whilst calcium reference intervals that were derived by this study in general agree with other studies, the harmonisation conclusion by the Pathology Harmony can be acceptable for three out of four analytical platforms. The same is not true for albumin. Differences between reference intervals for albumin are significant and suggesting harmonisation may not be appropriate for albumin reference intervals, however, further studies are required before arriving at a firm conclusion.

	Calcium (mmol/L)	Albumin (g/L)
UK Pathology Harmony (Berg et al., 2014)	2.20-2.60	35-50
Australian Adult RI (Koerbin et al., 2015)	2.10-2.60	NA
Japan RI Harmonisation project (Yamamoto et al., 2012)	2.20-2.50	42-52
Nordic RI project (NORIP) (Rustad et al., 2004)	2.17-2.51	34.5-47.9
NUMBER RI Study (Netherlands) (Den Elzen et al., 2019)	2.18-2.55	BCG: 38-49 BCP : 33-44
Turkish Nationwide RI Study (Ozarda et al., 2014)	2.12-2.47	BCG: 40-49
Australian Study, Abbott Architect (Hughes et al., 2016)	2.14-2.67	NA
RI in 70 year-old (Carlsson et al., 2010)	Male: 2.17-2.66 Female: 2.18-2.70	Male: 35.6-46.9 Female: 35.8-46.3

Table 4.6: Published calcium and albumin reference intervals. RI= Reference Interval.

Chapter 5: Albumin reference interval using a data-mining approach

5.1 Background

There is emerging evidence shows that albumin concentration in serum changes with age and gender (Kallner et al., 2000) (Weaving et al., 2015). Albumin is an important clinical marker for many diseases such as monitoring liver disease, renal disease, malnutrition and acutely ill patients. Its use in the staging of myeloma patients and as a prognostic marker is well established. Calcium is a largely albumin-bound compound. Knowledge about albumin tests and assay performance would shed light on the clinical application of albumin, particularly in the context of a calcium equation. For instance, population albumin mean is a factor that determines the regression of albumin on calcium outcome, thus equation performance. In fact, the practice of using equations that adjust calcium to a single albumin concentration, regardless of age or gender was recently criticised (Weaving et al., 2015).

A reference interval represents a distribution of test values for a number of predefined disease free individuals. Derivation of reference intervals is often a laborious and costly task. Reference interval derivation involves collecting samples from carefully selected individuals and the use of strict protocols defining the pre-analytical, analytical and statistical methods used. Often this approach suffers from a lack of inclusion of extreme age groups such as elderly and paediatrics. Recently, the International Federation of Clinical Biochemistry (IFCC) evaluated a less laborious approach for reference interval establishment (Jones et al., 2019). This alternative approach, also known as an indirect approach, makes use of routine retrospective laboratory data for the derivation of reference intervals. In this approach, the reference interval is determined statistically based on identifying a reference population in the midst of the data set. In contrast to the traditional approach (also called the direct approach), this method does not require an assessment of

individual participating subjects, does not require ethical approval and is less expensive than the direct approach. Nevertheless, exclusion and inclusion criteria also need to be clearly defined to filter out extreme results that may influence the reference interval in question (Grossi et al., 2005).

In the previous section (chapter 4) albumin reference intervals were derived using data collected from healthy participants aged 18-65 years old. In routine clinical practice, albumin is measured by two methods (BCG and BCP) on several analytical platforms. The previous study covered only four reference intervals. In the present study, the aim is to use an indirect approach to cover wider combinations of methods and analytical platforms that were not covered in the previous chapter.

5.2 Materials and methods

Retrospective and anonymised biochemical data for albumin was collected from the four most commonly used analytical platforms in the UK. These are; Roche Cobas (Roche Diagnostics Ltd, West Sussex, UK), Siemens Advia (Siemens Healthcare Diagnostics, Surry, UK), Abbott Architect (Abbott Diagnostics, Kent, UK) and Beckman Olympus or DXI (Beckman Coulter Ltd, High Wycombe, UK). An invitation for participation was sent to the UK laboratories. Participant laboratories were asked to collect data from their laboratory information system (LIMS) according to a circulated protocol. The protocol specified collecting data from a primary care setting for a period of 2-3 months. Primary care extracted data included the following parameters age (>18 Y), gender, hospital number, albumin, ALP, ALT and eGFR. Laboratories were asked to provide the name of the analytical platform, albumin methods, reference ranges for the collected tests and analytical performance indices of the albumin method.

5.2.1 Statistical analysis

Statistical analysis was undertaken using Analyse-it Statistical Package (Microsoft Excel 2010) (version 2.10). Outliers were removed by iterative removal of results outside the interquartile range after log transformation as described by Inal et al (2010). Because of non-normalised distribution, the Box-Cox-log transformation method was used for reference interval derivation. Once the distribution was normalised, the reference interval was based on the central 95% range in accordance with IFCC recommendations (CLSI, 2008). The error chart was performed on the Graph Pad Prism statistical package.

5.3 Results

5.3.1 Excluded subjects

Data filtration was employed to exclude results from liver disease and from renal disease individuals; therefore, data were filtered to exclude those with abnormal liver enzymes and abnormal eGFR. Abnormal enzymes were defined by ALT and ALP > upper limit of reference interval and normal kidney function was defined biochemically by eGFR > 60 mL/min/1.73 m². Only patients with one set of results were included to reduce the number of diseased individuals, as usually only abnormal results are repeated.

5.3.2 Albumin reference intervals

The participating laboratories were grouped by analytical platform as described in table 3.1, chapter 3. No data for BCG albumin method from Siemens Advia XP were obtained. The mean and age distribution of the reference population is given in table 5.1. The 2.5 and 97.5 percentiles for the age group 18 - 70 years old are presented in table 5.2 and those for the > 70 years old age group are presented in table 5.3. Figure 5.1 presents all reference intervals in relation to the Pathology Harmony reference interval.

Reference intervals of BCG methods, irrespective of analytical platforms or age, are higher for both LRL and URL than those for BCP. Even with the same analytical platform and at

either side of the reference limits, a difference of 3-4 gram between BCG and BCP, with the exception of Roche BCG, was observed. The Roche BCG based reference interval was up to 6-7 g/L higher than Roche BCP based reference interval. In fact, the Roche BCG based reference interval is higher at both LRL and URL than all reference intervals from all analytical platforms. Reference intervals for the > 70 age group are lower than those for the younger population with a LRL and URL are almost lower by 1-2 g/L.

	Median	Mean	Range
Abbott BCG	63	60	18-101
Abbott BCP	58	57	18-103
Beckman BCG	67	65	18-99
Beckman BCP	61	59	18-100
Roche BCG	59	57	18-101
Roche BCP	52	52	18-98
Siemens BCP	51	51	18-103

Table 5.1: Age distribution (in years) for reference populations.

Platform	Albumin Method	LRL	95% CI		URL	95% CI		n
Abbott Architect	BCG	36.3	36.1	36.3	47.9	47.69	48.01	3726
Abbott Architect	BCP	33.7	33.6	33.7	44.1	44.07	44.23	12272
Beckman Olympus	BCG	37.4	37.3	37.4	47.9	47.77	47.96	7549
Beckman DXI	BCP	33.7	33.6	33.7	44.3	44.27	44.39	20741
Roche Cobas	BCG	40.3	40.2	40.4	51.9	51.79	51.99	9232
Roche Cobas	BCP	33.6	33.5	33.7	45.7	45.52	45.83	4215
Siemens Advia	BCP	33.7	33.5	33.7	45.5	45.42	45.65	6897

Table 5.2: Reference intervals for albumin methods on various analytical platforms for 18-70 years old cohort.

Platform	Albumin Method	LRL	95%CI		URL	95%CI		n
Abbott Architect	BCG	34.4	34.2	34.5	45.8	45.6	46.0	2546
Abbott Architect	BCP	30.1	30.0	30.2	43.0	42.9	43.2	5109
Beckman Olympus	BCG	35.4	35.3	35.5	46.1	46.0	46.3	3419
Beckman DXI	BCP	32.9	32.8	33.0	43.8	43.7	43.9	9023
Roche Cobas	BCG	38.0	37.9	38.1	50.3	50.1	50.4	3779
Roche Cobas	BCP	32.6	32.4	32.8	43.9	43.6	44.2	990
Siemens Advia	BCP	31.3	31.1	31.4	43.6	43.4	43.8	2257

Table 5.3: Reference intervals for albumin methods on various analytical platforms for >70 years old cohort.

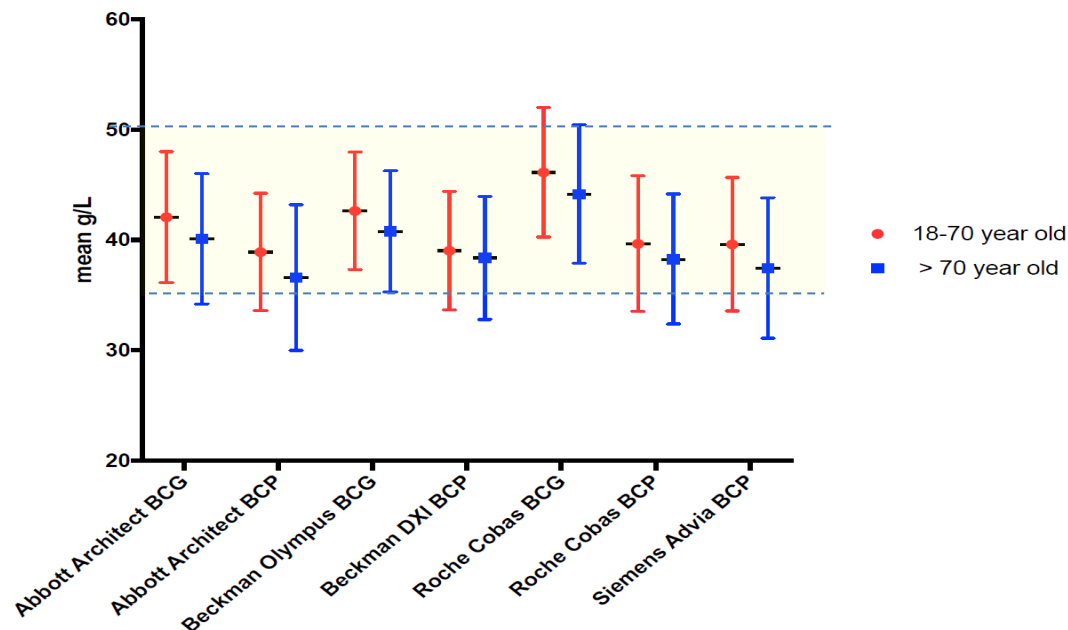


Figure 5.1: .Albumin reference range according to age and method compared to the UK Pathology Harmony recommended reference interval. The blue dashed line represents the Pathology Harmony reference interval.

In comparison to the Pathology Harmony reference interval, the younger population reference interval varied from the Pathology Harmony by up to 5.7 % at the LRL and up to 12% at the URL. In the older population, variation of up to 14% was observed at both LRL and URL. In general, the difference between age specific reference intervals and the Pathology Harmony reference interval, especially at the URL, was greater than the minimal acceptable analytical variation for albumin, which estimated to total error 6.1% (Westgard, 2014).

5.4 Discussion

The establishment of reference intervals is an important responsibility of laboratory medicine professionals. The recent promotion of the indirect approach made the reference interval

task less demanding (Jones et al., 2019). In the present study, the indirect approach has been used to establish method specific reference intervals for albumin.

This study found that the LRL in the younger population (18-70), for both albumin methods and irrespective of platforms (except Roche BCG LRL) was different from the Pathology Harmony by 1-2 grams. BCP based reference intervals were ≥ 1 g/L lower and BCG reference intervals were 1-2 g/L higher than the Pathology Harmony. The difference at the URL exceeds 4 g/L for both methods. The disparity becomes even larger (up to 5 g/L at the LRL and up to 7 g/L at URL) between reference intervals from >70 year age and the Pathology Harmony. Roche BCG based reference intervals for 18-70 and > 70 populations are not only higher than BCP based reference intervals, but also higher than all BCG based reference intervals from other analytical platforms participating in this study (table 5.2 and 5.3). In agreement with this finding, Coley-Grant et al (2016) reported that the albumin BCG method from Roche had a positive bias of 6 g/L compared with the Abbott BCP albumin method.

The difference between reference intervals obtained from BCG and BCP methods could be partly attributed to the lack of a direct traceability system transfer for routine albumin methods (Infusino and Panteghini., 2013), analytical variability between laboratories and method specificity (Infusino et al., 2011). Data from WEQAS showed that Roche BCG has a systematic bias positive to other analytical platforms (chapter 6). While this bias supports the findings of a higher BCG reference interval, it does not completely explain the size of the difference observed in this study. Two independent EQA schemes reported a BCG systematic bias of only 2 g/L or less (Jones et al., 2019) (chapter 6).

There is growing evidence that albumin concentrations decline with advanced age (Weaving et al., 2014) (Koerbin et al., 2019). In the present study, an inverse relationship between the reference interval limits and population age was observed. In support of previous studies,

derived reference intervals for those >70 years old were consistently lower than those for the 18-70 age group and the latter are also significantly lower than reference intervals obtained from a younger population in chapter 4. The mean age of the younger population in the current study was 51 to 67 years old, which was higher than the mean of population age in the direct reference interval study (chapter 4)(mean of 41.3 years). Weaving and colleagues (2016) presented a decline of nearly 1 g/L per a decade of age. This difference in the population age mean may add another layer of variation that contributes to the disparity between reference intervals from various analytical platforms.

Ryden and colleagues (2012) reported > 70 years old reference interval much higher than the one reported from this study (37.2 - 52.5 male and 38.2 - 51.1 for female), but also reported that their study employed a different analytical platform than those participating in this study, with reference standard material traceable to NIST SRM927c. This probably explains the observed difference. Another study that was performed on > 70 years old individuals reported albumin reference interval in agreement with the reference intervals obtained from this study (Carlsson et al., 2010).

Our data supports previous reports and shows through reference intervals a decline of albumin concentrations with age. These data challenge a common use of a calcium adjusted equation irrespective of age. Although Payne and Barth (1996) reported that differences in serum albumin by age were not significant in calculating serum adjusted calcium, this conclusion was contradictory to recent reports that showed albumin concentration differences due to age or clinical setting influenced calcium adjustment equations (Jassam et al., 2011) (Welsh and Jassam, 2012).

It is worth mentioning that our reference populations showed a small kurtosis and *P* values indicating a non-Gaussian distribution. This observation had led to age-partitioning of the data. While this partitioning slightly improved the Anderson Darling factor, this value

remained elevated suggesting further partition is needed. Data can be partitioned at a younger age, can be gender based partitioned or it is even possible to partition albumin reference population per a decade of age. We have not been able to further partition our data by a decade or by gender due to a small sample size number. Whilst the literature does not strictly prescribe the required reference sample number; however < 1000 and > 10000 data points are considered a small and a large population respectively (Jones et al., 2019). The size of our reference population fell mostly between these ranges. Therefore, a bigger data mining exercise is required to confirm the finding from this study and explore decade and gender specific albumin reference intervals and subsequently, age or gender specific calcium equations.

In conclusion, albumin reference intervals obtained from this study show large variation. This variation is probably attributed to the current traceability system, to analytical, age and probably gender variation. While reference intervals harmonisation is a desirable exercise, it is premature to embrace a common reference interval for albumin without further age and gender partitioning studies. The present study adds to the literature body that challenges the use of a common adjustment equation with single albumin concentration irrespective of population albumin mean.

Chapter 6: The impact of the analytical performance specifications of calcium and albumin on the adjusted calcium equation

6.1 Background

The generation of accurate and comparable biochemical results from different measurement procedures is a primary goal of Laboratory Medicine. Calcium and albumin measurements have wide clinical applications ranging from their use diagnostically, for monitoring of a wide array of diseases in addition to their use as prognostic markers in renal and myeloma diseases. Calcium and albumin are measured on a number of analytical platforms. Each analytical platform has its own analytical performance characteristics. These characteristics are determined at the design stage. Method specificity and precision are important markers of analytical performance as they directly impact on the diagnostic accuracy of biochemical tests. Several studies have attempted to define what constitutes a clinically acceptable analytical variation of biochemical data (Kallner et al., 1999). However, for assays with metrological traceability, achieving transferability of test results and harmonising reference intervals requires knowledge of analytical bias and allowable tolerance limit. For harmonising reference intervals the bias should be below the desirable limit which is expressed as $< 0.25\sqrt{(CVI^2 + CVG^2)}$, where CVI is within individual biological variation and CVG is between individual biological variation (Fraser and Pedersen, 1999). In this study the analytical performance of various routine methods for calcium and albumin measurement will be assessed to define the impact of the analytical bias of calcium and albumin on adjusted calcium equation performance and on reference intervals. In order to estimate bias, comparing results from various methods using a shared patient sample is essential. In fulfilment of laboratory regulations, laboratories are required to participate in an external quality assurance scheme (EQAS). National EQA schemes circulate samples (from a single pool) to all participating laboratories. Laboratories analyse these samples as patient samples and feedback results to EQAS. EQAS in turn compares results and issues

performance reports to the participated laboratories. In collaboration with the Welsh External Quality Assurance Scheme (WEQAS) anonymised national data for calcium and albumin was shared to enable bias estimation.

6.2 Material and methods

The data set was provided by WEQAS. These data were composed of six distributions during the period from December 2017 to April 2018. In the WEQAS scheme 24 samples are analysed for each general chemistry test in the course of a six month period, 4 samples per distribution. Four liquid human sera for albumin and calcium are circulated to the participating laboratories on a monthly basis. Sample are analysed and results are returned to WEQAS scheme for data analysis. A six months' worth of data was collected with the aim to cover a concentration range of clinical interest for both albumin and calcium. The data were initially grouped by analytical platforms/method.

This set of data was then analysed to identify the degree of bias in routine albumin and calcium methods in the UK. The analytical platforms of the manufacturers with analogous technology were grouped in a single category, eg. Abbott included all Architect models (Ci 6000, Ci8200, Ci 4000). Beckman analytical platforms included AU2700/AU5400/AU5800 and Beckman-Coulter (Olympus AU instruments). Roche analytical platforms included all Cobas instruments (Cobas 6000/8000) and a single group for Siemens Advia instruments. All calcium methods included in this study were colorimetric and all albumin methods included are turbidimetric. Biological variation desirable bias was calculated for total calcium, adjusted calcium and albumin using the following equation $0.25\sqrt{(CVI^2 + CVG^2)}$. Data for CVI and CVG were obtained from the European Federation of Laboratory Medicine (EFLM) website for biological variation data base <https://biologicalvariation.eu/> or Westgards data base for desirable biological variation (Westgard, 2013).

6.3 Results

Data for this phase were extracted from six WEQAS distributions during the period of December 2017 to April 2018. Each distribution was composed of results representing four samples for albumin and calcium measurements. Data were extracted for the main known analytical platforms and methods. Analytical platforms, calcium and albumin methods that were investigated in this study are presented in Tables 6.1 and 6.2 respectively.

Total calcium measurements from participating laboratories were compared to calcium reference values [defined by reference method]. Due to the unavailability of reference methods, serum albumin and adjusted calcium results were compared to all methods mean (AMM). AMM values for the BCG albumin method were derived from 108 laboratories. BCP albumin is available from the same manufacturers as BCG but was used by only 56 laboratories. Calcium AMM was derived from a total of 166 participating laboratories. The concentrations for calcium range from 1.2-2.9 mmol/L and the concentrations for albumin range from 25-49 g/L.

Analytical platform	No. of participated labs in WEQAS	Calcium Method
Abbott Architect	22	Arsenazo II
Beckman AU2700/AU5400/AU5800	19	Arsenazo III
Roche Cobas	18	O-Cresolphthalein
Roche Cobas	81	NM-BAPTA
Siemens Advia XP	4	O-Cresolphthalein
Siemens Advia XP	22	Arsenazo III

Table 6.1: Main analytical platforms and methods for calcium measurements and number of users in each method group.

Analytical Platform	BCG Method	BCP Method
	No. of participating labs	
Abbott Architect	3	21
Beckman AU2700/AU5400/AU5800	9	13
Roche Cobas	80	17
Siemens Advia XP	16	5

Table 6.2: Main analytical platforms for albumin analysis and number of users in each albumin method group.

Bias for albumin and adjusted calcium was calculated as the difference of $((X - AMM)/AMM) \times 100$, where X is either albumin or adjusted calcium and AMM is all methods mean. Bias for calcium was calculated as

$(\text{Measured calcium} - \text{calcium value measured by the reference method}) / \text{reference method}) \times 100$.

Data analysis of albumin BCG and BCP methods is presented in figure 6.1 A, B and C bias data for total and adjusted calcium is presented in figures 6.2 and 6.3 respectively. Data obtained from this study, were judged against the limits for bias derived from biological variation (Westgard data base).

Figure 6.1 A shows that BCG methods collectively are positively biased to BCP methods by an average of 8% or 2g/L. For albumin BCG methods (figure 6.1B), all analytical platforms with the exception of Roche Cobas, are negatively biased to the mean, with a bias range from -0.3% to - 6.7 %, which is exceeding the desirable bias of 1.4% and the minimum allowable bias for albumin of 2.1%. Roche Cobas BCG method has a positive 2% bias which appears within the minimal allowable bias from biological variation. Beckman BCP

method is also positively biased to the rest of BCP methods from different analytical platforms, with a bias range from 2.7 to 5.1%.

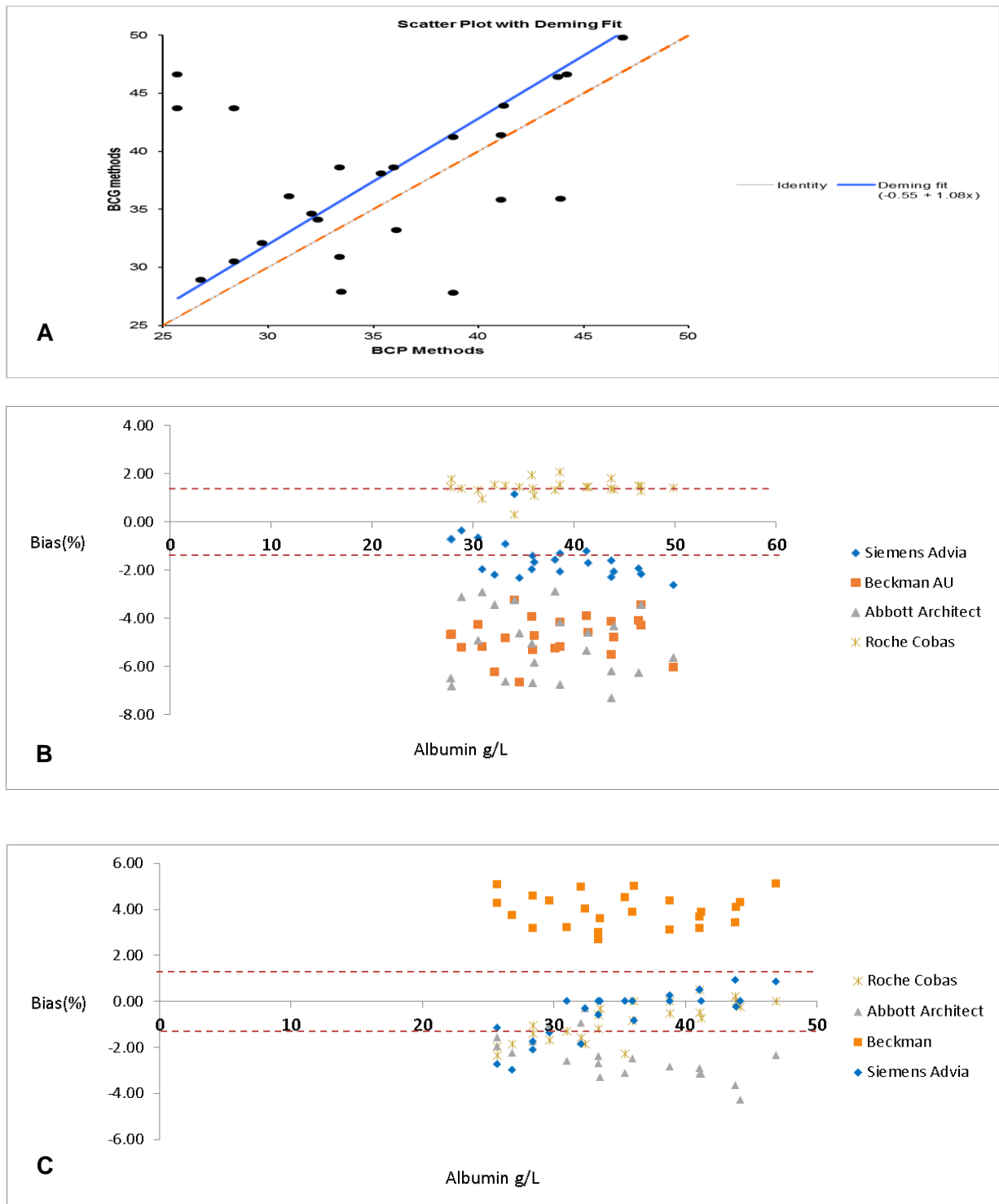


Figure 6.1: Albumin bias plot using data from WEQAS for the four serum pools distributed (A) Bias between BCG and BCP (B) is a sub-plot comparing all BCG methods. (C) is a sub-plot comparing all BCP methods. The red dashed lines represent the limit of desirable bias of 1.4% (EFLM biological variation database).

The rest of the analytical platforms showed a concentration dependent negative bias to the mean (range from 1 to -4.3%). The overall bias for BCP ranges from 5.1 to -4.3% and the overall bias for BCG methods is from 2 to -6.7%.

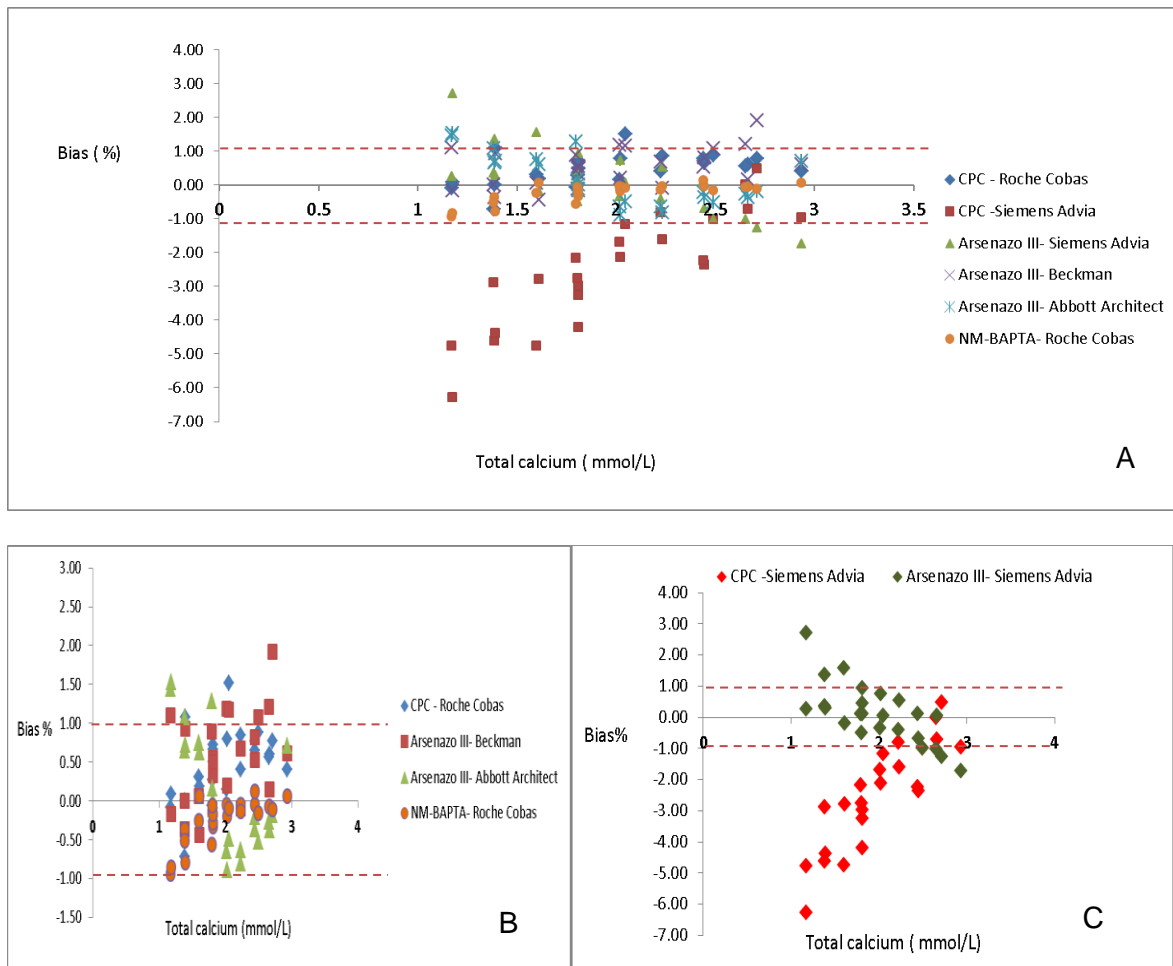


Figure 6.2: Total calcium bias plot using data from WEQAS for the four serum pools distributed. (A) Shows total calcium bias from all participated analytical platforms and methods. (B) Is a sub-plot comparing the Abbott, Roche and Beckmann calcium methods and (C) show a subplot comparing the two Siemens Advia calcium methods. The dashed line represents the limit for bias of $\approx 1\%$ (Westgard, 2013).

The comparability of calcium concentration within the reference range was moderate for Roche Cobas CPC and NM-BAPTA, Beckman Arsenazo III, Abbott Architect Arsenazo III, with overall bias ranges from 1.5 to -1% (figure 6.2A and B), [limits from biological variation for minimal allowable bias is 1.3%, and desirable allowable bias is $\approx 1\%$]. However, Siemens calcium methods CPC and Arsenazo III appear to suffer from concentration

dependent bias and this bias significantly worsens at the lower end of the reference limits of both assays. At calcium concentration of 2 mmol/L or below, however, the bias ranges from +3 to -6%, which exceeds even the minimal allowable limits (figure 6.2C).

Adjusted calcium shows significant total bias of 11% (range from +5 to -6%) (Figure 6.3).

Even with the exclusion of Siemens Advia, the scatter of adjusted calcium results exceeds that for total calcium (figure 6.2).

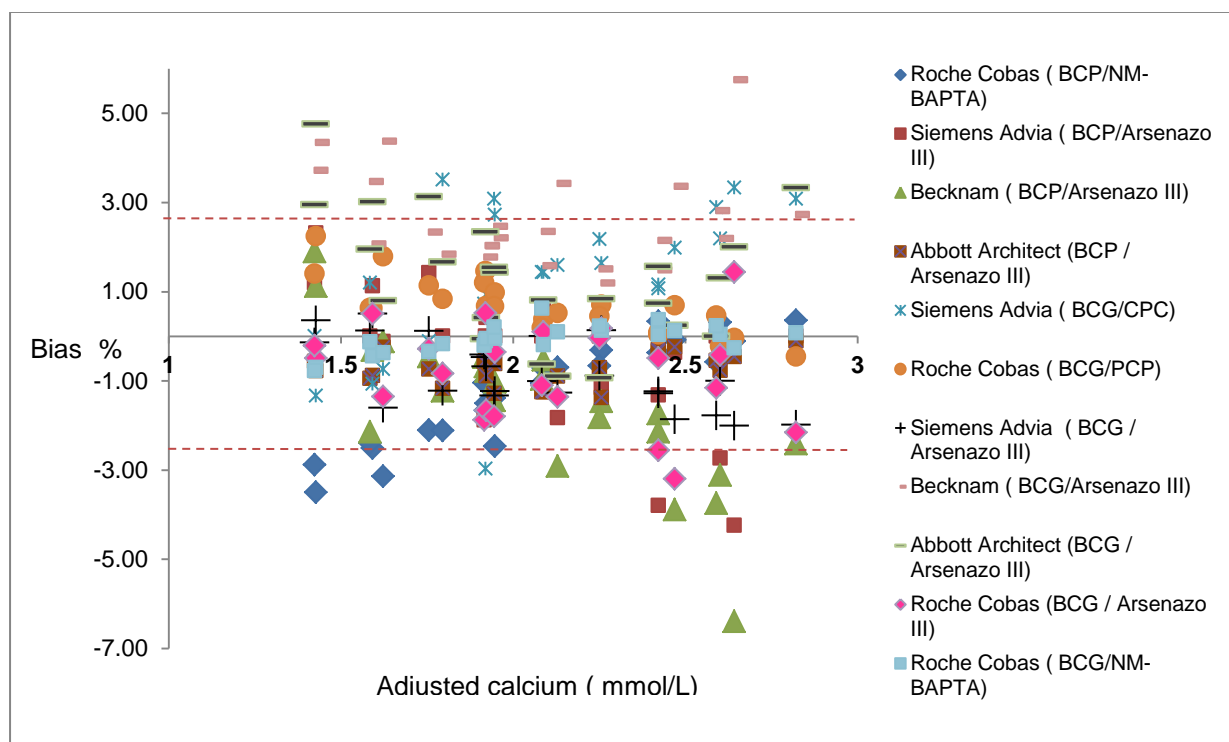


Figure 6.3: Bias plots of the adjusted calcium results obtained using WEQAS data for the four serum pools distributed. The red dashed line represents the limit for bias (2.6%, Westgard's Website).

6.4 Discussion

The analytical performance goal for biochemical tests was first published in 1999 and updated in 2015 (Kallner et al., 1999) (Fraser, 2015). The best scientific approaches follow a hierarchical order which starts from goals based on the impact of analytical performance

on clinical outcome, based on biological variation data, or based on the state of the art when all are not available (Fraser. 2015). There is a lack of studies that have evaluated the impact of albumin and calcium directly on the clinical outcome. Therefore, it is preferable to derive analytical goals from biological variation components of the albumin and calcium in serum. Calcium and albumin have narrow biological variation limits (Fraser and Pederson, 1999); therefore, to compare various analytical platforms, the minimal limits were used to define acceptable analytical performance in this study. However, for the sake of harmonisation of reference interval, the desirable bias was used as acceptable limits according to Fraser and Pedersen (1999). Desirable limits for albumin is 1.4%, for total calcium approximated to 1% and for adjusted calcium is 2.6%. The minimal bias and total error for total calcium are (1.3% and 3.6%), and for albumin are (2.1 %and 6.1%) respectively (Westgard, 2014).

In this study, the performance of all analytical platforms for calcium was moderately acceptable except for Siemens calcium assays which demonstrated significant concentration dependent bias. These data partially agrees with a previous Norwegian study that used a fresh-frozen single donation to assess the standardisation status for common analytical platforms (Van Houcke et al., 2012). Unlike this study, Van Houcke and colleagues reported “moderately” acceptable performance for all participated calcium methods, including Siemens. This difference could be related to the use by Van Houcke et al of a target value calculated from AMM which usually is less accurate than using a target value obtained from a reference method. Nevertheless, the Van Houcke et al study is superior to the current study because the design of the study ensured research sample commutability. In contrast to the Van Houcke study, calcium variation in the present study is not unique. A pilot study by Jansen and colleagues (2014), presented a large variation of calcium results obtained from various analytical platforms despite the use of proven commutable samples. A very recent multinational study confirmed the Jansen et al study and reported even larger total calcium error variation of up to 6.5% across a larger number of various analytical platforms (Weykamp et al., 2017). Total calcium is one of those biochemical assays that have a well-

established traceability system. Yet, the performance from these studies drove these authors to call for an urgent optimisation of routine calcium assays. In concurrence with these studies, we also support further optimisation of the calcium assay as removal of bias is of vital importance to enhancing diagnostic accuracy and transferability of calcium results.

The current study shows that BCG based albumin methods can be up to 8% (or 2 g/L) positively biased compared to the BCP based albumin methods. For BCG albumin assays, the difference between the 108 methods under review ranges from -6.7% to +2%, which is higher than the allowable desirable or even minimal bias for albumin. The absolute difference among BCP albumin based assays is similar to that seen with the BCG (which is +5.1% to -4.3%). Whilst our data showed that Roche Cobas BCG and Beckman BCP had a positive bias of < 2% and 4% respectively, we believe this is misleading as we used a target value calculated from the mean of all BCG methods to calculate the bias for albumin methods. The majority of this group were Roche Cobas users which amount to 75% (80/108) of the total participating laboratories. Therefore, higher results from Roche Cobas BCG shifted the overall mean of all the methods, which made the rest of the analytical platforms appear negatively biased. The same is not correct for the Beckman BCP method, because there was an almost even distribution among method users.

An Australian study presented an identical BCG method bias of 2 g/L to the one we reported from this study (Koerbin et al., 2019). In support of our data, another study by Bachmann and colleagues (2017) showed that the Roche BCG method had larger mean biases when compared to BCP methods and that none of the BCG methods met the minimum analytical performance defined by the biological variation. Albumin overestimation by BCG methods was found to be of clinical significance in several clinical conditions (Ueno et al., 2016) (Coley-Grant et al., 2016).

The unsatisfactory performance of albumin methods is not surprising, because unlike calcium, the reference system for albumin is problematic. Although, a nephelometric-based

method, (BCR-470) and ERM-DA470k/IFCC materials, were accepted by the Joint Committee on Traceability in Laboratory Medicine (JCTLM) as a reference measurement system for albumin (Zegers et al, 2010), albumin standardisation and traceability transfer was found to require further optimisation (Infusino and Panteghini, 2013). A number of issues related to albumin standardisation have been identified that contributed to overall unsatisfactory albumin performance. The stock of albumin reference material (BCR-470) was exhausted by 2008 and manufacturers are expected to establish traceability to ERM-DA470k/IFCC reference. However, manufacturers still refer to (BCR-470) reference material for establishing traceability of routine albumin assays (Roche Cobas ALB2 Gen 2 BCG, P 2/4) which makes ensuring albumin method traceability a difficult task. Even with the direct traceability to ERM-DA470k/IFCC reference material; it was found that the reference value transfer was associated with high uncertainty (Infusino and Panteghini, 2013). Reports also showed that all routine albumin assays inherently suffer from large imprecision (Van Houcke et al., 2012). Poor selectivity of some albumin assays such as BCG methods (Infusino, 2011) adds a random variation to an already uncertain metrological system and imprecise field assays. All these factors contributed to the overall variable methods that fail to satisfy the analytical goal needed for clinical applications. Our findings regarding albumin methods are in keeping with a large Australian study which involved circulating commutable EQA samples to participated laboratories (Koerbin et al., 2014). Koerbin and colleagues undertook this study on behalf of the reference interval committee for the Australian Association of Clinical Chemistry (AACC). On the basis of this study, the AACC has not proposed a harmonised reference interval for albumin and has recommended that laboratories should consider the use of the BCP albumin method for their routine use (Koerbin et al., 2014). Furthermore, the modified-BCP albumin assay, which is superior even to the current BCP albumin method, has been released but is not yet widely used (Ueno et al., 2013).

With regards to the adjusted calcium equation, harmonisation of total calcium methods would certainly reduce the degree of variation. But analytical variation in adjusted calcium

from this study exceeds that from total calcium which suggests a further source of variation (figure 6.3). This large bias is likely to represent the use of various calcium equations in addition to calcium method variation. This bias was translated into adjusted calcium variation within a single serum pool to amount to 0.31 mmol/L (2.47-2.79). This difference is clinically unacceptable and efforts should be focused to remove this variation.

WEQAS showed evidence of considerable variation in the way UK laboratories derive and apply adjusted calcium equations. This will be due in part to a proportion of laboratories using a literature equation rather than an in-house derived equation (WEQAS, 2011). Thus, harmonisation of calcium equation derivation is a way to minimise variation within adjusted calcium results, but is unlikely to eradicate it. It is clear that analytical variation in both serum calcium and serum albumin are critical in determining the regression equation.

Variation of albumin regression on calcium factor is a product of variation in calcium and albumin assays. Low least square R^2 values, which are usually seen with albumin regression on calcium, reflect firstly a poor predictability of Y from X, but also reflect poor precision and inaccuracy of the methods used too (Forst , 2019). Calcium method precision has significantly improved over the last 3 decades; however, it is reported that most of the albumin assays fail to meet the minimum allowable limits (Infusino, 2011). Therefore, further standardisation optimisation or improved assay design may improve calcium albumin regression equations and reduce variability in adjusted calcium results.

In conclusion, in this small study, the analytical performance of albumin and calcium was assessed. The findings from this study showed wider than acceptable analytical variation that may indirectly contribute to overall adjusted calcium equations variation and invalidate the application of a harmonised reference interval for calcium and albumin.

Chapter 7: The effect of different equations on calcium status

7.1 Background

Payne's calcium adjusted equation is the most widely used equation (K/DOQI ,2003). This equation was derived using a well-defined population of hospitalised patients with low albumin (Payne et al., 1979). Payne's data collection criteria include patients with creatinine of $\leq 200 \mu\text{mol/L}$, but excluded patients with diseases affecting calcium metabolism. Recently, renal function for chronic kidney disease has been assessed by estimated glomerular filtration rate (eGFR) which is a calculated marker that includes creatinine amongst other variables (age, sex, race), rather than creatinine alone. A creatinine of $\leq 200 \mu\text{mol/L}$ includes patients with severe renal failure and eGFR as low as $10 \text{ mL/min/1.73m}^2$. Patients with advanced CKD stage 3-5 are known to have metabolic bone disease. A population including all those with creatinine $\leq 200 \mu\text{mol/L}$ lends itself to include patients with disturbed calcium metabolism.

Normal kidney function is now defined as $\text{eGFR} > 60 \text{ mL/min/1.73m}^2$ (CKD stage 2 and 1). There is an abundance of literature criticising the performance of Payne's calcium equation in patients with advanced chronic renal failure (Dickerson et al., 2004) (Obi et al., 2018). It must be noted, that eGFR measurement is only valid for primary care patients with stable chronic renal disease (KDIGO, 2013). Its use in the acute setting has not been validated. Therefore, this study can only be applied to the primary care adjusted calcium equation. In chapter 2 an adjusted calcium equation for ambulant patients was derived using Payne's criteria and this equation was validated against ionised calcium. In the study described in this chapter, a new community equation will be calculated, using the proposed modified criteria of $\text{eGFR} > 60 \text{ mL/min/1.73m}^2$. The new equation will be compared to the community equation which used traditional Paynes' criteria. It is hypothesised that in the primary care

population, an equation derived using the modified criteria of $eGFR > 60 \text{ mL/min/1.73m}^2$ can outperform an equation derived using Payne's criteria.

7.2 Materials and methods

In this study the same biochemical data sets of primary care patients that were collected in section 3.2 were used. The collection of eGFR data from the primary care setting was built into the circulated protocol that was used in section 3.2. The primary care data were collected using Payne's inclusion/exclusion criteria plus eGFR data.

The mathematical equation derivation for the $eGFR > 60$ population followed the same method that was described in section 2.2.7. Community data were further filtered to exclude patients with $eGFR < 60 \text{ mL/min/1.73m}^2$. The equation with a population consistent with $eGFR > 60 \text{ mL/min/1.73m}^2$ will be referred to as "Paynes' modified criteria equation".

7.2.1 Statistical Analysis

All data were analysed by a statistical package, Analyse it (Microsoft Excel 2010) (version 2.10). The comparison of the two equations was presented using Deming regression. The t-test was used to test the statistical significance of differences between the adjusted calcium mean of the newly derived equation and the routine community equation. The Z test was used to test the statistical significance of differences between the albumin, calcium and creatinine mean from both populations.

7.3 Results

7.3.1 Community equation using the modified criteria

The community equations using Paynes' criteria and the modified criteria for Harrogate (Roche 1) are presented in Table 7.1. The outcome of the community equation derived

using Payne's criteria compared against the community equations derived using the modified criteria of $eGFR > 60 \text{ mL/min/1.73m}^2$ gave a correlation equation of $Y = 1.00 X + 0.00$, $p = 0.9946$, figure 7.1. The same analysis was conducted for all the participating laboratories and the same observation was seen for all community equations that were derived from different analytical platforms (data presented in table 7.2).

Equation Name	Regression Coefficient	Intercept	Mean Ca mmol/L	Calcium Equations	n value
Community Equation (Payne's criteria)	0.014	1.77	2.378	Adj.Ca=T.Ca- 0.014 (Alb. - 44.9)	6062
Community Equation (Modified criteria)	0.014	1.76	2.380	Adj.Ca=T.Ca- 0.014 (Alb. - 45.2)	5725

Table 7.1: Shows the community adjusted calcium equation derived using Payne's criteria and the modified criteria. Adjusted calcium and total calcium (mmol/L), albumin (g/L).

Table 7.2 presents the modified criteria equations for 13 participating laboratories with varying analytical platforms and calcium and albumin methods. We used the Z-test to assess the difference in creatinine mean for the two populations studied here. The mean creatinine using Paynes' exclusion criterion was $85.0 \mu\text{mol/L}$ and $76.2 \mu\text{mol/L}$, $p = 0.2254$ for the modified criteria. It was found that despite the significant difference in mean creatinine in the two populations, mean calcium and mean albumin of the two populations remains unchanged (table 7.3).

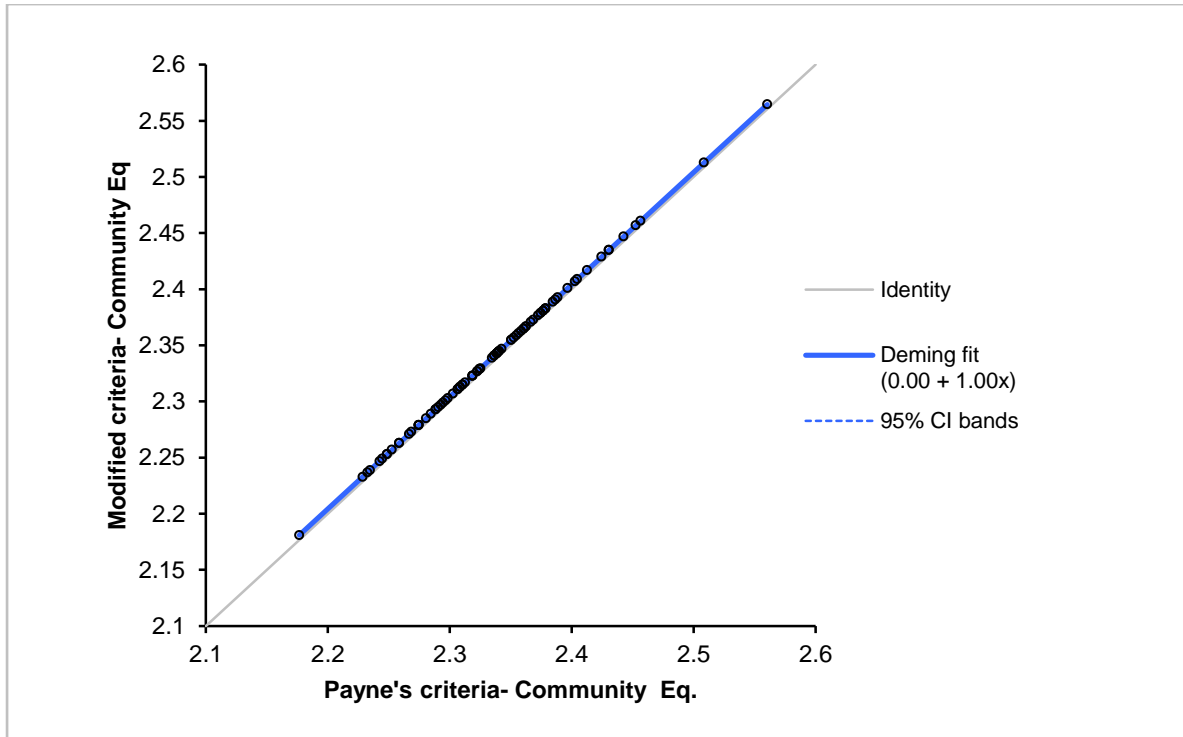


Figure 7.1: Comparison of the outcomes of the community equation that was derived using Payne's exclusion criteria and the community equation derived using the modified criteria of (eGFR > 60 ml/min/1.73 m²).

Similar to the Harrogate laboratory finding, mean creatinine was significantly different between the two community populations but neither mean calcium nor albumin were clinically different. This finding suggests the two populations are not different despite removing those with advanced renal diseases. Data for mean calcium, albumin and creatinine are presented in table 7.3.

Hospital	Paynes' Criteria	Modified Criteria	Deming fit	
			Constant	Proportional
Abbott 1	Adj. Ca= TCa-0.014(Alb-39.0) n=7647	Adj. Ca= TCa-0.014(Alb-39.0) n=7013	0.00 No p value	1.00 p= 0.6320
Abbott 2	Adj. Ca= TCa-0.014(Alb-41.2) n= 4562	Adj. Ca= TCa-0.015(Alb-41.8) n=4120	0.01 p<0.0001	1.00 p=0.3016
Abbott 3	Adj. Ca= TCa-0.014(Alb-38.0) n=9580	Adj. Ca= TCa-0.015(Alb-38.0) n=8466	0.00 p=0.6590	1.00 p 0.8431
Abbott 4	Adj. Ca= TCa-0.019(Alb-41.4) n=6089	Adj. Ca= TCa-0.017(Alb-41.2) n=5721	0.00 p=0.4664	1.00 p=0.0281

Hospital	Paynes' Criteria	Modified Criteria	Deming fit	
			Constant	Proportional
Beckman 1	Adj. Ca= TCa-0.014(Alb-39.4) n= 11666	Adj. Ca= TCa-0.015(Alb-39.0) n=10333	0.00 No p value	1.00 p= 0.2184
Beckman 2	Adj. Ca= TCa-0.016(Alb-41.5) n= 13077	Adj. Ca= TCa-0.016(Alb-41.8) n= 11000	0.00 No p value	1.00 p= 0.9949

Hospital	Paynes' Criteria	Modified Criteria	Deming fit	
			Constant	Proportional
Roche 2	Adj.Ca=T.Ca- 0.016 (Alb. – 45.0) n=2938	Adj.Ca=T.Ca- 0.016 (Alb. – 45.4) n=1761	0.01 p<0.0001	1.00 p=0.9982
Roche 3	Adj. Ca= TCa-0.015(Alb-40) n=5934	Adj. Ca= TCa-0.016(Alb-40) n=5253	0.00 p=0.8693	1.00 p=0.2610
Roche 4	Adj.Ca=T.Ca- 0.013 (Alb. – 45.5) n=5134	Adj.Ca=T.Ca- 0.014 (Alb. – 45.8) n=4009	0.00 p=0.2333	1.00 p=0.1384
Roche 5	Adj.Ca=T.Ca- 0.015 (Alb. – 44.0) n=1991	Adj.Ca=T.Ca- 0.015 (Alb. – 44.3) n=1568	0.00 no p value	1.00 p=1.0000
Roche 6	Adj.Ca=T.Ca- 0.013 (Alb. – 45.5) n=5133	Adj.Ca=T.Ca- 0.014 (Alb. – 45.7) n=4611	0.00 p=0.0109	1.00 p=0.8474

Hospital	Paynes' Criteria	Modified Criteria	Deming fit	
			Constant	Proportional
Siemens	Adj.Ca=T.Ca- 0.015 (Alb. – 39.0) n=9428	Adj.Ca=T.Ca- 0.014 (Alb. – 39.0) n=8142	0.00 p=0.4679	1.00 p=0.6048

Table 7.2: shows the derived equations for each analytical platform and a comparison between community equations derived using Payne's exclusion criteria and community equation derived using the modified criteria (eGFR > 60 mL/min/1.732 m²) presented as Deming fit constant and proportional factors following Deming fit equation as $Y = C + \alpha X$, whereas C is the constant factor and α is the proportional factor.

Location	Analytical Platform	Calcium Mean Mmol/L In Payne's criteria and the modified criteria respectively	Creatinine mean $\mu\text{mol/L}$ In Payne's criteria and the modified criteria respectively	Albumin mean g/L In Payne's criteria and the modified criteria respectively
Roche 1	Roche Cobas 702 BCG	2.379 and 2.377 $p=0.2554$	85.0 and 76.2 $p<0.0001$	44.5 and 44.9 $p<0.0001$
Roche 2	Roche Cobas 702 BCG	2.359 and 2.360 $p=0.5005$	81.2 and 80.0 $p<0.0001$	45.1 and 45.4 $p<0.0001$
Roche 3	Roche Cobas 702 BCG	2.389 and 2.391 $p=0.1414$	83.0 and 75.4 $p<0.0001$	45.4 and 45.8 $p<0.0001$
Roche 4	Roche Cobas 702 BCG	2.338 and 2.340 $p=0.9017$	81.6 and 74.0 $p<0.0001$	44.0 and 44.3 $p<0.0001$
Roche 5*	Roche Cobas 702 BCG	2.343 and 2.342 $p=0.6424$		45.1 and 45.5 $p<0.0001$
Roche 6	Roche Cobas 702 BCP	2.356 and 2.355 $p=0.5364$	75.9 and 72 $p<0.0001$	38.8 and 39.0 $p<0.0001$
Roche 7	Roche Cobas 702 BCP	2.339 and 2.41 $p=0.3758$	79.2 and 73.6 $p<0.0001$	39.1 and 39.4 $P<0.0001$
Beckman* 1	Beckman Olympus BCG	2.353 and 2.350 $p=0.6424$		38.7 and 38.7 $p=0.9680$
Beckman 2	Beckman Unicel DXI BCP	2.343 and 2.342 $p=0.6424$	87.6 and 80.1 $p<0.0001$	41.4 and 41.7 $p<0.0001$
Abbott 1	Abbott BCG	2.360 and 2.359 $p=0.1686$	74.5 and 71.1 $p<0.0001$	41.2 and 41.3 $p<0.0001$
Abbott 2	Abbott BCP	2.363 and 2.363 $p=0.1686$	72.9 and 69.4 $p<0.0001$	38.5 and 38.6 $p<0.0001$
Abbott 3	Abbott BCP	2.352 and 2.350 $p=0.8433$	77.3 and 71.5 $p<0.0001$	37.4 and 37.6 $p<0.0001$
Abbott 4	Abbott BCG	2.328 and 2.300 $p=0.7509$	75.6 and 71.1 $p<0.0001$	40.9 and 41.1 $p<0.0001$
Siemens 1	Siemens Advia XP	2.361 and 2.360 $p=0.3131$	73.0 and 69.2 $p<0.0001$	39.7 and 38.9 $p<0.0001$

Table 7.3: Albumin, calcium and creatinine mean in the original Payne's criteria $< 200 \mu\text{mol/L}$ and the modified criteria ($\text{eGFR} > 60 \text{ mL/min/1.73m}^2$). Two laboratories marked with *, these labs filtered data according to the Payne's criteria but did not provide the data.

7.4 Discussion

Disorders of mineral and bone metabolism are prevalent in patients with chronic kidney diseases ($\text{eGFR} < 60$ or stage 3-5) (Damasiewicz and Nickolas, 2018). In chapter 2, it was shown that the primary care equation correctly identified calcium status in only 91% of the studied population. In the current study, it was hypothesised that Payne's inclusion criteria of creatinine $< 200 \mu\text{mol/L}$ may contribute to the less than optimal diagnostic accuracy of the

albumin-adjusted equation in ambulant patients, because creatinine $<200 \mu\text{mol/L}$ is usually associated with eGFR of $90 -10 \text{ mL/min/1.73m}^2$. We therefore, proposed a modified inclusion criteria consistent with patients with $\text{eGFR} > 60 \text{ mL/min/1.73m}^2$.

In this study, the comparison of calcium equations using Payne's inclusion criteria (which may include patients with severe renal failure) and the modified criteria of $\text{eGFR} > 60 \text{ mL/min/1.73m}^2$ yielded identical regressions. This outcome was replicated in all participating laboratories irrespective of calcium, albumin methods or platforms.

Severe renal failure is associated with acid-base disturbances and uraemia. Uraemia alone was excluded as a factor effecting calcium albumin binding (Leme and Silva, 1977). However, the mechanism by which renal failure affects the adjusted calcium equation is attributed to the acid-base imbalance in this group of patients due to alteration in albumin binding affinity, thus altering the albumin regression coefficient (Jain et al., 2008). This is in agreement with another study which reported on the performance of adjusted calcium in a large cohort of patients with CKD stages from 3-5 (Gauci et al., 2008). Interestingly, Gauci and colleagues reported that adjusted calcium poorly predicted the correct calcium status in CKD 3–5 patients and found that the risk of misclassification of adjusted calcium was independently increased by low albumin concentration and low bicarbonate concentration (Gauci et al., 2008), suggesting that acid-base in the context of renal failure is probably as an important factor for the adjusted calcium equation as low albumin.

These above studies could provide a plausible explanation to the agreement between Payne's criteria and the modified criteria. The agreement suggests that the percentage of patients with acid-base disturbance in those with creatinine $\leq 200\mu\text{mol/L}$ population is minimal. This is the most likely explanation because n value shows that the percentage of $\text{eGFR} < 60 \text{ mL/min/1.73m}^2$ excluded is small in relation to the overall patient cohort (table 7.3), therefore, the overall impact of those with severe renal failure on the equation was

negligible. Further data analysis showed that although mean creatinine was significantly different in the two populations, calcium and albumin mean did not differ between populations. This supports the fact that the excluded population was small enough to not alter calcium and albumin mean and suggests that calcium and albumin means in a population are more important factors impacting the derivation and performance of the calcium equation than creatinine concentration.

The conclusion from this study is that excluding severe renal failure patients from the population used to derive adjusted calcium equation had no impact on the newly derived community equation. Therefore, it is safe to conclude that Payne's criteria remain the best approach for derivation of the adjusted calcium equation in the community setting until further evidence becomes available.

Chapter 8: Effect of standardisation of derivation of adjusted calcium equation on calcium status

8.1 Background

Evidence from the UK suggests wide variability in derivation and reporting of adjusted calcium (WEQAS, 2012). A WEQAS survey undertaken in 2012 showed that 42.6% of laboratories reported adjusted calcium using a published adjustment equation. Among those who derive their own equations there is variability in how the adjustment equation is derived. It is evident that this variability between adjustment equations would result in variability in calcium reporting. This variability relates to case-mix as well as other factors such as differences in measurement methods. To reduce the variation resulting from case-mix, the ACB working group (ACB WG) on harmonisation of adjusted calcium suggested exclusion criteria slightly modified from Payne's criteria (O'Kane et al., 2015). The ACB WG excluded those with total calcium concentration <2.0 and >2.7 mmol/L. This item was not defined in the original Payne's exclusion criteria. The ACB WG also recommended the use of a calcium mean of 2.4 (representing the midpoint of a nationally standardised reference interval for calcium) rather than a population mean as originally described (Barth et al., 1996).

In this study the additional criteria will be assessed against the original Payne's criteria. For this purpose we will derive an equation for each criterion and we will compare these equations to an equation derived using the original criteria as described by Payne et al (1973).

8.2 Materials and methods

Retrospective anonymised biochemical data for calcium equations derivation was collected from laboratories employing the four most commonly used analytical platforms in the UK as

described in chapter 3 (section 3.2). The participating laboratories, analytical platform and methods for albumin and calcium are given in table 3.1.

The hospitalised patients data set was further filtered to exclude calcium outside the range of 2.0 – 2.7 mmol/L, as recommended by the ACB best practice guidelines. The derivation of an equation from the filtered data followed the same principles described in section 2.2.7.

However, the regression equations were calculated for each data set but instead of using population calcium mean, a standardised mean of 2.4 mmol/L was used in equation 1 (section 2.2.7). This gives rise to the following equation:

$$\text{Adjusted calcium} = \text{Total Calcium} - (\text{slope} \times \text{albumin}) + (2.4 - \text{intercept})$$

(Adjusted calcium and total calcium measured in mmol/L, albumin in g/L).

This method means that each laboratory has two ACB equations (see below), in addition to in-patient equations that were originally derived from the in-patient data set and according to the original Payne's criteria. The ACB equations are:

1. Equation using the ACB exclusion criteria as described in the ACB's position paper in (2015). This equation will be referred to as "ACB exclusion criteria equation"
2. Equation using the mid-point of the calcium reference range of 2.4 as described in the ACB's position paper (2015). This equation will be referred to as the "ACB standardised equation"

These two equations were then applied to the in-patient data set from each laboratory and adjusted calcium from each equation compared to the in-patient traditional equation. The Pathology Harmony reference interval of (2.2-2.6 mmol/L) was used to define the limits for hypo/hypercalcaemia.

8.2.1 Statistical analysis

All data were analysed by a statistical package, Analyse it (Microsoft Excel 2010) (version 2.10). The Z test was used to test the statistical significance of differences between the calcium mean from the in-patient populations and the modified criteria (<2.0 and >2.7 mmol/L) populations.

8.3 Results

Table 8.1 presents the slope (regression coefficient) and constant values for the ACB exclusion criteria equation and the ACB standardised equation for the 13 participating laboratories.

For example Roche 1 (Harrogate lab) equations are;

Adjusted calcium = Total Ca – regression coefficient x (Alb - constant)

The ACB exclusion criteria: Adjusted calcium = Total Ca - 0.015 (Alb - 39.4)

The ACB standardised equation: Adjusted calcium = Total Ca - 0.015 (Alb - 46.4)

Table 8.1 shows that the constant values from the standardised equation were higher than the constant values in the ACB exclusion criteria equation. The constant value is a mathematically derived value; ideally it reflects the albumin mean of a population. These two equations were derived from the same population. This difference in the constant value is attributed to the fact that the population calcium mean used to drive the ACB exclusion criteria equation is lower than the recommended mean of 2.4 mmol/L (table 8.2).

The impact of the ACB calcium equations on the prevalence of hypo/hypercalcaemia in hospitalised patients is presented in figure 8.1A and figure 8.1B. In this study it was found that equations derived using the ACB exclusion criteria yielded a prevalence of hypocalcaemia in hospitalised patients almost the same as that obtained from Payne's

traditional equation. While the ACB standardised equation lowered the prevalence of hypocalcaemia several fold depending on analytical platform or method used.

Roche Cobas

Hospital	Regression Coefficient	Constant ACB Excl. Criteria	Constant ACB St. Eq.	<i>n</i>
Roche 1	0.015	39.4	46.4	1074
Roche 2	0.014	36.7	47.8	1831
Roche 3	0.015	40.0	45.5	8734**
Roche 4	0.015	36.6	47.2	1310
Roche 5	0.015	35.4	46.6	1522
Roche 6	0.015	38.0	42.0	6473
Roche 7	0.014	35.6	44.8	892

** Separate set of data

Beckman DXI and Olympus

Hospital	Regression Coefficient	Constant ACB Excl. Criteria	Constant ACB St. Eq.	<i>n</i>
Beckman 1	0.016	36.1	42.4	1820
Beckman 2	0.017	39.4	44.4	4833

Abbott Architect

Hospital	Regression Coefficient	Constant ACB Excl. Criteria	Constant ACB St. Eq.	<i>n</i>
Abbott 1	0.015	36.0	42.0	6037
Abbott 2	0.011	36.3	39.0	1322
Abbott 3	0.018	36.3	44.2	1333

Siemens Advia

Hospital	Regression Coefficient	Constant ACB Excl. Criteria	Constant ACB St. Eq.	<i>n</i>
Siemens 1	0.014	36.2	43.6	3208

Table 8.1: The ACB exclusion criteria equations and the ACB standardised equations for all participated laboratories, grouped by analytical platform.

In the same manner, the exclusion criteria equation performances paralleled the original Payne equations, while the ACB standardised equation increased the prevalence of hypercalcaemia in the in-patient population up to 3-4 fold higher than the hypercalcaemia rate given by the original Payne's equation.

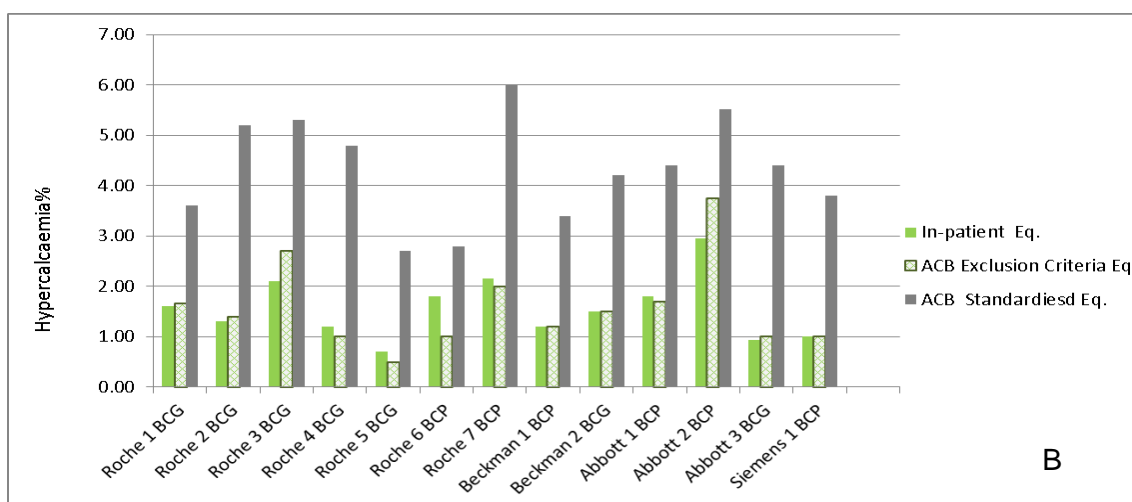
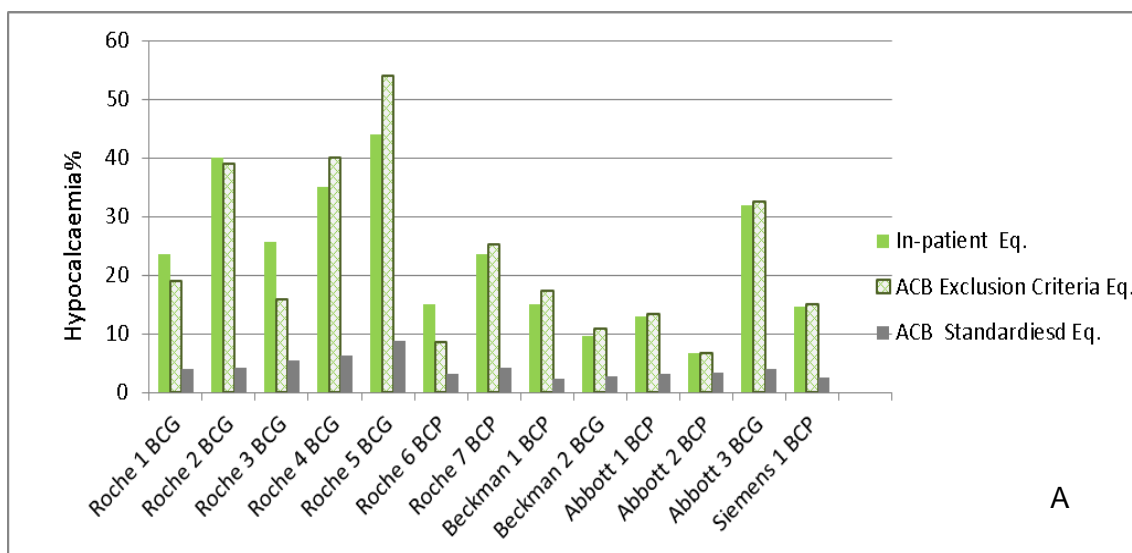


Figure 8.1: shows the impact of various analytical methods and their equations on the prevalence of hypocalcaemia (A) and hypercalcaemia (B) in hospitalised patients

Analytical platforms	Calcium mmol/L Payne's criteria	Calcium mmol/L The ACB criteria	P value
Roche 1	2.279	2.295	$P=0.0006$
Roche 2	2.224	2.246	$P<0.0001$
Roche 3	2.269	2.320	$P<0.0001$
Roche 4	2.192	2.240	$P<0.0001$
Roche 5	2.170	2.220	$P<0.0001$
Roche 6	2.266	2.270	$P<0.0001$
Roche 7	2.159	2.204	$P<0.0001$
Beckman 1	2.227	2.300	$P<0.0001$
Beckman 2	2.300	2.316	$P<0.0001$
Abbott 1	2.30	2.312	$P<0.0001$
Abbott 2	2.355	2.371	$P=0.2683$
Abbott 3	2.225	2.260	$P<0.0001$
Siemens 1	2.278	2.292	$P<0.0001$

Table 8.2: Calcium mean derived for each laboratory using Payne's criteria and the ACB exclusion criteria.

Furthermore, mean total calcium for the ACB exclusion criteria equation was significantly different from Payne's criteria equation (with the exception of Abbott 2), at $p < 0.0001$, table 8.2. This observation remained constant despite platform and methodological differences. The mean range of total calcium (2.159 -2.35 mmol/L) was lower than the mean total calcium for the ACB exclusion criteria (2.2-2.37 mmol/L) and that in turn was lower than the value of (2.40 mmol/L) suggested by the ACB. The difference in calcium means suggests that the removal of < 2.0 and > 2.7 total calcium as described by the ACB exclusion criteria contributed to the rise in calcium mean of these populations.

8.4 Discussion

In this comparative study attempts were made to show the impact of the change from the original method of derivation of adjusted calcium equation as described by Payne's on calcium classification status.

Payne's inclusion/exclusion criteria define the population that will be used to derive the equation. To date, and despite many criticisms of Payne's equations, no viable replacement to estimate calcium has been proposed. Various studies have assessed the diagnostic accuracy of Payne's equation in several clinical settings (Ijaz et al., 2006) (James et al., 2008). The data presented in chapter 2 is one example of many in the literature that attempted to improve the equation prediction of the patient's calcium status.

In an earlier study (chapter 3), it was shown that the in-patient population mean calcium was approximately ≈ 2.2 mmol/L which is significantly lower than the ACB proposed figure of 2.4 mmol/L. Given that the "regression factor" and the "constant value" in an equation are mathematically derived values that usually vary between populations, it is hardly surprising that the arbitrary use of a higher constant value would produce a significant change in calcium classification status.

The ACB exclusion criteria equation gave a performance closer to the in-patient equation than did the ACB standardised equation. The WEQAS 2012 survey showed significant equations variation among laboratories using identical analytical platforms and methods. Part of the variation can be due to patient mix. It is the experience of this author that errors in data collection are not uncommon. Indeed, the ACB proposed truncated total calcium criterion (2.0-2.7 mmol/L) seems a promising approach and can be seen as a method for a standardisation data collection exercise. Payne's calcium equation is widely used and deeply rooted in the current clinical practice. Therefore, a change to this practice would be expected to have a major clinical impact. Therefore, it is recommended that such a change to Payne's original criteria is validated against ionised calcium measurement before putting this approach in clinical practice.

The mean calcium for the in-patient population collected according to Payne's criteria was lower than the suggested value of 2.4 mmol/L. A previous study (chapter 3), showed that the calcium mean of a population has a major impact on the outcome of the adjusted calcium equation. Moreover a significant difference in calcium mean was demonstrated to be a marker of population differences.

It was also observed that the ACB exclusion criteria produced an equation with a lower coefficient regression compared to in-patient Payne's equations, since these criteria produce different equations; they need to be validated against ionised calcium to ensure their diagnostic accuracy.

In conclusion of this study, it has been shown that imposing a calcium mean other than the one obtained from its population would yield a significant misclassification of calcium status. The suggested exclusion criteria have also led to a smaller change, but this change should be assessed against the reference method.

Chapter 9: Conclusions

In clinical practice, the most widely used equations for calcium estimation were originally derived by Payne et al in 1973. Since they were developed, many inherent problems with applying calcium equations to adjust total calcium measurements across different patient groups and clinical settings have been described. The accuracy of adjusted calcium results is of pivotal importance to guarantee the accuracy required for optimal clinical use. Reducing variability and improving accuracy of adjusted calcium results would positively impact on patients' clinical outcome and healthcare resources. This current series of studies attempted to elucidate the reasons behind variation in reporting adjusted calcium and its suboptimal performance as well as exploring new concepts to improve adjusted calcium performance through the derivation and validation of a population specific equation for ambulant patients.

In this study (chapter 2) adjusted calcium from the newly derived population specific equation was compared to ionised calcium. It was shown that an equation for adjusted calcium derived from ambulant patients and applied to those patients out-performed Payne's traditional equation in a primary care setting. Moreover, this study showed that the population specific equation reduced the magnitude of variation in hypo/hypercalcaemia between communities suggesting that the population specific equation would potentially harmonise reporting of adjusted calcium in the primary care setting (chapter 3).

In chapter 3, the validity of a primary care population equation was extended from a single analytical platform (Roche Cobas) and N-MPABTA calcium method and BCG albumin method to the majority of commercially used analytical platforms and calcium and albumin methods. The application of the same concept to different analytical platforms has shown results followed the same pattern that has been obtained from Roche Cobas, BCG and NM-BAPTA methods, suggesting that this concept is valid irrespective of platform or method.

This study (chapter 3) demonstrated that albumin and calcium concentrations in ambulant patients are significantly different from that in hospitalised patients; hence calcium equations from these populations differed. This concept may open the door to addressing other populations that differ in their characteristics from the original Payne's equation population. Therefore, age or gender related adjusted calcium equations should be explored. The change in the practice from the application of a single equation to the application of multiple population specific equations relies on the capacity of laboratory information systems (LIMS) to deliver this change; therefore, one of the major limitations of this work is the limitation of some of the current LIMS.

It was found that one of the most important factors contributing to the variability in adjusted calcium results was related to the analytical variability of both calcium and albumin routine assays. This study revealed a performance difference between BCG and BCP albumin methods. Albumin methods (BCG or BCP) accounted for significant differences in the classification of adjusted calcium results (chapter 3). This difference is attributed to variation in albumin concentrations measured by these assays and mean albumin of these assays was consistently higher for BCG than BCP methods irrespective of platform (chapter 6).

The implementation of measurement standardisation in laboratory medicine is the only way to ensure the equivalence of results produced from different measurement procedures. Despite the claims of assay standardisation of albumin and calcium methods, the data showed that for albumin methods, assay specific reference interval is a safer practice because the current harmonised reference interval for albumin may mislead users away from the concentration difference between these methods. Due to the problematic standardisation of albumin methods, coupled with the widespread use of poor specificity assays (e.g. BCG), no harmonisation for albumin is recommended until the presence of bias is eliminated and the BCG albumin assay specificity improved (Chapter 4, 5 and 6). To improve adjusted calcium equivalence of results, laboratory medicine professional bodies

are called to lead on the implementation of a more specific form of albumin assays. The case of albumin is similar to a recently demonstrated case of creatinine, where the use of a non-specific method (Jaffe) made standardisation of creatinine measurements difficult, or almost impossible.

Reference interval is another factor that contributes to misclassification of calcium status. With the exception of Siemens assays, calcium from Roche Cobas, Abbott Architect and Beckman showed close analytical agreement reflected by almost identical reference intervals (chapter 4 and 6). Current UK practice of harmonisation of calcium reference intervals contributes to the misclassification of calcium status. These studies results (chapter 4 and 6) indicate that calcium is subject to harmonisation for those three analytical platforms only, or widening the current harmonised calcium reference interval to 2.1- 2.60 mmol/L to include Siemens's calcium assays.

This study (chapters 7 and 8) reviewed a number of alterations to the clinical inclusion/exclusion criteria for the original Payne's equation as an attempt to further improve the diagnostic value of the newly derived primary care equations. However, data showed the original criteria by Payne were confirmed as the most valid approach up to date. The ACB attempts to standardise the derivation of Payne's adjusted calcium equation by imposing total calcium of 2.4 as a population mean negatively impacted on the calcium classification in comparison to the traditional equation in the hospitalised setting. However, the standardised data collection method proposal may have merit, but only if it has been validated against ionised calcium.

Finally, for accurate classification of calcium status, it's important that the causes of variability in adjusted calcium are identified. The literature review has addressed a number of variables (summarised figure 1.5) that may impact on calcium status classification such as population, age, gender, reference range etc. This study described other factors that would contribute to variation in calcium reporting. For example the impact of albumin and calcium

methods traceability and the siftings criteria used for post data collection handling have not been reported before in the context of adjusted calcium results equivalence.

The large number of variables in the adjusted calcium derivation process makes it important that all future adjusted calcium studies are described in sufficient details to allow readers to fully understand the process and identify any weaknesses/strengths and assess the impact on calcium classification. Further to this, a checklist for publication has been developed to support authors, editors and reviewers in this process, see below.

Factors affecting adjusted calcium equations variability	
Calcium	<ul style="list-style-type: none"> • Methods: Arsenazo III, CPC, NM-PABTA • Method traceability • Analytical performance • Reference interval
Albumin	<ul style="list-style-type: none"> • Methods: BCG vs. BCP • Method traceability • Analytical performance and method selectivity • Age and gender
Data collection and sifting criteria	<ul style="list-style-type: none"> • Payne's criteria • ACB criteria
Population	<ul style="list-style-type: none"> • Hospitalised vs. ambulant population • Children , adult and elderly • Various patient groups and case mix
Equation	<ul style="list-style-type: none"> • Payne's equation (UK) • Method of derivation and statistics • Other equations

Table 9.1: Minimum requirements for publication of adjusted calcium equation studies.

Taken together, these series of studies show that standardising adjusted calcium equations and reducing variability in adjusted calcium results is not currently attainable without eliminating albumin and calcium calibration issues. Until these issues are resolved, laboratories should continue the use of method dependent equations according to Payne's described method. However, the introduction of a population specific equation in combination with the ACB recommended data collection approach is a step forward towards reducing variability in adjusted calcium reporting.

9.1 Recommendations

In order to reduce the variability in adjusted calcium measurement and reporting, the following is recommended:

1. Laboratories continue to derive their own equation
2. Professional bodies to promote the use of a population specific equation. However, a confirmatory study comparing other analytical platforms may be advised before national implementation.
3. To review the use of the Pathology Harmony reference interval for albumin and calcium
4. Recommend that the modified BCP method, not the BCG method, be used for adjusted calcium estimation.
5. Professional bodies to promote the profile of the modified-BCP method in a similar manner to that used to raise the profile of the enzymatic creatinine assay.
6. To explore the age related adjusted calcium equation.
7. To assess future adjusted calcium studies against a checklist that includes all the variables that may affect calcium classification.

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Appendices

Appendix 1a: National Research ethic Committee approval letter.



Health Research Authority

Mrs Nuthar Jassam
Consultant Clinical Biochemist
Harrogate and District NHS Foundation Trust
Blood Sciences Department, Pathology
Fewston Wing
HG2 7SX

Email: hra.approval@nhs.net

23 February 2017

Dear Mrs Jassam

Letter of HRA Approval

Study title:	Derivation of adult population-dependent calcium adjusted equations
IRAS project ID:	205694
REC reference:	17/NI/0010
Sponsor	Harrogate and District NHS Foundation Trust

I am pleased to confirm that **HRA Approval** has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications noted in this letter.

Participation of NHS Organisations in England

The sponsor should now provide a copy of this letter to all participating NHS organisations in England.

Appendix B provides important information for sponsors and participating NHS organisations in England for arranging and confirming capacity and capability. Please read *Appendix B* carefully, in particular the following sections:

- *Participating NHS organisations in England* – this clarifies the types of participating organisations in the study and whether or not all organisations will be undertaking the same activities
- *Confirmation of capacity and capability* - this confirms whether or not each type of participating NHS organisation in England is expected to give formal confirmation of capacity and capability. Where formal confirmation is not expected, the section also provides details on the time limit given to participating organisations to opt out of the study, or request additional time, before their participation is assumed.
- *Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria)* - this provides detail on the form of agreement to be used in the study to confirm capacity and capability, where applicable.

Further information on funding, HR processes, and compliance with HRA criteria and standards is also provided.

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Population-Specific Calcium Adjustment Equation

INVITATION

The Blood Sciences Department at Harrogate and District NHS Foundation Trust are interested in updating the current calcium estimation method. The new method will help our laboratory and doctors decide whether patient results are abnormal. To do this we need healthy volunteers to donate blood samples. We are inviting you to help us with a research project which we hope will enable us to derive the new equation for calcium. The following letter explains the research in more detail and what it would involve for you. Please take the time to read through the following information carefully and discuss it with friends, relatives or your GP if you wish. Please contact us if you find anything that is not clear or if you would like more information.

WHAT IS THE PURPOSE OF THE STUDY?

The current equation for calcium measurement was developed more than 40 years ago. Recent changes in technology and information indicate that reviewing the equation may improve the estimation of calcium and therefore improve the diagnosis of patients with bone disease. We aim to calculate and assess the performance of the new population-specific equations. We need you to help by providing a blood sample to help assess the performance of the equation.

WHY HAVE I BEEN CHOSEN?

We are inviting healthy people to take part in our study. You must be over 18 years of age not taking any medications not breast feeding or pregnant and not suffering from any medical condition such as diabetes cancer or heart disease.

WHAT WOULD TAKING PART INVOLVE?

We need you to help by donating a blood sample to help assess the performance of the equation. A maximum of three blood samples (2 blood tubes 5 ml each and 1 tube of 2.5 ml) is required. We analyse your blood samples and we use your data to assess the new equation performance. **You will be sent a copy of your blood results with the interpretation.** If you agree to take part in the study we expect you to attend a session at the Blood Sciences laboratory in Harrogate District Hospital for blood collection.

In the occasion where abnormal test results were found a letter from the investigators of this study which includes a copy of these results and their interpretations will be sent to your GP. Your GP may wish to further investigate these abnormal results.

WHAT ARE THE POSSIBLE RISKS OF TAKING PART?

By taking part in the study you may experience a minimal discomfort or bruising at venepuncture site. No other risk is anticipated.

DO I HAVE TO TAKE PART?

We do hope you will be able to find the time to help us but you are under no obligation to take part in this research. If you do decide that you would like to help with the study then you change your mind you will be free to leave it at any point and without having to give a reason.

WILL MY INFORMATION BE KEPT CONFIDENTIAL?

Patient confidentiality will be safeguarded during and after the study. We will destroy all direct identifiers and store only fully anonymised data in the longer term. All study data will be stored securely on an NHS server and only the main researcher will have access to your data. All personal data will be destroyed after the end of the study and only anonymised data will be kept for up to 10 years.

Your blood samples will be labelled with a code and only the main researcher will be able to link the samples and results of the tests to your name and other data. Your samples will be used for analysis including measurement of calcium albumin vitamin D parathyroid hormone and ionised calcium. Surplus samples will be destroyed once analysis is complete.

WHO HAS REVIEWED THIS STUDY?

All research in the NHS is looked at by an independent group of people called a Research Ethics Committee to protect your interests. This study has been reviewed and given favorable opinion by Health and Social Care Research Ethics Committee A (HSC REC A).

WHO SHOULD I APPROACH IF I AM UNHAPPY WITH THE STUDY?

If you have any concerns about the study and your involvement in it or you would like to make a complaint you may directly contact the Research and Development Department (R&D) in Harrogate Hospital. Contact details are: Research and Development manager (Dr James Hughes) Tel: 01423 555697 or email: research@hdft.nhs.uk.

GENERAL INFORMATION:

There are a number of web resources available which would give further information of calcium and its measurement. Such as www.labtestsonline.org.uk. For further information about this research study you may contact:

Nuthar Jassam

Consultant Clinical Biochemist

Head of Blood sciences Department Harrogate and District NHS Foundation Trust

Nuthar.jassam@hdfn.nhs.uk

Or

Dr Julian Barth

Consultant Chemical Pathologist

Leeds Teaching University Hospitals Trust

Julian.barth@nhs.net

Harrogate and District 
NHS Foundation Trust

Calcium Project

Centre Number:

Study Number:

Patient Identification Number:

CONSENT FORM

Name of Researcher:

Please tick box below:

1. I confirm that I have read and understand the information sheet dated for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.....
2. I understand that as a volunteer and I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.....
3. I agree to my GP being informed about my care during the study
4. I understand that relevant sections of all the research notes and data collected during the study may be looked at by individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.....
5. I agree to take part in the above study.....

Name of Patient, Date & Signature

Name of Person taking consent, Date & Signature (if different from researcher)

Researcher Date & Signature

Harrogate and District NHS Foundation Trust



Health Questionnaire- Calcium Study

1. Patient Demographics

Title Mr Mrs Miss Ms Dr Professor (delete as appropriate)
 Surname
 Forename
 Date of birth:
 Male or Female (delete as appropriate)
 Current Contact Address:

 Occupation
 Contact telephone number:
 Email:

GP name and address:

2. General Health Screening

Have you been unwell within last 1-2 weeks?	Yes/No (delete as appropriate) If yes please specify.....												
Do you have any medical conditions including any of the following?	<table border="0"> <tr> <td>Myeloma</td> <td>Yes/No</td> </tr> <tr> <td>Chronic Kidney Disease</td> <td>Yes/No</td> </tr> <tr> <td>Metabolic Bone Disease</td> <td>Yes/No</td> </tr> <tr> <td>History of Cancer</td> <td>Yes/No</td> </tr> <tr> <td>Liver disease</td> <td>Yes/No</td> </tr> <tr> <td>TPN feed</td> <td>Yes/No</td> </tr> </table> <p>Other (please specify)..... </p>	Myeloma	Yes/No	Chronic Kidney Disease	Yes/No	Metabolic Bone Disease	Yes/No	History of Cancer	Yes/No	Liver disease	Yes/No	TPN feed	Yes/No
Myeloma	Yes/No												
Chronic Kidney Disease	Yes/No												
Metabolic Bone Disease	Yes/No												
History of Cancer	Yes/No												
Liver disease	Yes/No												
TPN feed	Yes/No												
Have been sick within the last 4 weeks	Yes/No If yes, describe illness.....												
Do you use tobacco	Yes/No												

	If yes, have you smoked in the last hour
Are you taking any prescribed or over the counter medications including vitamin supplements and cod liver oil?	Yes/No If yes please specify.....
Did you take part in strenuous exercises in the last 24 hour?	Yes/No
Have you had a alcohol in the last 24 hour	Yes/No If yes, How much did you drink in a typical week..... For how many years this has been typical.....

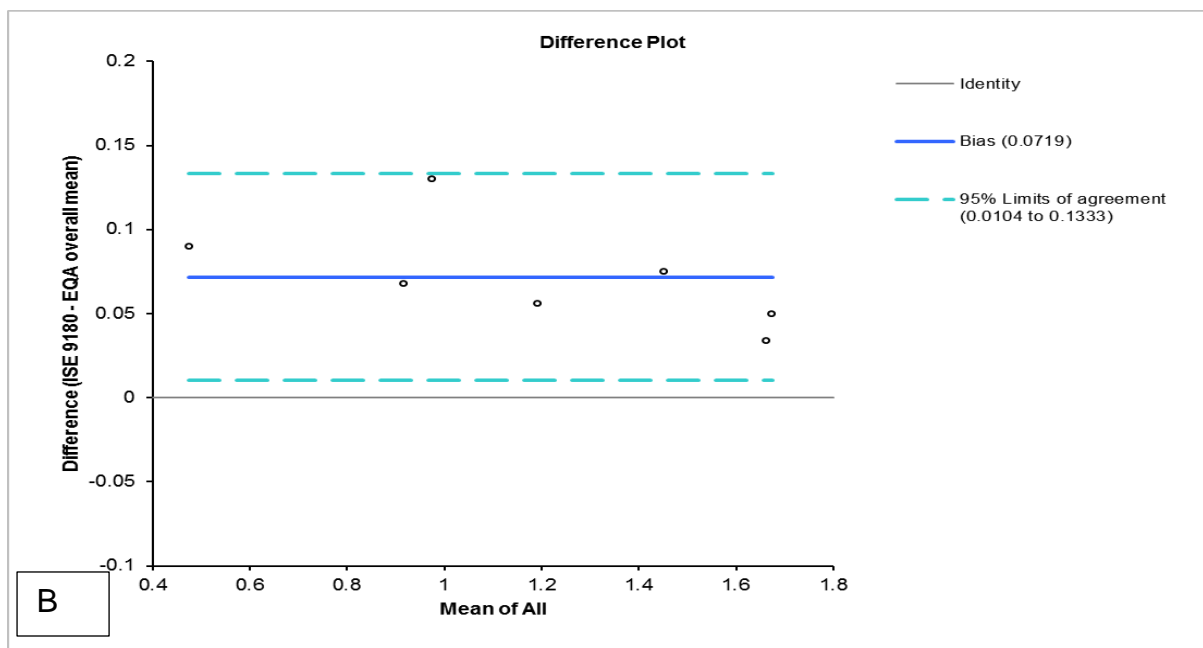
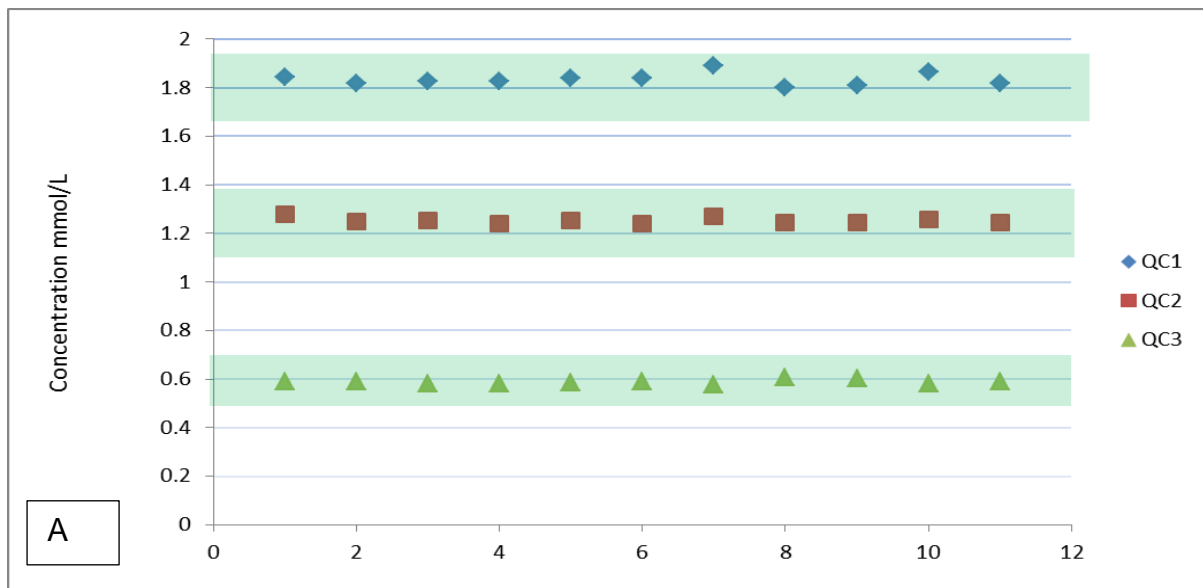
3. **Ethnicity Monitoring** (please select most appropriate option)

A: White	D: Black or Black British
British	Caribbean
Irish	African
Any other white background.....	Any other black background.....
.....
B: Mixed	E: Chinese
White & Black Caribbean	Chinese
White & Black African	Any other.....
.....
White & Asian	
Any other mixed background.....	
.....	
C: Asian or Asian British:	Not stated
Indian	Not stated
Pakistani	
Bangladeshi	
Any other mixed background.....	
.....	

Appendix II: Analytical precision (CV %) of calcium albumin PTH and vitamin D methods

Analyte	CV% for QC1	CV% for QC2	Acceptable limit
Calcium	1.24	0.85	2% State of the art
Albumin	3.33	2.72	4% State of the art
PTH	11.5	6.55	12.5% Desirable
Vitamin D	7.06	5.60	10% state of the art

Appendix III: Analytical performance for ionised calcium analyser ISE9180 during the period of study (A) ISE9180 CV% green shades indicate the acceptable limits as sets by the manufacturer. (B) Bias data given as a comparison with overall mean obtained from WEQAS



Appendix III: In house-derived reference interval for ionised calcium

Healthy volunteers were recruited from among members of staff and visitors to the Harrogate Hospital. This phase of the study was approved by the National Research Ethics Committee of Northern Ireland (Ref 17/NI/0010) Appendix Ia.

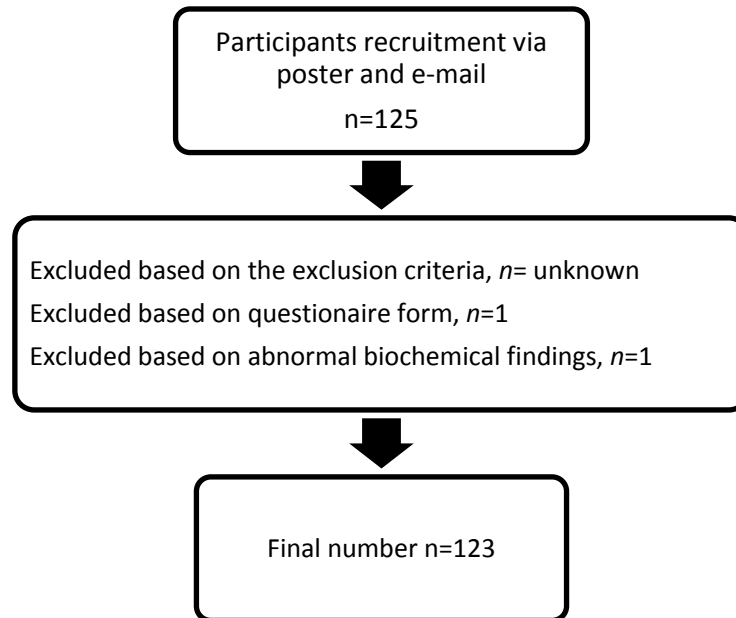


Figure 1: Flow chart of exclusion process.

N	123
Gender	96 female 27 male
Exercise in the last 24 hour	90% no exercise 9% mild level 1% strenuous exercise.
Ethnicity	93% white 7% Asian and black
PTH (1.6-7.0 pmol/L) Roche Cobas	86% within reference interval 14% within 7.3-14 (median = 8)

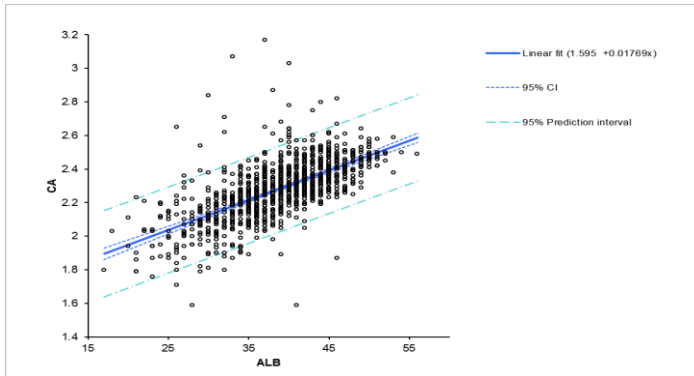
Table 1: Reference population characteristics

Platform	95% Interval				Normality
	LRL	90% CI	URL	90%CI	Anderson Darling
9180 Electrolyte Analyser (Roche)	1.183	1.178 to 1.199	1.33	1.326-1.347	1.2 p= 0.0037

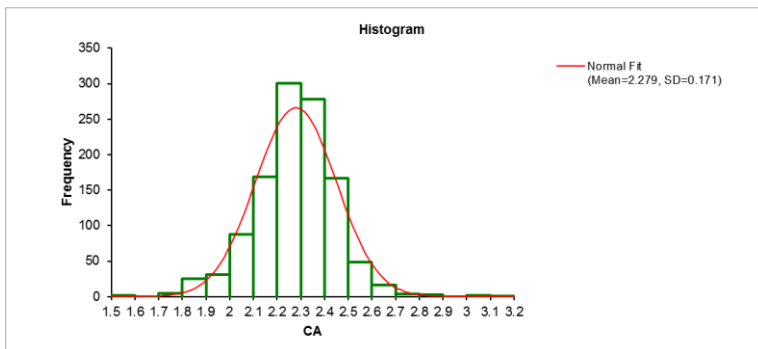
Table 2: ionised calcium reference interval. Ionised calcium was suitable for parametric analysis due to normal distribution.

Appendix IV: Adjusted calcium derivation equation

1. Using analyse-it perform Deming regression (x variable albumin and y variable Ca). This analysis gives an intercept or non- protein bound calcium and slope. See example below



2. Perform continuous summary descriptive on measured calcium data. This analysis gives the mean calcium for this population of data. Subtracting the intercept the non- protein bound calcium from mean Ca gives the average protein –bound calcium.



3. Enter intercept slope and mean Ca values into this equation:

$$\text{Adj calcium} = \text{total calcium} - (\text{slope} \times \text{albumin}) + (\text{mean calcium} - \text{intercept calcium}) \dots \dots \dots 1$$

From above analysis we find that:

Calcium mean = 2.279 mmol/L

Intercept = 1.5950

Slope = 0.01769 this figure rounded to (0.0177)

Place these values in equation 1: Adjusted calcium abbreviated as; Adj Ca Total calcium abbreviated as; T.Ca Albumen abbreviated as; Alb

$$\text{Adj. Ca} = \text{T. Ca} - (0.0177 \times \text{Alb}) + (2.279 - 1.595)$$

$$\text{Adj. Ca} = \text{T. Ca} - (0.0177 \times \text{Alb}) + 0.684$$

Divide both sides of the equation by 0.0177 and re-arrange.

$$\text{Adj. Ca} / 0.0177 = \text{T. Ca} / 0.0177 - (0.017 \times \text{Alb} / 0.0177) + 0.684 / 0.0177$$

$$\text{Alb} = (\text{T. Ca} - \text{Adj. Ca}) / 0.0177 + 39.0$$

Re-arrange to give the final adjustment equation.

$$\text{Adj. Ca} = \text{T. Ca} - 0.0177 (\text{Alb} - 39)$$