Evaluation of MITRA™ Volumetric Absorptive Microsampling (VAMS) as a Sampling Technique for β-Isomerized Carboxy-Terminal Telopeptide (β-CTX) and Type 1 Procollagen Amino-Terminal-Propeptide (P1NP) Determination Using Electro-Chemiluminescent Immunoassay (ECLIA).

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ABSTRACT

Introduction

Type 1 collagen forms >90% of the organic matrix of bone. Bone is continually being remodelled, where type 1 collagen is broken down and renewed. During this, fragments created by the synthesis of new type 1 collagen or the breakdown of old type 1 collagen are released into the blood. The most widely used fragments for assessment of bone turnover are the synthesis product type 1 procollagen amino-terminal propeptide (P1NP), and the degradation product β-isomerized carboxy-terminal telopeptide (β-CTX). The concentrations of these molecules are conventionally assessed in serum or plasma obtained by venepuncture. Here I assess whether the concentrations of total P1NP and β-CTX can be ascertained from whole blood samples collected by volumetric absorptive microsampling (VAMS).

Methods

VAMS and K_3-EDTA plasma samples were collected from military subjects (n = 44) enrolled in the UK Ministry of Defence's ADAPT study at three timepoints. The microsamples and K_3-EDTA plasma samples were analysed for P1NP and β-CTX by electrochemiluminescent immunoassay (ECLIA). The haematocrit (HCT) of the samples was assessed by automated cytometry on baseline whole blood K_3-EDTA samples. P1NP and β-CTX concentrations for microsamples were back-calculated to plasma equivalent values (PEVs), based on the HCT obtained.

Results

PEVs for β-CTX showed positive linear correlation with a Pearson's R of 0.900 when compared with conventional sampling. However, Bland-Altman analysis demonstrated

the wide variability among this sample set. VAMS did not yield any valid results for P1NP due to assay interference from haemolysis and analyser sampling errors.

Conclusions

With improved processing of the microsamples, VAMS could be employed for β-CTX measurement. Further work is required to optimise the sample collection procedure to eliminate haemolysis of the sample and re-evaluate VAMS as a collection method for the analysis of β-CTX and P1NP.

CHAPTER 1

Literature Review

Introduction

Bone is not a static, inert structure, as once thought, but a functionally diverse, metabolically active and dynamic tissue. Not only does it provide the biomechanical properties for locomotion, support for the rest of the body and the protection of vital internal organs, it is the environment where haematopoiesis occurs, has a role in both calcium and phosphate homeostasis, and also contributes to acid-base balance via hydrogen ion buffering (Clarke, 2008). Additionally, some studies have implied that bone also has an important role in endocrine function including fertility, muscle function, appetite regulation and energy metabolism (Oldknow et al., 2015, Mera et al., 2017, Mera et al., 2018, DiGirolamo et al., 2012). Most countries are experiencing an ageing population, with a concomitant increase in the prevalence of bone and mineral metabolism disorders, escalating the interest in, and the need for, effective techniques for screening, diagnosis and follow-up of patients with such pathologies in routine clinical practice and also in research. In the assessment of metabolic bone health, one of the most important tools available to researchers and/or clinicians is biochemical analysis of cellular and extracellular components of the bone matrix; biochemical markers that give an indication of the status of this dynamic tissue.

Collagen in bone

Collagen is a major structural component of bone and, in conjunction with the mineral hydroxyapatite, provides the required mechanical properties of the skeleton. Collagen is a superfamily of macromolecules (biopolymers) consisting of 28 distinct

types, classified into several groups, determined by the structure they form on maturation. The majority (>90%) of organic bone matrix consists of type 1 collagen, a fibril-forming collagen which comprises of three polypeptide chains wound into a triple helix tertiary structure. The triple helix is a heterotrimer made up of two identical α1(1) helices and one α2(1) helix, being held together by pyridinium cross-links. In adult bone, the majority of collagen is mineralised; a composite of predominantly calcium and phosphate which, together with hydroxyl groups, forms hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). This provides bone with the biomechanical properties required for load bearing, torsional stiffness, and tensile strength, (Fledelius et al., 1997, Sherman et al., 2015, Gelse et al., 2003).

In healthy individuals, bone is being continuously remodelled by tightly regulated anabolism of type 1 collagen in bone and catabolism of mature bone matrix. This occurs at identifiable, well-defined areas at the surface of both cortical and trabecular bone, known as bone remodelling units. This process is driven by the activity of osteoblasts (formation), osteocytes (maintenance) and osteoclasts (resorption). The resorption phase usually takes around 10 days to complete, followed by the formation phase that can last for as long as 3 months under normal conditions (Figure 1). Several biochemical markers (defined by The Biomarkers Definitions Working Group as "*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"* (Biomarkers Definitions Working Group, 2001)) are produced during this remodelling, reflecting the metabolic process they are considered to be derived from, which can be detected in serum, plasma and/or urine. The bone formation markers are either

consequences of collagen synthesis or incorporation of type 1 collagen into the matrix, matrix proteins or osteoblastic enzymes, whereas the resorption markers are degradation products of type 1 collagen breakdown or the osteoclastic enzymes that drive this process. By determining the concentration of bone resorption and formation markers (Table 1), osteoclast and osteoblast activity, and therefore bone turnover, can be estimated (Seibel, 2005).

Formation Markers	**Resorption Markers**
Bone-specific alkaline phosphatase	Hydroxyproline
Osteocalcin	Hydroxylysine-glycosides
C-terminal propeptide of type 1 procollagen (P1CP	Pyridinoline (PYD)
N-terminal propeptide of type 1 procollagen (P1NP).	Deoxypyrodinoline (DPD)
	Carboxyterminal cross-linked telopeptide of type 1 collagen (α- and β-isomerised forms) (α-CTX & β-CTX)
	Aminoterminal cross-linked telopeptide of type 1 collagen (NTX-1)
	Bone sialoprotein (BSP)
	Osteocalcin fragments (OC)
	Tartrate-resistant acid phosphatase, 5b (TRAP5b)

Table 1: Biochemical markers of bone turnover used in clinical practice.

Whilst there are several bone turnover markers shown in the above table, the decision was made to concentrate on the collagen formation and breakdown products for this project, as collagen is the main component of bone matrix.

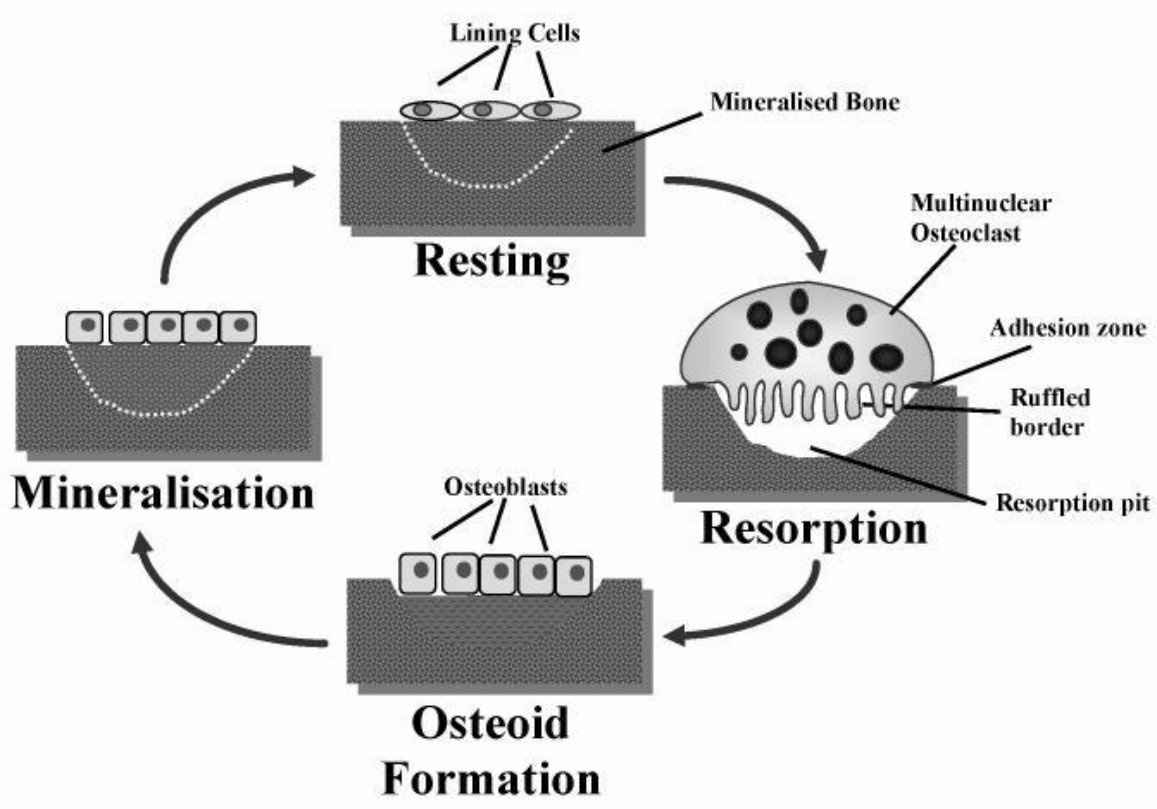


Figure 1. The bone remodelling cycle. In healthy individuals, the osteoclast-driven resorption phase takes approximately 10 days, followed by an osteoblast-driven formation phase that can last for up to 3 months. (Reproduced from Seibel MJ. Biochemical markers of bone turnover: part I: biochemistry and variability. The Clinical biochemist. Reviews. 2005 Nov;26(4):97-122.)

Type 1 collagen is ultimately derived from type 1 procollagen synthesized by fibroblasts and the bone forming cells, the osteoblasts (Burgeson, 1988). Similar to other secreted proteins, collagen polypeptides are synthesized by ribosomes on the rough endoplasmic reticulum (RER). The polypeptide chain then travels through the RER and the Golgi apparatus, where post-translational modifications occur, before being secreted. Post-translational modifications include hydroxylation of the proline (Pro) and lysine (Lys) residues, forming hydroxylproline (Hyp) and hydroxylysine (Hyl) respectively, and addition of the carbohydrates galactose and glucose. Galactosyl- and glucosyl-

residues, catalysed by the enzymes hydroxylysyl galactosyltransferase and galactosylhydroxylysyl glucosyltransferase, are transferred to the hydroxyl groups of Hyl. Type 1 procollagen contains both N-(amino) and C-(carboxy) terminal extensions. These extensions (propeptides) help to align the three polypeptide chains correctly and prevent the three chains from aggregating prematurely. On secretion from the cell, the propeptides are removed by procollagen N-proteinase and procollagen C-proteinase respectively during the conversion of procollagen to tropocollagen. The propeptides are named type 1 procollagen carboxy-terminal-propeptide (P1CP) and type 1 procollagen amino-terminal-propeptide (P1NP). The tropocollagen then aggregates into a microfibril and undergoes pyridinium cross-linking to produce the mature collagen fibre. The mature collagen fibre is subsequently incorporated into the bone matrix (Prockop et al., 1998). Whilst neither P1CP nor P1NP are 100% bone-specific in origin, studies on pigs have suggested that the concentration of P1CP (and therefore the same can be assumed for P1NP) in the serum that has entered the circulation (via the subclavian vein) from lymph draining from the collagen-containing soft connective tissues such as the skin is negligible, so the majority of P1CP and P1NP in the serum has been contributed by the metabolism (formation) of the organic bone matrix (Jensen et al., 1990).

Type 1 Procollagen Amino-Terminal-Propeptide (P1NP)

Type 1 procollagen amino-terminal-propeptide (P1NP) is used as biochemical marker of bone formation. P1NP was originally identified in humans from amniotic fluid and was referred to as foetal antigen 2 before it was discovered that it was in fact a homomer of the α1 chains of P1NP (Teisner et al., 1992). P1NP is a specific indicator of type 1 collagen deposition in bone tissue and therefore can be defined as a true bone

formation biomarker (Orum et al., 1996). During type 1 collagen formation P1NP is released into the intracellular space and eventually into the circulatory system. P1NP is thought to be released as a trimeric structure (derived from the triple helix structure of type 1 collagen) but is rapidly broken down to a monomeric form by thermal degradation effects, as the trimeric nature of P1NP is only maintained by non-covalent forces and the structure is labile at 37^{o}C (Jensen et al., 1998, Brandt et al., 1999). Assays that measure both the trimeric (high molecular weight) and monomeric (low molecular weight) forms of P1NP are known as "total P1NP" assays.

β-Isomerized Carboxy-Terminal Telopeptide (β-CTX)

β-Isomerized Carboxy-Terminal Telopeptide (β-CTX) is used as biochemical marker of bone resorption. As mature type 1 collagen is degraded, small fragments pass into the bloodstream, eventually being excreted by the kidneys. In physiological or pathological conditions, characterised by elevated bone resorption (e.g. post-menopausal bone loss, bowel and joint inflammatory diseases, or osteoporosis), type 1 collagen degradation is increased, and there is a corresponding rise in the plasma concentration of collagen fragments in the blood and urine. The non-helical β-isomerized C-terminal telopeptides (β-CTX) are fragments with high specificity for the degradation of mature type 1 collagen (Bonde et al., 1994). This spontaneous isomerisation occurs post-translationally and non-enzymatically in the polar, charged aspartic acid residue in the 8 amino-acid (8AA) sequence of the C-terminus of the α1 chain of type 1 collagen (EKAHβDGGR), the C-terminal polar, charged arginine being essential (Bonde et al., 1997). These β-isomerized telopeptides are highly specific for the degradation of type 1 collagen predominantly present in mature bone as the degree of

isomerization increases with the biological age of collagen (Gineyts et al., 2000). Therefore, serum β-CTX concentration can be used to assess bone degradation.

Clinical Utility of β-CTX and P1NP as Markers of Bone Turnover

β-CTX and P1NP are, respectively, the most commonly utilised markers of bone resorption and formation used in both basic and clinical research and in patient care. Firstly, for any biochemical marker to be of clinical utility, the change in the concentration of the marker in response to a clinical intervention or disease process must exceed the minimum significant change (MSC) (also known as least significant change (LSC)). This represents a change significantly large enough to be unlikely to be caused solely by a combination of biological variability within an individual and analytical variation in the assay, and thus reflecting change as a result of the clinical intervention or change in metabolism (Hannon et al., 1998, Rosen et al., 2000). Sources of biological variation in bone turnover markers include age, gender and race. There is also a strong diurnal variation in their concentrations. Concentrations of bone turnover markers in childhood tend to correspond to their growth rate, for example reflecting the high rate of postnatal growth seen in the first month of life and the growth spurt seen in adolescence (Mora et al., 1997, Mora et al., 1999, Tsukahara et al., 1999, Tsukahara et al., 1996). As adulthood progresses, bone turnover marker concentrations decrease to a nadir between 30 and 50 years of age, then increase with progression into old age. This increase is markedly higher in women than in men during the peri-menopausal and early post-menopausal years. Whilst men do not go through the equivalent of the menopause, they do experience a decrease in bioavailable (non-sex hormone-binding globulin (SHBG)-bound) testosterone and oestrogen as age progresses, which correlates

with an increase in bone resorption markers and bone loss (Resch et al., 1994, Kelly et al., 1989, Kuwana et al., 1988, Khosla et al., 1998, Khosla et al., 2001). These changes over the life-course reflect the cessation of longitudinal growth, the completion of bone mass consolidation, followed by the onset of the menopause in women at around 50 years and ageing in both women and men. Several studies have shown that ethnicity also has an effect on the incidence of osteoporosis, being lower in populations with African heritage than in those with Caucasian ancestry. There is an approximately 10% higher bone mineral density (BMD) at the lumbar spine, radius and proximal femur associated with a lower rate of bone turnover in black versus white children. Similar findings have also been shown in adults, with white adults consistently having lower BMD than their equivalent African-American counterparts (Slemenda et al., 1997, Ettinger et al., 1997, Meier et al., 1992). Bone turnover and therefore bone markers show significant diurnal variations, the highest concentrations being obtained during the early morning hours, decreasing to the lowest concentrations in the afternoon and evening (Eastell et al., 1992, Greenspan et al., 1997, Mautalen, 1970, Schlemmer and Hassager, 1999). This is especially true for β-CTX with one study reporting daily amplitude differences of up to 66% (Wichers et al., 1999).

Elevated serum concentrations of β-CTX have been reported for patients with increased bone resorption in conditions such as osteoporosis or hyperparathyroidism. The serum concentrations return to normal during antiresorptive therapy (Bonde et al., 1995, Ravn et al., 1996, Rosenquist et al., 1998, Christgau et al., 1998). Determination of β-CTX concentration in serum is therefore recommended for clinically monitoring the efficacy of antiresorptive therapy in osteoporosis or other bone diseases. Such

antiresorptive therapies include bisphosphonates, such as alendronic acid and zolendronic acid, receptor activator of nuclear factor-κB (RANK) ligand (RANKL)-inhibiting antibodies, such as denosumab, or hormone replacement therapy (HRT) using Selective Estrogen Receptor Modulators (SERMs), such as raloxifene. By monitoring patients' β-CTX concentrations throughout a treatment regime, therapy-induced changes can be demonstrated after just a few weeks (Rosenquist et al., 1998).

Whilst it is important to highlight that some of the above studies investigated other bone turnover markers such as urinary cross-linked amino-telopeptides (uNTX), urinary pyridinoline (uPYD), urinary deoxypyridinoline (uDPD), bone-specific alkaline phosphatase (BSALP) and osteocalcin (OC) and do not necessarily assess β-CTX or P1NP in their analysis, it is a fair assumption that, as β-CTX and P1NP are also markers of bone turnover, they would also follow the same biphasic pattern as age progresses.

Conventional Sampling Techniques

As indicated above, bone turnover markers can be found in either urine, serum or plasma. Whole blood can be divided into three portions: the plasma (clear extracellular fluid) containing platelets (required for clotting); the white blood cells (leucocytes); and the red blood cells (erythrocytes). The haematocrit (HCT) is the proportion of whole blood that is made up of erythrocytes and is sometimes referred to as the packed cell volume (PCV). The most widespread blood collection method for routine biochemical analysis is phlebotomy to collect sufficient blood (usually between 5 and 10 mL) to yield an appropriate volume of either plasma (where an anticoagulant in the collection tube has been used) or serum (obtained post-coagulation i.e. plasma

minus platelets) upon centrifugation. What is deemed an 'appropriate volume', depends on various factors such as the number of analytes being assessed, the analytes of interest themselves and the analytical technique employed. Depending on the stability and/or nature of the analyte of interest, anticoagulants may be employed. These include tri-phosphate ethylene-diamine-tetra-acetic acid (K_3-EDTA), sodium citrate and fluoride oxalate. Much work has been carried out to standardise sample collection, to minimise or eliminate the pre-analytical issues mentioned above. Whilst there are certain sources of biological variation which, of course, cannot be mitigated for, such as gender, age and race, standardisation of sampling times can certainly reduce variability produced by the diurnal nature of bone turnover markers. This is also true of bone markers found in urine, with timed urine collections such as first or second morning void or 24 hour collections preferred, depending on the analyte to be assessed. If the biochemical marker is affected by diet, or the timing of food intake, then appropriate instruction for fasting needs to be given to patients prior to venepuncture. Consideration of the effects of acute exercise shortly prior to blood collection also needs to be taken, as this can cause some markers to rise by 30-40% of their baseline concentrations (Woitge et al., 1998, Fujimura et al., 1997).

Conventional blood collection requires specialist staff and equipment, and if there is requirement for the sample to be transported long distances between collection and analysis, this usually occurs refrigerated or frozen post-centrifugation, which can be expensive. Precautions also need to be taken to minimise potential infection via needle stick incidents and/or other accidents.

Microsampling

Microsampling refers to the collection of a small amount of blood (typically <50 μL), usually from a finger-prick in adults and a heel-prick in neonates. The most widespread method of microsampling collects blood from the finger or heel-prick onto a paper-based substrate which is allowed to dry for storage / shipping and are known as dried blood spots (DBS). It is considered more patient-friendly due to its relative non-invasiveness when compared with conventional venepuncture. The low sampling volume requirement is ideally suited for use in paediatric practice and in the elderly population in which conventional venepuncture can prove to be difficult (Lehmann et al., 2013), and in which bone turnover monitoring is important for overall health and well-being. It is also suited to other patient groups in which venepuncture may be challenging, for example in those with limited mental capacity. A phlebotomist or member of nursing staff is not required to perform venepuncture on patients capable of self-sampling as minimal training is required, which reduces the time and the inconvenience of attending hospital/GP appointments for phlebotomy as the sampling can be done at home.

The reduced volume and the fact that DBS do not need to be shipped refrigerated or frozen also means there are financial benefits to microsampling and allows patients to mail their samples at ambient temperature, streamlining the process of transporting samples to the laboratory and improving efficiency, as clinicians can be in possession of the results in advance of routine appointments. This is also an advantage for sampling in remote communities and communities with limited technical infrastructure such as laboratories and cold-chain logistics (Judd et al., 2003).

Additionally, there is evidence that dried blood samples carry a reduced risk of infection from certain pathogens than a conventional wet sample as some viruses are deactivated upon desiccation due to disruption of the viral capsid (Déglon et al., 2012, Resnick et al., 1986). All of these benefits also make this method of sample collection attractive for clinical study recruitment. However, conversion of measured analyte concentrations in DBS samples to their plasma equivalents has hindered the implementation of microsampling in routine patient care. This is because the viscosity of the patient's blood (the higher the HCT, the more viscous the sample) has an impact on how a DBS sample spreads through the absorbent substrate, and therefore, decisions on whether to take sub-punches or use the entire blood spot can have a major influence on the precision and accuracy of analytical results. Depending on the analyte of interest and the absorbent substrate used for microsampling, HCT values can cause significant assay bias (Spooner et al., 2015b, Denniff and Spooner, 2010, O'Mara et al., 2011). This is widely known as the "haematocrit issue".

Volumetric Absorptive Microsampling (VAMS)

Mitra™ is an innovative volumetric absorptive microsampling (VAMS) system developed by Neoteryx (Torrance, CA, USA) (Spooner et al., 2015a). They are polymer-based tips containing a network of capillaries with a fixed internal capacity, and so have the advantage over more traditional, paper-based DBS because they absorb a consistent volume of whole blood sample (20 μL), regardless of the patient's HCT. Previous studies have used VAMS as a sampling technique where conventional serum / plasma samples would ordinarily be used (Denniff and Spooner, 2014, Denniff et al., 2015, Parker et al., 2015, Mercolini et al., 2016, Kip et al., 2017). Whilst these studies claim to overcome the

HCT bias, they involved direct interpolation from standard curves generated from matrix-matched calibrators, which would be impractical to achieve on platforms where it is necessary for the manufacturer's calibrators to be used. These previous studies do not address a scenario where the calibrators must be, by the nature of the analytical method, in a different matrix, as described herein. It is also worth noting that these previous studies did not address the issue of whether the analytes assessed undergo erythrocyte-plasma partitioning, which, if it occurs, HCT variation would exert a greater influence on final determined concentrations.

Two of the formats designed by Neoteryx are the 'butterfly' and 'clamshell' cartridges, containing two and four microsampling devices respectively, which can be distributed to patients for sampling and subsequent shipping within a sealable foil pouch, with or without a sachet of desiccating material. This design can be augmented by addition of vials of appropriate liquid to keep the sample hydrated if necessary. This liquid would need to be suitable, so no interference is observed in the analytical method employed. For studies with access to a central laboratory, there is also a 96-tip format which can interface with automated and semi-automated sample processing platforms, for high throughput analysis for the purposes of therapeutic drug monitoring and drugs of abuse screening. An illustration of the Neoteryx Mitra^{TM} VAMS system in use is shown below.

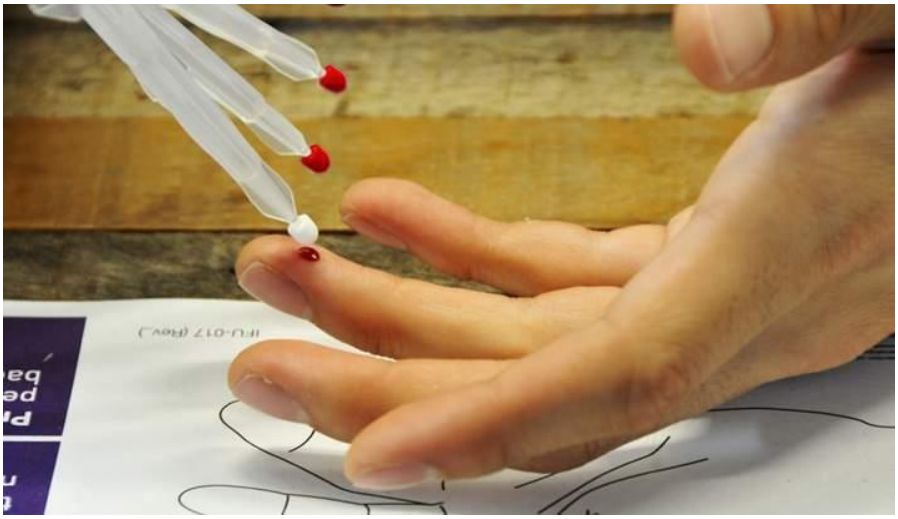


Photo: Courtesy of Neoteryx, LLC.

Detection Methods

Current methods for the detection of β-CTX and total P1NP in serum and plasma include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and electrochemiluminescent immunoassay (ECLIA), with ECLIA being the most widespread in routine laboratories. Spectrophotometry is also widely used in colorimetric and turbidimetric assays.

Electrochemiluminescence

Electrochemiluminescence (ECL) describes any emission of light from a chemical solution when it relaxes from an electrically excited state to its lower-level state after experiencing electron-transfer reactions. Electrochemiluminescent Immunoassay (ECLIA) utilises this property in conjunction with an immunoassay (Richter, 2008).

Roche Diagnostics GmbH (Mannheim, Germany) have refined this into an automated process on their COBAS platform, where the analyte of interest is incubated with a biotin-conjugated antibody raised against the analyte of interest, along with a

316 ruthenium compound (Tris(2,2'-bipyridyl)ruthenium(II) complex $[Ru(bpy)_3]^{2+}$)-
317 conjugated antibody to a separate epitope on the same analyte of interest, creating a
318 sandwich complex. This immunologically-created sandwich complex is further incubated
319 with para-magnetic microparticles, coated with streptavidin. The biotin-streptavidin
320 binding ensures that any captured analyte from the first incubation is further complexed
321 to the para-magnetic microparticles. The solution is then transferred to the measuring
322 cell of the COBAS platform, where the para-magnetic microparticles are subjected to a
323 magnetic field and immobilised. ProCell M™ (Phosphate buffered solution containing
324 tripropylamine (TPA) at 180 mmol/L and a small concentration of detergent (<0.1%)) is
325 then flushed through the measuring cell, removing any unbound compounds and
326 providing the reaction environment with the co-reactant (TPA) required for the
327 excitation/relaxation to occur when an electrical potential difference (voltage) is
328 applied. The wavelength of the photon emitted as the $[Ru(bpy)_3]^{2+}$ decays to its lower-
329 level state is around 620 nm and this emission is detected by a photomultiplier. The
330 intensity of the emission is directly proportional to the concentration of the analyte of
331 interest in the original sample, and is quantified by comparing to a two-point calibration
332 curve, generated prior to analysis. Assay performance is assessed by analysing quality
333 control (QC) materials with known target concentrations of analyte, with acceptable
334 performance being determined by whether or not the concentration obtained falls
335 within pre-defined range from the target concentration (±2 standard deviations (SD)
336 from the mean).

337 The COBAS serum P1NP ECLIA utilises monoclonal mouse antibodies raised
338 against P1NP to detect both the trimeric and monomeric fractions of P1NP present in

blood and is therefore called Total P1NP. The COBAS serum β-CrossLaps ECLIA is specific for crosslinked β-isomerized type 1 collagen fragments, independent of the nature of the crosslink (e.g. pyrrole, pyridinolines etc) (Te Koppele, 1998). The assay specificity is guaranteed by using two monoclonal antibodies against the linear β-8 amino acid octapeptides (EKAHD-β-GGR) epitope. The COBAS ECLIA β-CrossLaps serum assay therefore quantifies all type 1 collagen degradation fragments that contain two of the β-isomerized octapeptide (Rosenquist et al., 1998, Christgau et al., 1998).

Spectrophotometry

Spectrophotometry is one of the most widely used techniques employed in biochemistry and molecular biology, measuring the absorption of light in the visible and ultraviolet regions of the spectrum. Two fundamental principles govern the light absorption of a solution. Firstly, the absorption of light passing through a solution is exponentially related to the number of molecules of the absorbing solute (i.e. the solute concentration), and secondly the absorption of light passing through a solution is exponentially related to the length of the absorbing solution. Spectrophotometry applies the Beer-Lambert Law which is a combination of these two principles and states that the relationship between the concentration and the absorbance of a solution is linear, enabling the concentration of the solution to be calculated by measuring its absorbance. In its simplest form, the equation defining the Beer-Lambert law is $A = \varepsilon cl$, where A = absorbance, ε = absorption coefficient, c = concentration of the analyte of interest and l = the length of the light path through the sample being analysed (Atkins and De Paula, 2006).

Haematocrit Determination

Conventional methods to measure the HCT include Wintrobe tubes, where whole blood is centrifuged or allowed to sediment in heparinised, graduated capillary tubes and the red blood cell fraction directly measured. More recently developed methods are the automated cytometric methods where calculation of the HCT is achieved by multiplying the number of red cells by the mean corpuscular volume (MCV) (Billett, 1990). Sysmex (Kobe, Japan) have developed the XN-series automated haematology analyser that utilises these cytometric methods with various innovations in waveform transformations and unique digital technology and algorithms to improve full blood count analysis (Arbiol-Roca et al., 2018, Briggs et al., 2012, Seo et al., 2015) The accepted reference range for HCT in the UK is 0.40 – 0.50 L/L for men and 0.35 – 0.45 L/L for women (Osei-Bimpong et al., 2012). However, in addition to gender, there is also variation between individuals depending on age, physiology (such as disease state, pregnancy or high-altitude training), and season can also have an effect (Thirup, 2003).

CHAPTER 2

Proposal & Methods

Scientific Rationale & Outline of Project Objectives

Microsampling has received increased attention in recent years, with articles returned by searching "microsampling" on PubMed rising from 5 in 2003 to 58 in 2019. Currently, searching "microsampling AND bone markers" returns zero results. The COBAS 6000 e601 module (Roche Diagnostics GmbH, Mannheim, Germany) uses 50 µL of sample to analyse β-CTX and 20 µL to determine total P1NP. This provides the opportunity to investigate whether a microsample could be used instead of a conventional plasma sample to assess an individual's β-CTX & total P1NP concentrations. The project undertaken was to evaluate Mitra™ VAMS devices as a blood sample collection method for subsequent quantification of β-CTX and total P1NP as microsampling has a number of advantages over conventional venepuncture, as set out previously in Chapter 1.

To assess the β-CTX and total P1NP concentrations in a VAMS sample, and convert those to clinically meaningful values, the haematocrit (HCT) of the sample also needs to be determined, to calculate the original plasma content of the same volume of a matched conventional whole blood sample. Any calculation of β-CTX and total P1NP would have to take this HCT value into consideration, as the higher the HCT, the lower proportion of plasma in a sample meaning the β-CTX and total P1NP concentrations obtained would be commensurately reduced.

Overcoming the disparity between the volume of sample collected (20 µL) and the volume required for analysis (120 µL (50 µL for β-CTX + 20 µL for total P1NP + 50 µL

dead volume)) necessitates a dilution which, in turn, presents the issue of conceivably decreasing the measurable concentration to below the detection limits for the assay. The lower limits of detection for β-CTX & total P1NP are 0.010 µg/L and 5.00 µg/L, which are around 3% and 13% of the average expected values respectively (Roche Diagnostics, 2019, Roche Diagnostics, 2020), suggesting dilutions of up to 1:30 for β-CTX and 1:7 for total P1NP could be successfully employed during extraction for subjects in the normal range. However, these would need to be revised for individuals with suspected lower concentrations of β-CTX and total P1NP, and assessed more thoroughly due to the already reduced plasma content of a VAMS sample as a result of the HCT. Another challenge will be the possible effects desiccation has on the stability of β-CTX and total P1NP. If the collagen products denature further upon drying, then the specificity of the assays will inevitably be reduced.

Therefore, the objectives of the project were three-fold:

1. To optimise extraction of β-CTX & total P1NP from whole blood samples collected by the Mitra™ VAMS system, and to quantify their concentrations using ECLIA.
2. To determine HCT from dried blood samples collected by VAMS using spectrophotometry.
3. To generate an algorithm for the conversion of whole blood β-CTX & total P1NP concentrations into plasma-equivalent (and therefore clinically relevant) β-CTX & total P1NP concentrations based on the VAMS-obtained concentration and HCT value.

Methods

Residual K_3-EDTA whole blood samples collected by venepuncture from routine analysis at the Norfolk & Norwich University Hospital (NNUH) Department of Laboratory Medicine were used for the optimisation of extraction of β-CTX & total P1NP from whole blood collected by Mitra™ VAMS system (Objective 1) and validation of the spectrophotometric measurement of HCT (Objective 2). All samples were anonymised at point of access and processed in accordance with generic ethical approval for assay development (UK Department of Health, 2011). To accomplish Objective 3, samples would be provided by the ADAPT (LongReach) study in conjunction with the Ministry of Defence (MoD) in accordance with ethics review reference 931/MODREC/18

Mitra™ VAMS in the 'butterfly' cartridge format collect two matched microsamples. I initially proposed to use one of these to assess β-CTX & total P1NP concentration, and the other to determine HCT however, as explained in more detail below, this protocol was changed due to insufficiencies in suitable equipment at the MoD sampling centre.

Total P1NP & β-CTX Determination from Dried VAMS

I proposed to extract β-CTX & total P1NP from whole blood samples collected by the Mitra™ VAMS system, and to quantify their concentrations using electro-chemiluminescent immunoassay (ECLIA) technology on the e601 module of a COBAS 6000 platform. This technology has been validated for serum/plasma β-CTX & total P1NP concentrations, necessitating the conversion of whole blood (VAMS) concentrations to

plasma equivalent concentrations. For this novel microsampling and extraction technique to be useful in the clinical/research setting, whole blood β-CTX & total P1NP concentrations need to be converted into clinically relevant values, otherwise normal reference intervals would need to be defined for this sampling technique, which would require a much larger number of samples, across all population characteristics to achieve. I hypothesised that whole blood concentrations from VAMS samples would be significantly lower than plasma concentrations due to the presence of cellular material (predominantly erythrocytes) in a whole blood sample.

Given the majority of cellular material in whole blood consists of the volume of erythrocytes (i.e. HCT), which varies between individuals depending on several factors (Thirup, 2003), this proposal also included a method of estimating HCT from dried blood samples collected by VAMS, in order to accurately and precisely determine the plasma equivalent β-CTX & total P1NP concentrations. The average HCT is around 45% for males and 40% for females (Osei-Bimpong et al., 2012). The expected β‑CTX & total P1NP concentrations in healthy individuals are shown below in Table 2.

β-CTX & total P1NP concentrations would be determined by first rehydrating the dried VAMS sample to reflect the physiological state and volume of the original whole blood sample. The analytes would then need to be extracted off the absorbent tip of the VAMS device before analysis by ECLIA on the COBAS platform. However, considerations in the extraction method include the requirement for sufficient volume for the COBAS technology to adequately pipette the sample twice (once for each analyte), without diluting the sample beyond the lower technical limits of both detection and

quantification, and for there to be no interference with the biochemistry of the ECLIA technology from the extraction medium. To address the latter consideration, a proprietary sample diluent routinely used on the COBAS was selected as the preferred extraction medium. As explained earlier, the minimum total volume required for analysis would be 120 µL; 50 µL for β-CTX, 20 µL for total P1NP and 50 µL for the dead volume. Therefore, adding 120 µL of sample diluent to the dried sample would simultaneously reconstitute the sample and provide sufficient volume for analysis. Once this extraction was optimised (see Preliminary Work, below), results obtained from the conventional plasma samples from the MoD cohort were statistically compared with matched VAMS values from the same cohort to generate an algorithm allowing laboratory staff, researchers, clinical biochemists and/or clinicians to mathematically transform VAMS β-CTX & total P1NP results to plasma β-CTX & total P1NP results, negating the necessity to redefine reference intervals when using this sampling technique and fulfilling Objective 3.

β-CTX	N	Mean (ng/mL)	SD (ng/mL)
Men			
30-50 years	165	0.300	0.142
50-70 years	109	0.304	0.200
>70 years	365	0.394	0.230
Women			
Premenopausal	254	0.299	0.137
Postmenopausal	429	0.556	0.226

Total P1NP	N	Mean (ng/mL)	Range (5^{th} – 95^{th} percentile)
Men (Jenkins et al., 2013)	1143	47.50	15.00-80.00
Women			
Premenopausal	129	30.10	15.13-58.59
Postmenopausal (on HRT)	154	31.74	14.28-58.92
Postmenopausal (no HRT)	290	45.05	20.25-76.31

Table 2. Expected β-CTX & total P1NP concentrations in healthy individuals (Adapted from COBAS pack inserts (Roche Diagnostics, 2020, Roche Diagnostics, 2019), except (Jenkins et al., 2013))

Preliminary Work

Initial experiments to determine β-CTX and total P1NP from a dried Mitra™ VAMS device used a proprietary diluent from Roche Diagnostics (Universal Diluent) to reconstitute the dried blood sample before analysis on the COBAS platform. The proprietary diluent was used to minimise interferences with the biochemistry of the assay. For β-CTX and total P1NP measurement from whole blood, the dried tip was placed into a 200 µL Eppendorf tube with 120 µL of the Universal Diluent. This represents a 1:5 dilution – 20 µL to replace the liquid lost through desiccation and an additional 100 µL to ensure sufficient volume for the COBAS platform to pipette the sample. The Eppendorf was agitated at 1400 rpm at 25^{o}C for 30 minutes to ensure complete reconstitution before being analysed for β-CTX and total P1NP as per standard protocols.

The β-CTX results obtained from these initial experiments were confounding as the concentrations measured were consistently the same (~0.74 µg/L) across all QC materials and samples, regardless of the matched plasma/QC material result. This suggested there was an interfering factor within the diluent. This was tested and proved to be the case, as when the neat diluent was assayed, it delivered a β-CTX concentration of 0.70 µg/L. The diluent was replaced by another proprietary diluent from Roche Diagnostics (MultiAssay Diluent) which was tested as a blank and returned an undetectable result for both β-CTX and total P1NP.

This approach produced satisfactory results as a proof of concept design, however, deviations from expected results were deemed to be due to the red blood cell content of the resulting solution interfering with the assay. It is not advised to run haemolysed samples for β-CTX or total P1NP as haemoglobin interferes with the assays

(Roche Diagnostics, 2020, Roche Diagnostics, 2019). It is worth noting that haemolysis does not interfere with certain analytes, such as the ones mentioned in Chapter 1 (Denniff and Spooner, 2014, Denniff et al., 2015, Parker et al., 2015, Mercolini et al., 2016, Kip et al., 2017). It was also noted that the Mitra™ tip retained a pink hue, suggesting that some material had remained inside the capillary network of the tip, which could conceivably be a source of variability within any results. Further experiments were carried out with a modified extraction technique, namely, the wet tip was placed into a spin column including a filter (Bio-Rad, UK) with 120 µL of the MultiAssay Diluent, followed by agitation at 1400 rpm at 25°C for 30 minutes to ensure the sample had been extracted from the tip thoroughly. The spin column with the bottom snapped off was then placed into a collection tube and centrifuged at 15000 rpm for 5 minutes at room temperature. This separated the red cell material allowing the supernatant to be carefully removed and analysed. This extraction technique also left the Mitra™ tip without the pink hue previously observed, suggesting all the material had been removed from the tip, minimising this as a potential source of pre-analytical variability within the results (Figure 2).

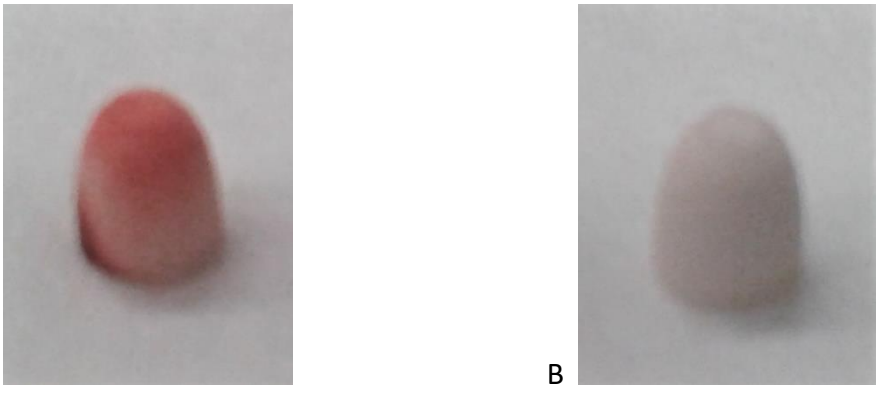


Figure 2. Mitra™ tips post-extraction. (A) example of tip when initial sample allowed to dry (showing pink hue); (B) example of tip when initial sample not allowed to dry (pink hue absent).

These experiments yielded very encouraging data for β-CTX, however, many of the samples analysed for total P1NP returned results that were undetectable. It was reasoned that the 1:5 dilution of the sample was too high for accurate determination of total P1NP. This led to dilution experiments being performed to assess the linearity of dilution for both β-CTX and total P1NP, where high calibrators and two levels of QC material (PreciControl Varia 1 (PCV1) & PreciControl Varia 2 (PCV2)) were serially diluted using MultiAssay Diluent before analysis. The results of these dilution experiments are shown in Figure 4 in Chapter 3.

Haematocrit Determination

To address the second objective of the project, spectrophotometry was used to estimate HCT. A standard curve was created from a whole blood sample with known HCT; diluting this known sample appropriately gave a standard curve from which an unknown blood sample's HCT could be determined. HCT determination allowed the plasma proportion of that sample to be calculated, as the VAMS device absorbs a fixed volume of blood. This was intended to be used to back-calculate the β-CTX & total P1NP concentrations from a VAMS sample to a plasma equivalent value, which would correspond to established reference ranges for these analytes.

Calibration curves for HCT with relevant working ranges were consistently generated by taking a conventional K_3-EDTA whole blood sample with known volume and HCT measured by standard analytical methods, centrifuging at 4200 rpm for 5 minutes at room temperature and then removing and discarding the resultant plasma

equivalent to half the original sample volume. This sample is then re-homogenised on an end-over-end rotary mixer set at 20 rpm for 5 minutes at room temperature, resulting in a whole blood sample with a haematocrit double that of the original sample (Figure 3).

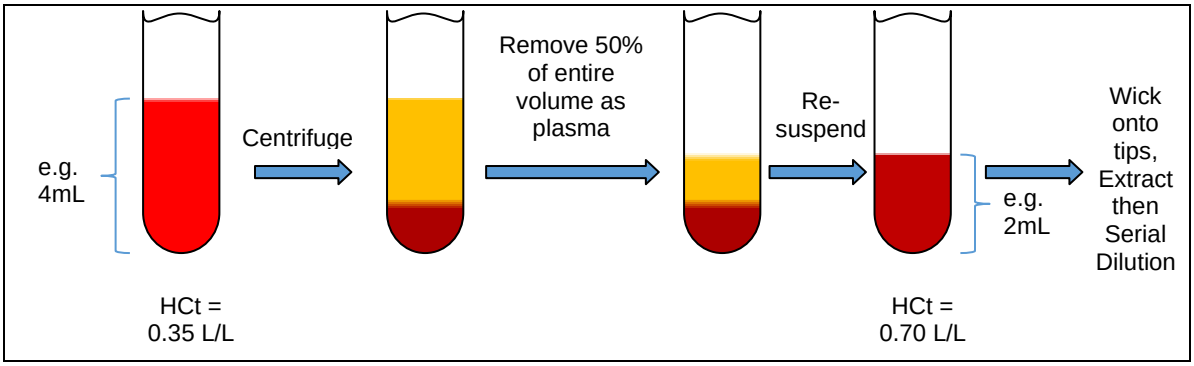


Figure 3. Method for preparing top standard for HCT determination.

This sample is absorbed onto a Mitra™ VAMS device and allowed to dry for a minimum of two hours, as per the manufacturer's instructions for use. The absorbent tip is removed from the device, placed in a 1.5 mL Eppendorf tube and 500 µL haemolysing reagent (A. Menarini, UK) added. The sample is agitated on an orbital microtube shaker at 1400 rpm for 30 minutes at 25°C. The resulting solution serves as the top standard for the calibration curve and is diluted appropriately with the haemolysing reagent to create the other four standards, with the haemolysing reagent acting as the zero standard, generating a 6 point calibration curve, as shown in Results Chapter, Figure 5. Test samples and quality control (QC) materials (Randox Biosciences, UK) are processed in the same manner, without the subsequent dilution. Fifty microlitres of each standard, QC and test sample are pipetted into a designated position of a 384-well microplate and read at 576 nm on a Thermo MultiSkan (Thermo Scientific, UK). This wavelength represents the wavelength at which maximum absorbance (λ_{max}) occurs in such solutions, as determined by a previous spectral analysis of 25 different samples with a range of haematocrit values prepared in this way (data on file). The calibration curve is

562 generated by plotting the HCT of the standards (values based on the HCT of the top
563 standard and subsequent dilutions) on the abscissa against the optical density (OD)
564 obtained on the ordinate axis. A line of best fit is determined by the MultiSkan software
565 and the HCT of each QC and test sample is determined by direct interpolation of this
566 calibration curve. The results generated by spectrophotometry were then compared
567 against results obtained by routine full blood count analysis at NNUH on the Sysmex
568 haematology analyser.

569 A total of 106 test samples were assessed to validate this protocol, with the
570 results shown in Figure 6. The total allowable error between analytical methods for
571 haematocrit has been defined as ± 6% by the Clinical Laboratories Improvement
572 Amendments 1988 (CLIA '88) guidelines and by the American Association of Bioanalysts
573 (AAB) (Innovations).

574 ## Application of the theory – ADAPT Study

575 To address the third objective of the project, the ADAPT (LongReach) study, in
576 association with the Ministry of Defence (MoD), involved taking VAMS samples
577 concurrently with conventional whole blood and plasma samples from subjects
578 undergoing a five day intensive military training exercise. VAMS and conventional
579 plasma samples from these subjects were to be taken at baseline (before the military
580 exercise), at day 5 on completion of the military exercise, and after a 3-day period of
581 recovery (day 8). Baseline whole blood samples were also collected for full blood count
582 analysis. β-CTX & total P1NP concentrations assessed according to standard protocols in

583 the matched plasma samples would provide the results for comparison with results
584 obtained from the VAMS samples.

585 However, limitations in the appropriate equipment at the sampling centre (no
586 agitator) meant that the method of agitating the VAMS tip with the MultiAssay Diluent
587 before centrifuging was not possible. Instead, it was decided that six VAMS tips collected
588 from each individual (equivalent to 120 µL whole blood) would be collected in one spin
589 column at each time-point and these would be centrifuged, the filtrate aliquotted into a
590 separate tube before being frozen and sent to the laboratory for analysis.

591 For the baseline samples only, 250 µL haemolysing reagent was added to the
592 remaining red blood cells for spectrophotometric HCT analysis. Only baseline samples
593 were collected for HCT analysis as within 8 days HCT does not vary significantly in
594 healthy individuals. This HCT was to be applied in any subsequent calculation of plasma
595 equivalent values, regardless of the time-point at which the microsample was collected.

596 The extraction from six tips was tested prior to commencement of the study
597 using residual K_3-EDTA whole blood samples from routine analysis at the NNUH
598 Department of Laboratory Medicine and yielded clear plasma from the centrifuged
599 sample. It is worth noting that samples extracted in this way are similar to conventional
600 venepuncture samples as the cellular material in the sample has been removed before
601 analysis, and assuming there is no other interference in the biochemistry of analysis,
602 results obtained from these samples would only require a simple dilution factor to be

applied in order to determine the true analyte concentrations, without the need to assess the HCT.

In practice however, the capillary blood collected from the MoD cohort had no anticoagulant present, and subsequently began to clot almost immediately upon sampling and contact with air. When these were centrifuged, it was observed that the serum collected was grossly haemolysed. It is postulated that upon clotting, there was an irreversible interaction between the erythrocyte cell walls and the polymer of the sampling device meaning that when the tips underwent centrifugation post-collection, the erythrocytes experienced catastrophic physical disruption, resulting in the haemolysis observed. It was decided that as the samples contained red cell material that the HCT would indeed need to be taken into consideration in order to convert the β-CTX and total P1NP concentrations to plasma equivalent values.

On receipt of the aliquotted VAMS samples from the MoD study, 120 µL MultiAssay diluent was added and the sample vortexed. The resulting solution was then centrifuged at 15000 rpm, for 5 minutes and pipetted into COBAS micro-cups and centrifuged again at 15000 rpm for 5 minutes at room temperature to spin down any sample that may have adhered to the side walls of the COBAS micro-cup, to minimise any cellular material that may be suspended in the sample as a result of the erythrocyte disruption upon collection/pipetting and also to remove air bubbles. These steps aimed to decrease the likelihood of sample aspiration errors (either short sampling or clot detection) by the COBAS 6000 automated platform. The conventional venous K_3-EDTA plasma samples were analysed for β-CTX and total P1NP in the established manner for

routine sample processing. The results generated from the VAMS protocol were then compared with the results from the corresponding conventional sample for each subject and time-point.

Calculation of Plasma Equivalent Values (PEV) from Whole Blood Samples

For this project, the plasma proportion of a whole blood sample is assumed to be equal to one minus the haematocrit value (Equation 1). It is also assumed that β-CTX and P1NP do not enter the erythrocytes, so the expected whole blood analyte concentration can therefore be deduced as the analyte concentration in a conventional venous plasma sample multiplied by the plasma proportion of the whole blood sample (Equation 2). Therefore, a plasma equivalent value (PEV) can be derived from an observed whole blood analyte concentration by effectively rearranging Equation 2 and dividing the observed whole blood analyte concentration by the plasma proportion (Equation 3):

$$Plasma\ proportion = 1 - HCT \quad (\textit{Equation 1})$$

$$WB[analyte]_{Expected} = Plasma[analyte]_{Conventional} \times (1 - HCT) \quad (\textit{Equation 2})$$

$$Plasma[analyte]_{Equivalent} = \frac{WB[analyte]_{Observed}}{(1-HCT)} \quad (\textit{Equation 3})$$

$$Plasma\ volume_{ADAPT\ VAMS} = 6\ tips \times 20\mu L \times (1 - HCT) \quad (\textit{Equation 4})$$

To compensate for the red blood cell debris and intracellular volume in the samples from the MoD study, it was recognised that for these samples an additional calculation would be required to determine the dilution factor generated by addition of the fixed volume of diluent to each sample. Individual dilution factors would be therefore calculated for each sample based on the HCT as determined by the baseline full blood counts measured on the Sysmex platform and the initial volume of whole blood collected. The calculation of the dilution factor used is therefore a modification of Equation 1 which also takes into consideration the original sample volume collected by VAMS for the ADAPT Study (Equation 4).

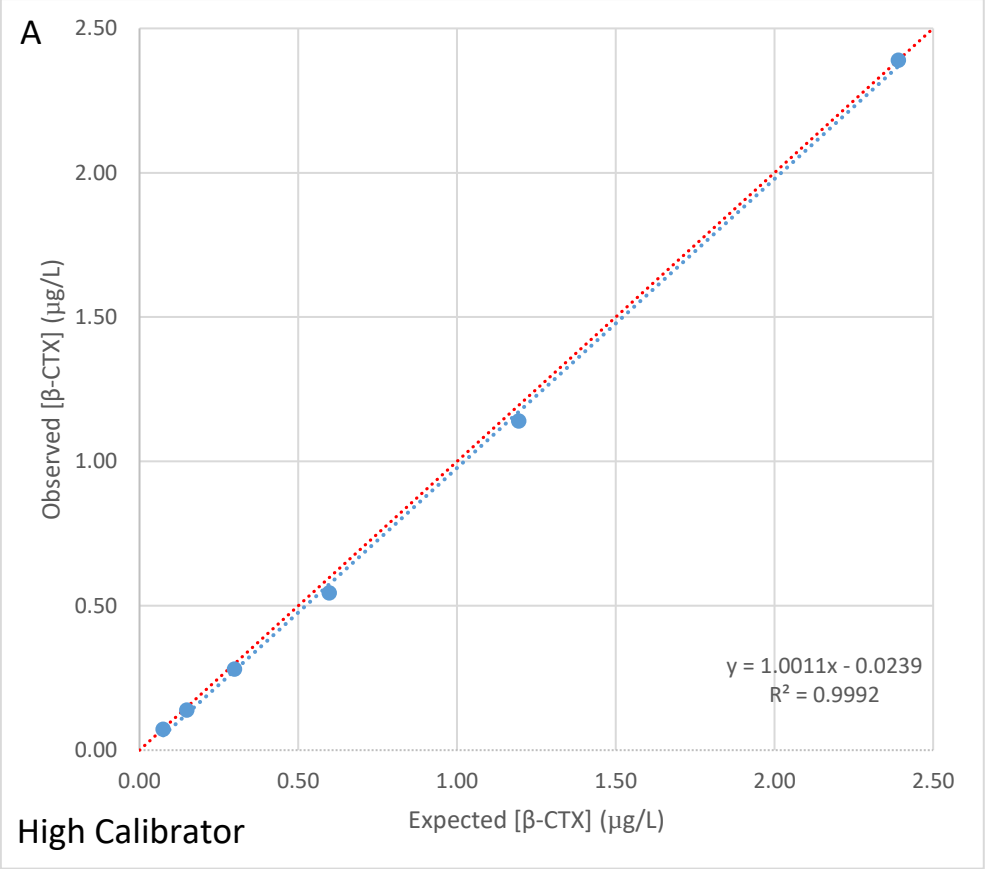
Results generated from the matched plasma samples analysed in the conventional manner according to standard protocols were then statistically compared with the calculated plasma equivalent values. Linear regression analysis was performed to calculate the Pearson's correlation coefficient (R) and coefficient of determination (R^2), the slope of the line of best fit, and the intercept of this line. Bland-Altman analysis was also performed to estimate the bias between the two methods, and to determine whether that bias was concentration dependent.

CHAPTER 3

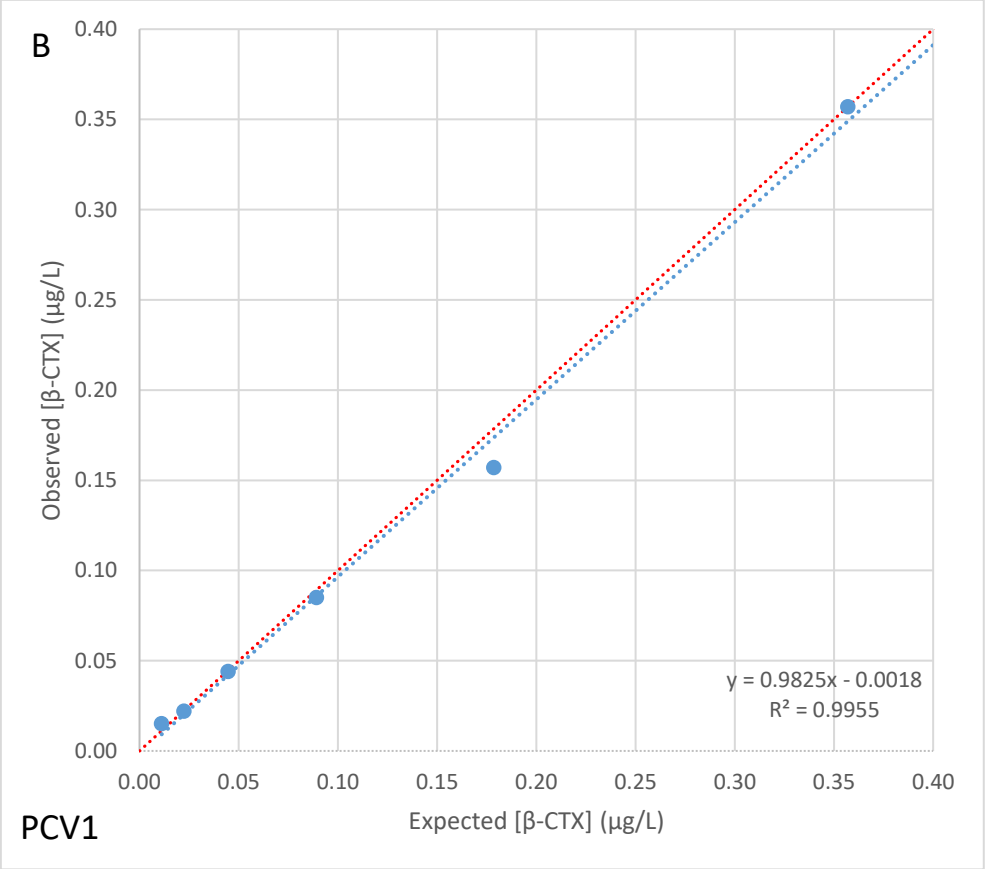
Results

Linearity of Dilution – β-CTX and Total P1NP

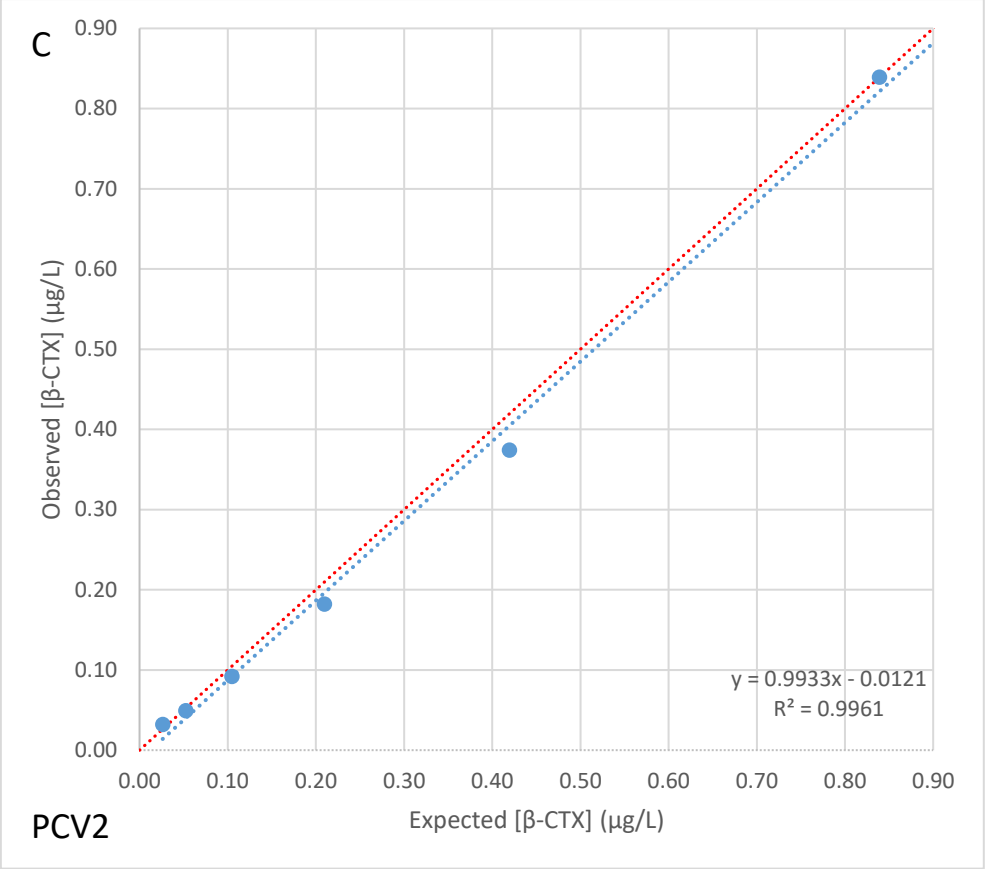
Since the comparison between VAMS samples and conventional samples require dilution, the linearity of dilution for β-CTX and P1NP were assessed before any other analysis. It was found that β-CTX dilutes in a linear manner, with coefficient of determination (R^2) values ranging from 0.9955 to 0.9992 (Fig 4 A-C). However, based on dilutions of the high calibrator and QC materials, there appears to be a curvilinear dilution profile for total P1NP, with R^2 values varying from 0.9828 to 0.9951 (Fig 4 D-F). Even though these R^2 values are acceptable, it was postulated that there may be some type of matrix effect of the calibration material from the manufacturing process interfering with the assay and consequently affecting the dilution linearity, so in addition plasma samples were serially diluted to evaluate this, returning an R^2 value of 0.9951 (Fig 4G). Based on the dilution profile and the R^2 value for the total P1NP samples assessed, it was concluded that plasma samples do dilute in a more linear manner than the calibrators and QC materials, and therefore the method could still be applied for total P1NP. It is worth noting there are fewer points on the total P1NP dilution curve for PCV1 (Fig 4E), reflecting the lower limit of detection (5 µg/L) for total P1NP, where higher dilutions returned concentrations below the lower limit of quantification.


A
2.50
2.00
1.50
1.00
0.50
0.00
Observed [β-CTX] (µg/L)
0.00
0.50
1.00
1.50
2.00
2.50
Expected [β-CTX] (µg/L)
y = 1.0011x - 0.0239
R² = 0.9992
High Calibrator

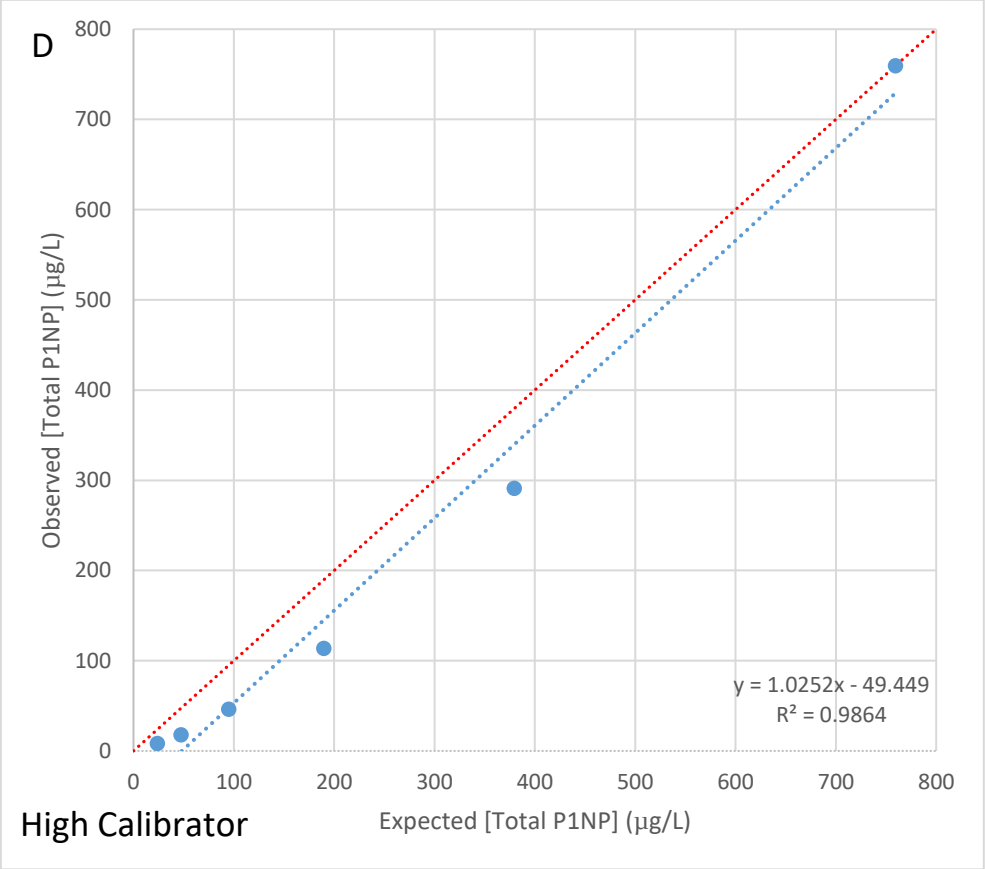
675


B
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Observed [β-CTX] (µg/L)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
Expected [β-CTX] (µg/L)
y = 0.9825x - 0.0018
R² = 0.9955
PCV1

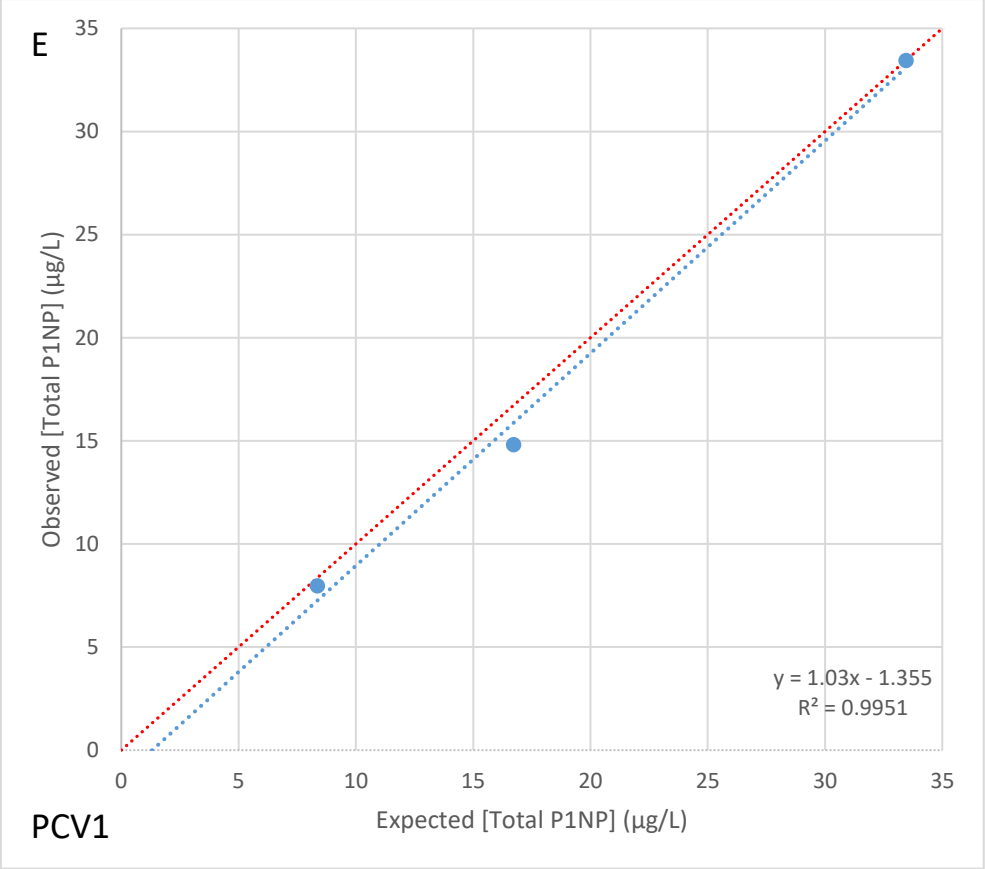
676


C
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Observed [β-CTX] (µg/L)
y = 0.9933x - 0.0121
R² = 0.9961
0.00 0.10 0.20 0.30 0.40 0.50 0.60 0.70 0.80 0.90
Expected [β-CTX] (µg/L)
PCV2

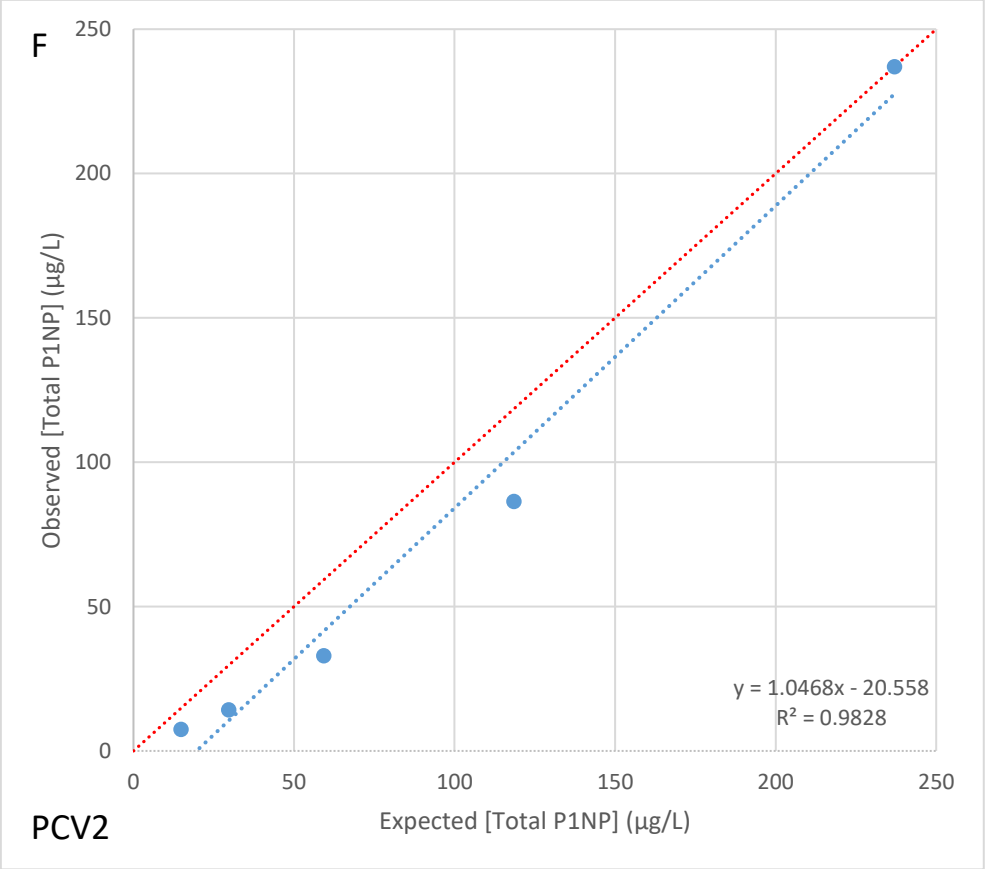
677


D
800
700
600
500
400
300
200
100
0
Observed [Total P1NP] (µg/L)
y = 1.0252x - 49.449
R² = 0.9864
0 100 200 300 400 500 600 700 800
Expected [Total P1NP] (µg/L)
High Calibrator

678


E
Observed [Total P1NP] (µg/L)
Expected [Total P1NP] (µg/L)
y = 1.03x - 1.355
R² = 0.9951
PCV1

679


F
Observed [Total P1NP] (µg/L)
Expected [Total P1NP] (µg/L)
y = 1.0468x - 20.558
R² = 0.9828
PCV2

680

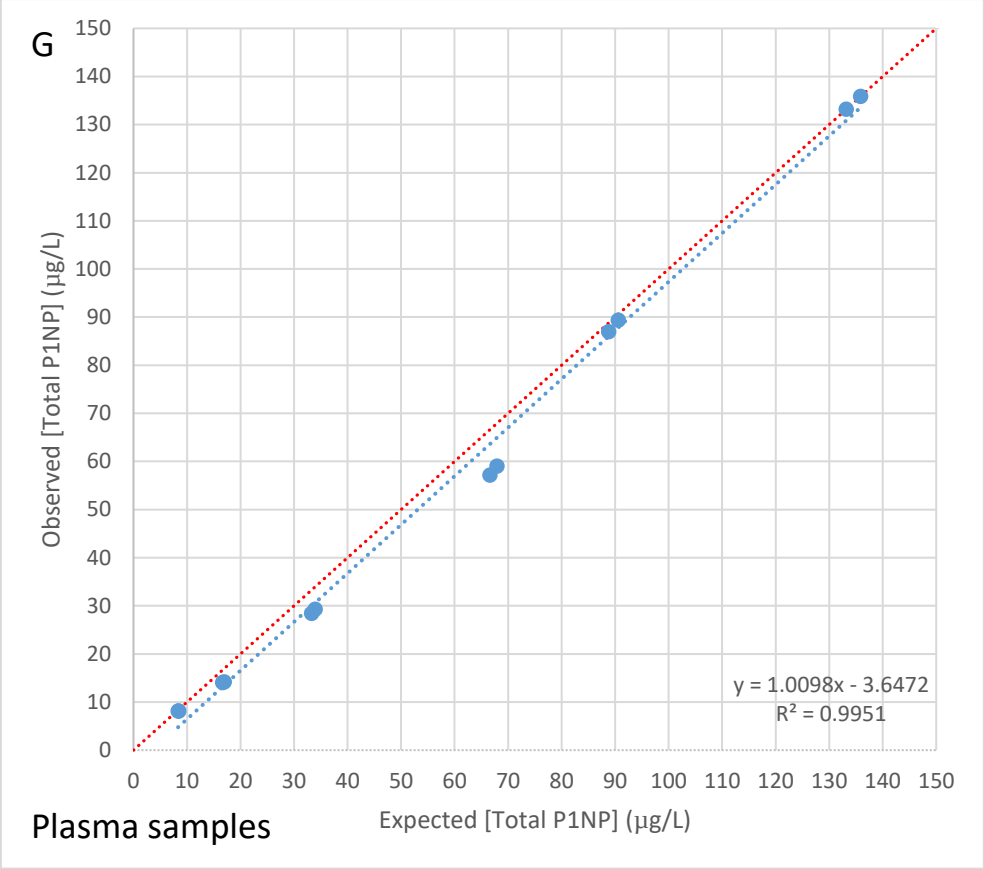


Figure 4. Linearity of Dilution for β-CTX in (A) high calibrator, (B) PreciControl Varia 1 (PCV1) (low QC), (C) PreciControl Varia 2 (PCV2) (high QC), and for total P1NP in (D) high calibrator, (E) PCV1, (F) PCV2 and (G) plasma samples. Red dotted line denotes identity, blue dotted line denotes linear trend line for data points.

Haematocrit

Standard curves were generated as described above and schematically in Figure 3. A typical standard curve is shown in Figure 5. The standard curve was linear, over the physiological range. Whilst acceptable calibration curves could be generated, when the extraction and analytical method is applied to practical samples prepared from EDTA whole blood obtained by venepuncture (n = 106), 40.6% of results fall outside the criteria for acceptable error in this evaluation (Figure 6).

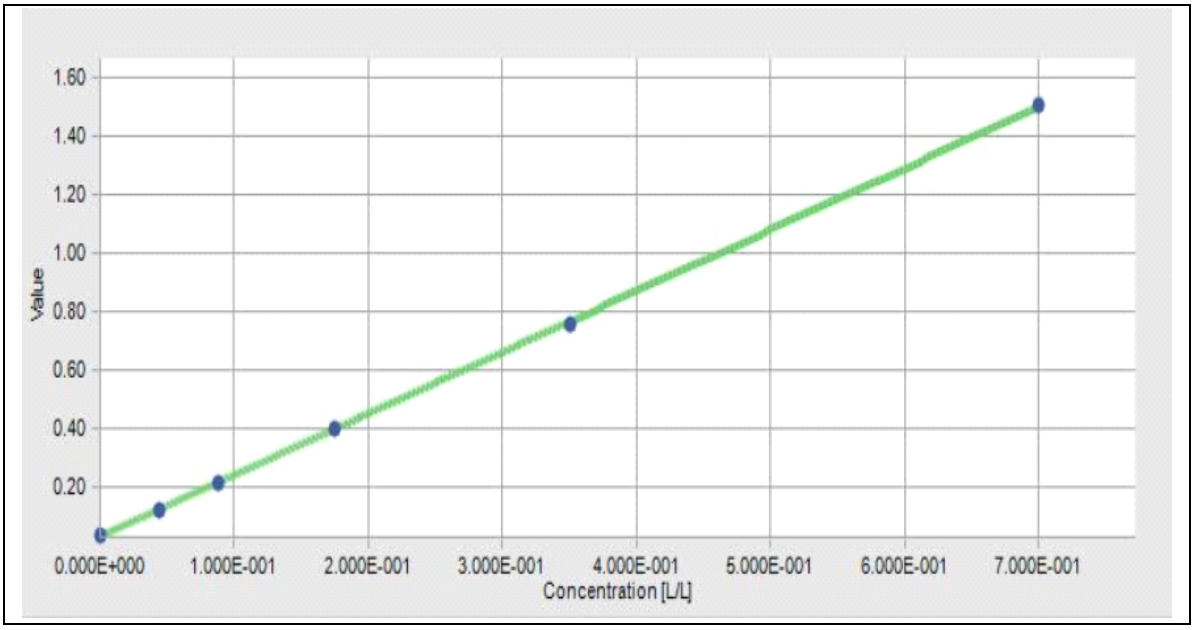


Figure 5. Typical standard curve prepared as in Fig. 3, R^2=0.9999.

Regression analysis of the HCT validation samples shows a Pearson's correlation coefficient (R) of 0.8716 and a coefficient of determination (R^2) of 0.7597, with the gradient of the line of best fit being 1.003 and the intercept 0.0045 (Fig 6). Bland-Altman (Bland and Altman, 1986) analysis of the HCT reveals there is an average bias of +1.4% between the two sampling methods (Fig. 7A), which equates to an absolute bias of +0.0065 L/L (Fig. 7B). Whilst this regression analysis and bias initially appear to be acceptable, these interpretations should be taken with caution, as the variability of the percentage differences is large, ranging from -18% to +28.4%, outside the CLIA '88 and AAB total allowable error between methods of 6% (Data Innovations, Online).

As only around 60% of the HCT results obtained using this protocol were within acceptable limits it was decided that HCT values generated from the spectrophotometric method described herein were invalid and that the HCT values obtained from standard

analysis by the Sysmex platform should be employed when calculating plasma equivalent values for β-CTX and total P1NP.

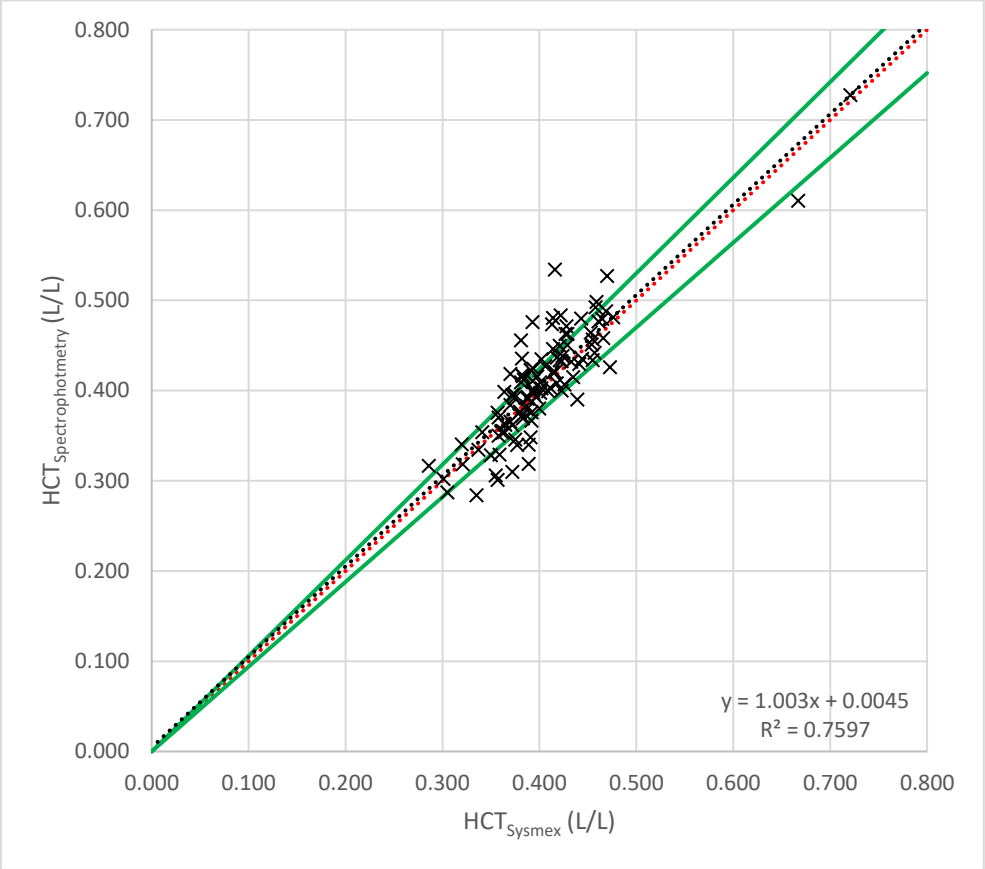
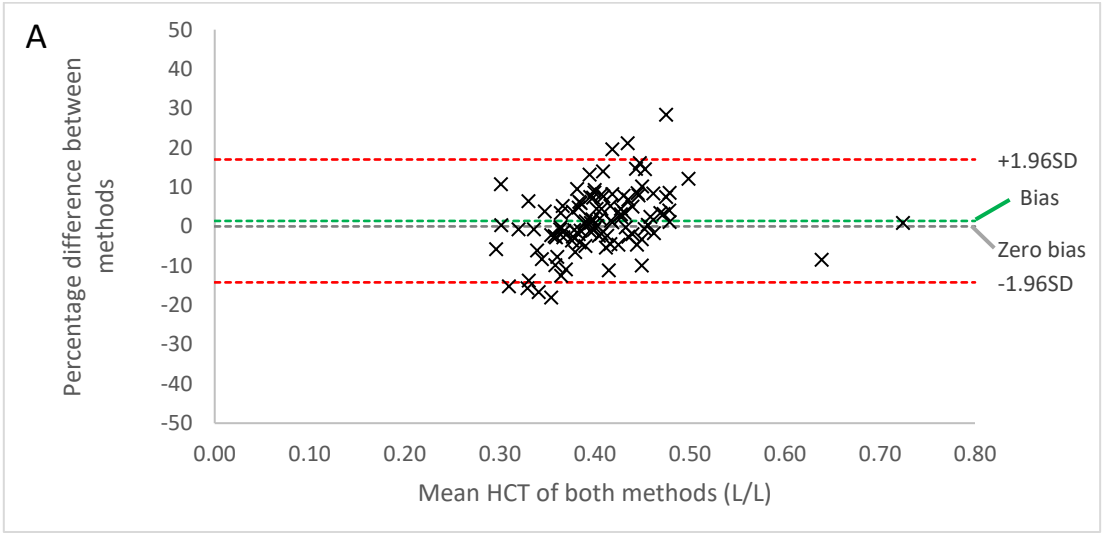


Figure 6. HCT Comparison. Comparison between conventional HCT determination by Sysmex versus spectrophotometry, n=106. Red dotted line specifies identity, black dotted line denotes linear trend line for data points (equation at bottom right corner) and green solid lines represent 6% deviation from identity (total allowable error between methods as defined by CLIA '88/AAB) (Data Innovations, Online). R^2=coefficient of determination.



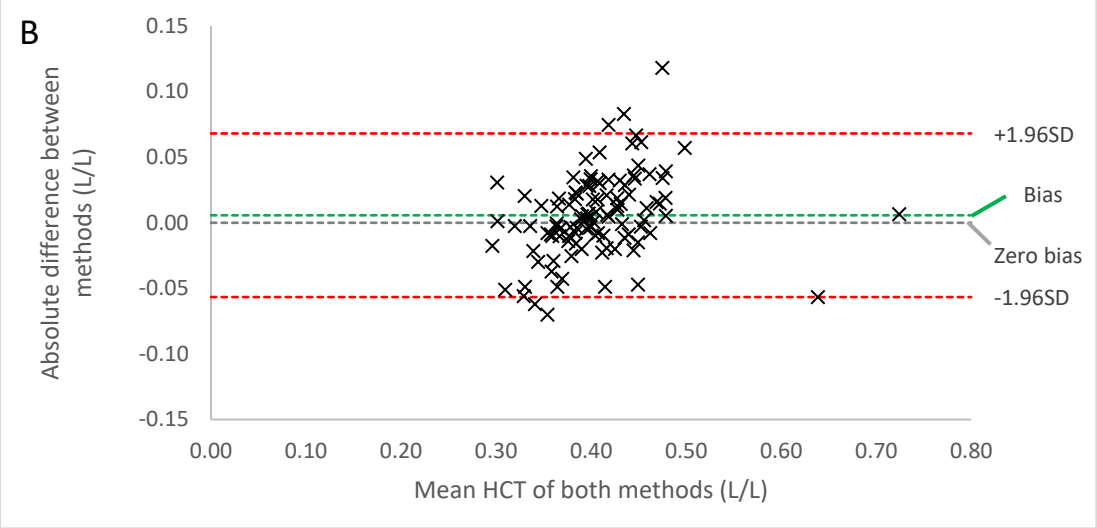


Figure 7. Bland-Altman plots for HCT comparison showing (A) percentage difference between conventional sampling and VAMS sampling and (B) absolute difference between both sampling methods. Green dotted line denotes average bias between the two methods, grey dotted line represents zero bias and red dotted lines indicate ±1.96 SD limits.

Application of the theory – ADAPT Study

The baseline VAMS and plasma samples were analysed first (n=44). For total P1NP only 2 out of 44 (4.5%) VAMS samples yielded results, 11 out of 44 (25%) were undetectable (<5 µg/L) and the remainder (70.5%) did not return a result at all. Whilst it was not quantified for each sample due to the low sample volume, the haemolysis observed will have affected the quality of any result obtained for total P1NP as a haemoglobin content greater than 0.1 g/dL will cause interference in the assay (Roche Diagnostics, 2019). The other reason for the lack of data is sampling errors on the COBAS, where either the sample volume was insufficient for analysis, or other aspiration errors, such as clot detection, occurred. Unfortunately, where any aspiration error occurs, the COBAS discards the conductive tip with any sample it has achieved to aspirate, meaning there was subsequently insufficient sample for a repeat attempt.

More acceptable results were obtained from the comparison between β-CTX results from VAMS samples versus conventional plasma samples (Figure 8A). Whilst each VAMS sample obviously had the same concentration of haemoglobin for both total P1NP and β-CTX analyses, β-CTX assay is unaffected by haemolysis up to a haemoglobin concentration of 0.5 g/dL, i.e. five times as much as for the total P1NP assay (Roche Diagnostics, 2020). The PEV calculated for β-CTX was based on the HCT assessed on the baseline whole blood samples analysed at the NNUH, and not determined by the spectrophotometric method described herein due to this method being invalid. The PEVs for 15 out of the 43 baseline samples measured (34.9%) were within the total allowable error limits (± 14.1%) for methods of β-CTX determination, as defined by the 2004 update of the Spanish Society of Clinical Chemistry and Molecular Pathology (SEQC)) (Data Innovations, Online).

Regression analysis of the baseline samples shows that the Pearson's coefficient of correlation (R) was 0.8469. Only a minority of the plasma equivalent values were within acceptable error limits and the correlation is below pre-defined acceptance criteria of $R^2 > 0.90$. This is most likely due to the differing degrees of haemolysis within each sample prepared in this way, attributable to the differing lengths of time between sampling and extraction, and therefore drying times experienced by each of the six VAMS devices for each subject. Whilst the β-CTX results were inadequate based on both the coefficient of correlation and the percentage of results within the acceptable limits, the linear trend-line did fall within acceptable limits over the range of expected concentrations of β-CTX. The slope of the trend-line was 1.1523 and the intercept was -0.0435. Based on these results, the decision was made to analyse the remainder of the

VAMS samples (total n = 129) for β-CTX only and not for total P1NP, primarily on the basis of considering the cost of the reagents versus the high likelihood of a poor return of results, if any for total P1NP.

Regression analysis for the post-exercise sample results shows a Pearson's correlation coefficient (R) of 0.9361 and a coefficient of determination (R^2) of 0.8763, with a slope of 1.0727 and an intercept of 0.0065 (Fig 8B). Analysis of the recovery sample results shows an R value of 0.9102 and an R^2 value of 0.8284, with a slope of 1.1371 and an intercept of -0.0463 (Fig 8C). Performing the regression analysis on the results from all time-points gives an overall R value of 0.9000 and an R^2 of 0.8101, with a slope of 1.115 and an intercept of -0.0233 (Fig 8D). Bland-Altman analysis (Bland and Altman, 1986) reveals that for all samples there is an average bias of +7.7% between the two sampling methods (Fig. 9A), which equates to an absolute bias of +0.05 μg/L (Fig. 9B). These interpretations should be taken with caution, as the spread of the percentage and absolute differences is large, ranging from -60.1% to +68% and -0.47 μg/L to +0.56 μg/L respectively.

Reproducibility

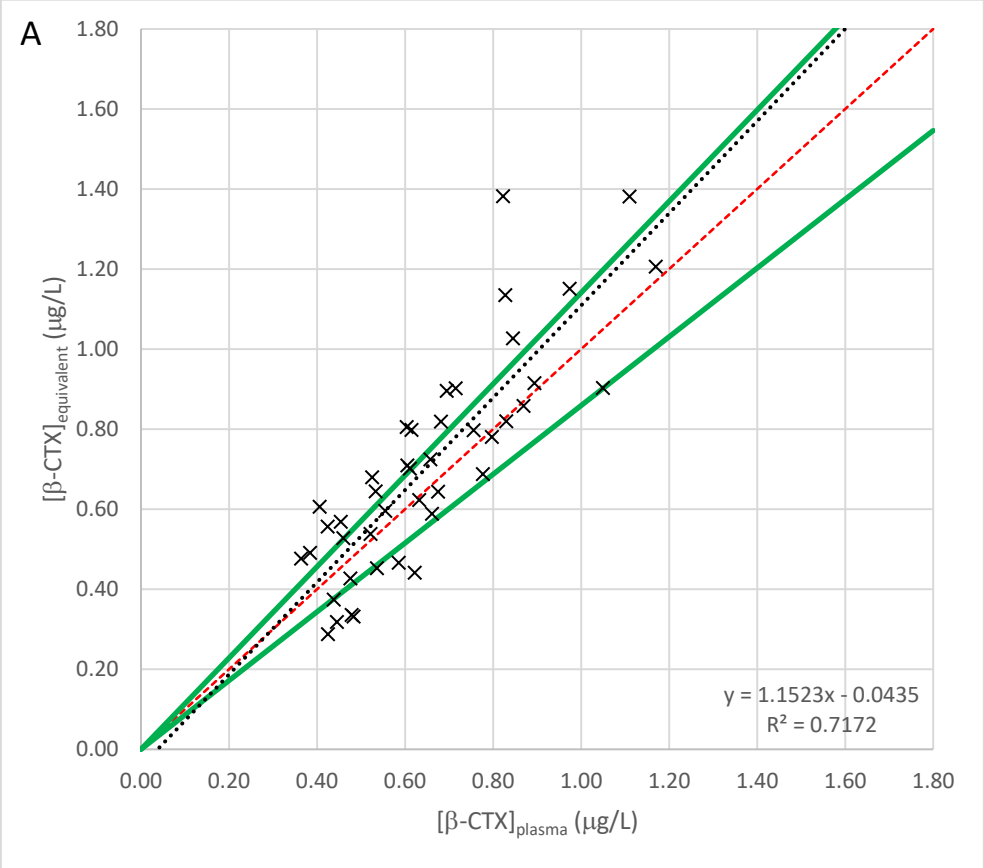
To assess the reproducibility of VAMS as a collection method for the assessment of bone turnover markers, PCV1 and PCV2 were wicked onto ten 20 μL Mitra™ tips each, not allowed to dry, and extracted with 80 μL MultiAssay Diluent. This represented a 1:5 dilution. Ideally, this experiment would have been done using whole blood (matrix-matched) QC materials, but as this does not exist for bone markers, the decision was taken to utilise the proprietary QC material. As it had been decided that this collection method in its current form was unsuitable for total P1NP measurement, only β-CTX was

776 assessed in the precision analysis. The precision data, along with target means and
777 standard deviations (SD) is shown in Table 3.

PC V1 (n=10)			
	Measured [β-CTX] (µg/L)	Calculated [β-CTX] (µg/L)	Target [β-CTX] (µg/L)
Mean	0.057	0.287	0.280
SD	0.003	0.016	0.030
CV%	5.53	5.53	

PC V2 (n=10)			
	Measured [β-CTX] (µg/L)	Calculated [β-CTX] (µg/L)	Target [β-CTX] (µg/L)
Mean	0.129	0.644	0.650
SD	0.008	0.038	0.060
CV%	5.84	5.84	

778 Table 3. Precision data for PCV1 and PCV2.



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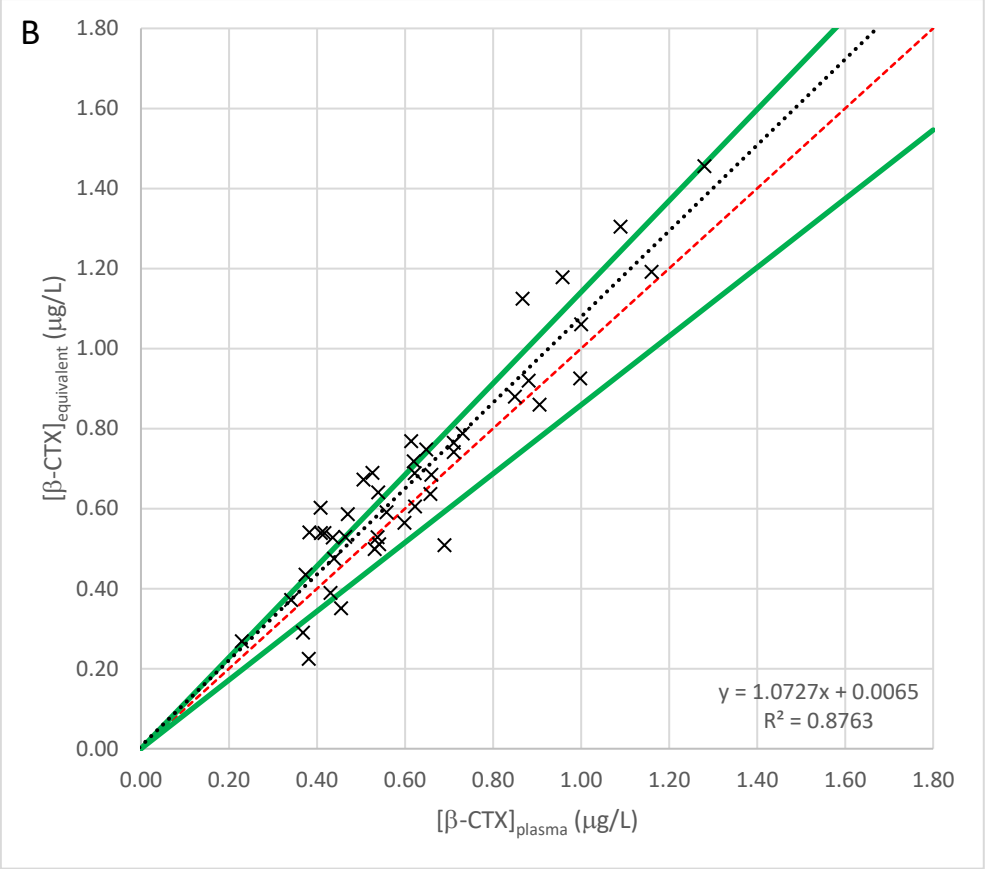
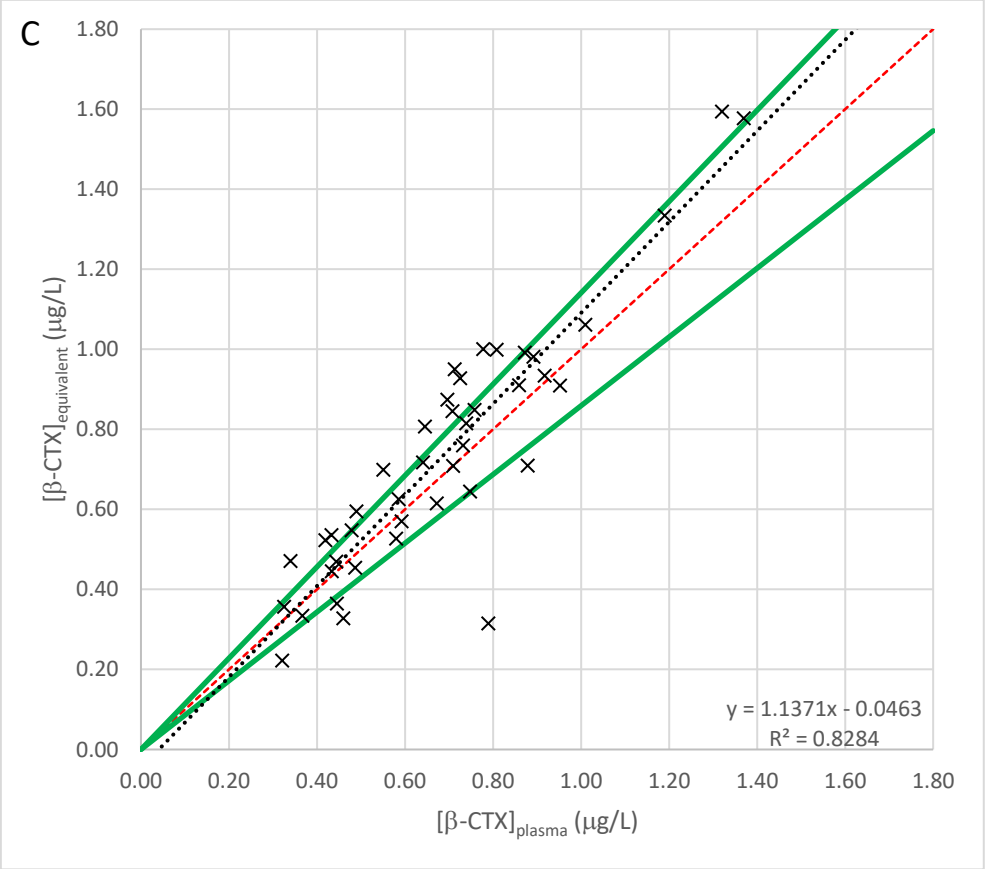
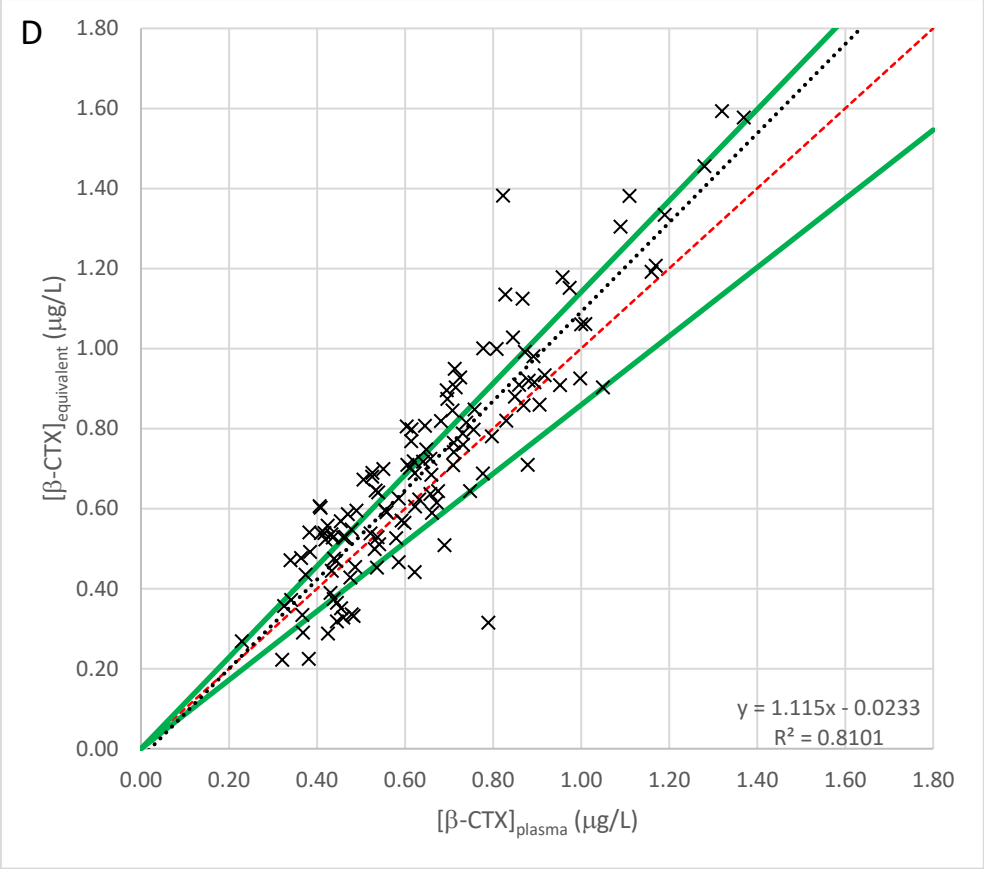

B
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
[β-CTX]equivalent (µg/L)
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
1.80
[β-CTX]plasma (µg/L)
y = 1.0727x + 0.0065
R² = 0.8763

780

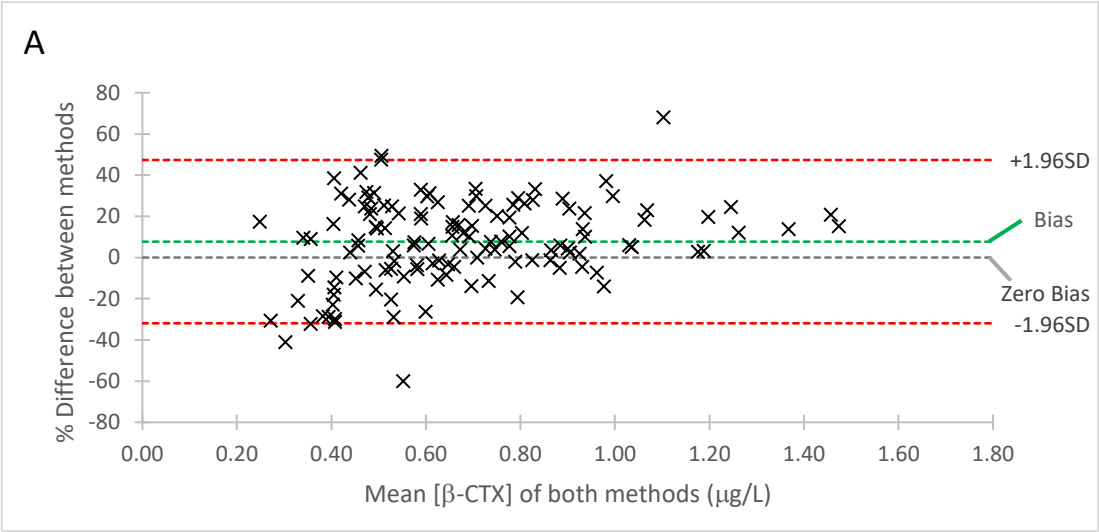

C
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
[β-CTX]equivalent (µg/L)
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
1.80
[β-CTX]plasma (µg/L)
y = 1.1371x - 0.0463
R² = 0.8284

781



782

783 Figure 8. β-CTX comparison. Comparison between conventional plasma β-CTX
784 concentrations and calculated plasma equivalent values using established HCT
785 measurement (n = 43). (A) Baseline samples, (B) Post exercise samples, (C) Recovery
786 samples and (D) all samples. Red dashed line specifies identity, black dotted line denotes
787 linear trend-line for data points (equation at bottom right corner) and green solid lines
788 represent 14.1% deviation from identity (total allowable error between methods as
789 defined by the 2004 update of the Spanish Society of Clinical Chemistry and Molecular
790 Pathology (SEQC)) (Data Innovations, Online). R^2=coefficient of determination.



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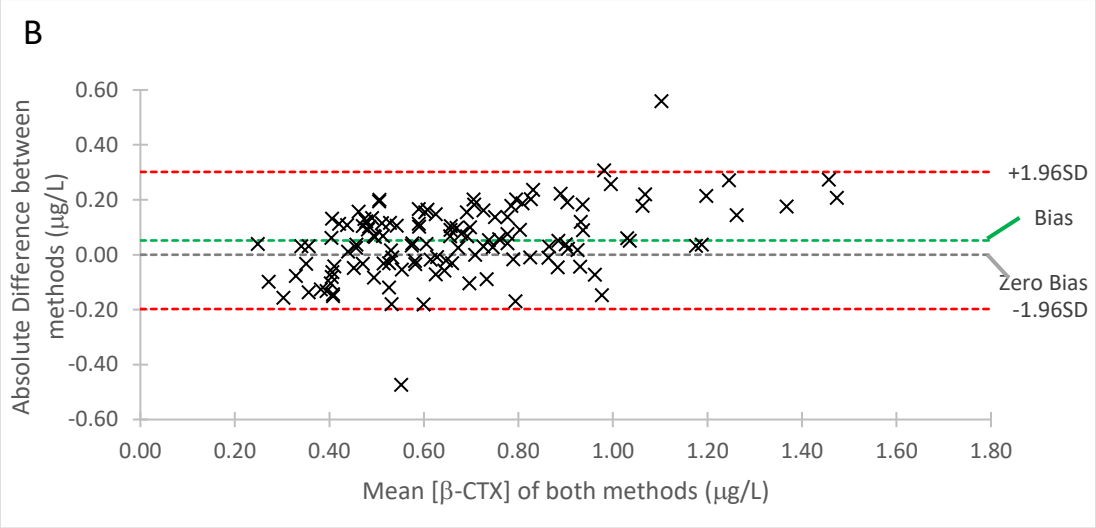


Figure 9. Bland-Altman plots for all samples showing (A) percentage difference between conventional sampling and VAMS sampling and (B) absolute difference between both sampling methods. Green dotted line denotes average bias between the two methods, grey dotted line represents zero bias and red dotted lines indicate ±1.96 SD limits.

Impact of adjusting for HCT

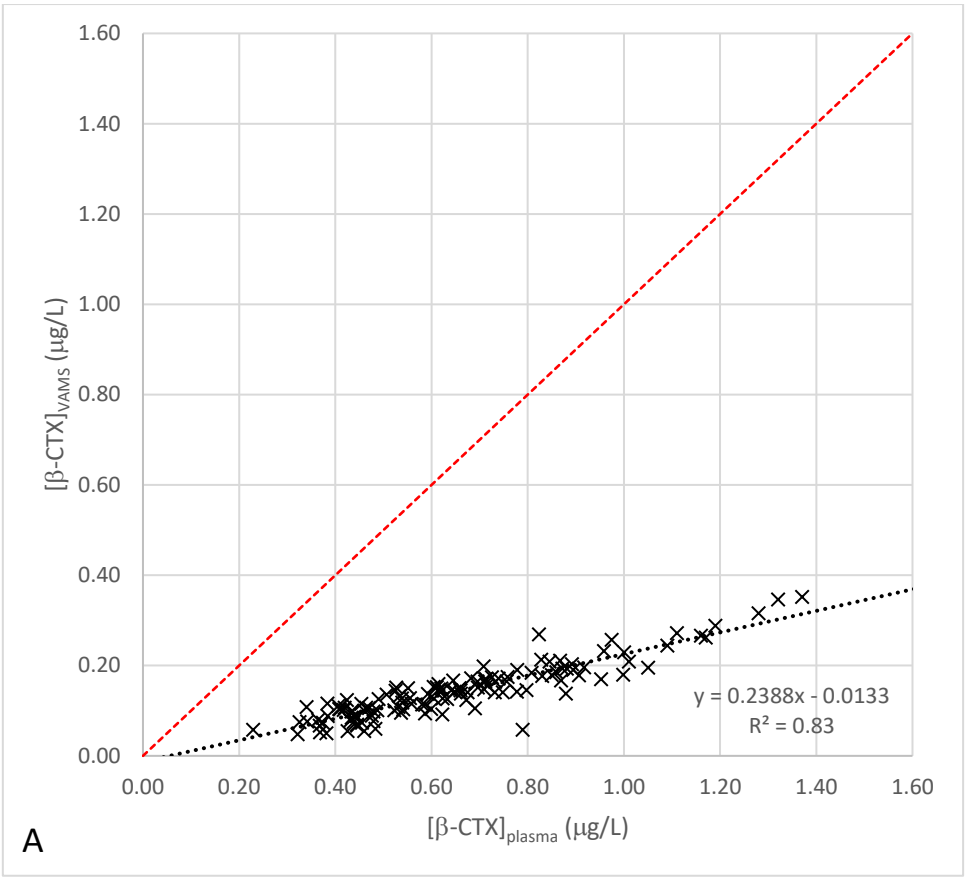
Given the complications surrounding the determination of HCT from the VAMS samples in this work, the decision was made to also make direct comparisons between the results from VAMS samples and conventional plasma samples, without correcting for HCT. The rationale for this was to remove any potential variability introduced by the mathematical correction, as any analytical variability in the HCT determination and subsequent transformation would be added to the existing variability in the measurements (such as pre-analytical and biological variability). Whilst there would be an expected bias, it was decided direct comparison was still deemed necessary in order to establish whether the underlying relationship between results obtained from VAMS and conventional sampling was any different without HCT adjustment.

The results of the data analysis without adjusting for HCT can be seen in Figure 10. Linear regression (Figure 10A) shows a marginally stronger correlation coefficient (R^2

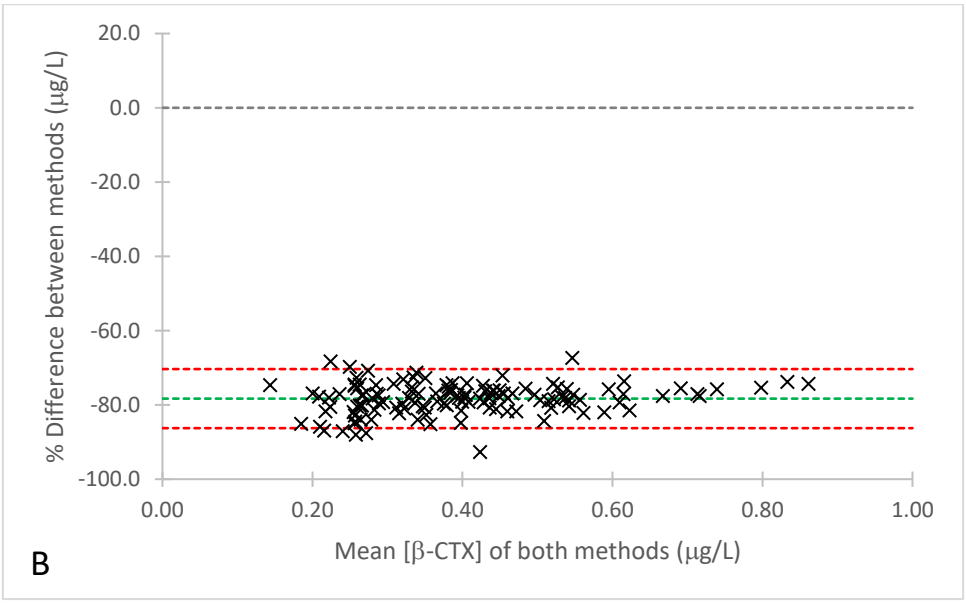
= 0.83 compared with R^2 = 0.8101 for HCT adjusted) and clearly demonstrates upon both visual inspection, and the equation for the linear trend-line for data points, the expected negative bias between the two methods of sample collection. This slightly weaker correlation in the adjusted results is expected due to the added variability introduced upon adjusting for HCT, as mentioned above. The negative bias is also strongly highlighted in the Bland-Altman plot in Figure 10B, showing the percentage difference between the two methods, indicating an overall negative bias in unadjusted β-CTX concentrations from VAMS samples of 78.3%.

Perhaps the most interesting finding is that when the unadjusted absolute differences between the two collection methods are compared (Figure 10C), there appears to be a concentration-dependent negative bias, where the negative bias increases as average β-CTX concentration increases. It is deduced that this is due to the differences in the range of values obtained between the two methods (0.05 – 0.35 μg/L for VAMS and 0.23 – 1.37 μg/L for conventional plasma samples), suggesting that as the mean concentration increases (primarily driven by the plasma result), the difference between the two values is disproportionately increased.

This demonstrates the importance of adjusting for HCT, as this concentration-dependent negative bias (if using absolute concentrations), and the very large non-concentration-dependent negative bias (if using percentage differences) are mitigated for (although not entirely eliminated) upon correction for HCT.


1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
[β-CTX]VAMS (µg/L)
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
[β-CTX]plasma (µg/L)
y = 0.2388x - 0.0133
R² = 0.83
A

830


20.0
0.0
-20.0
-40.0
-60.0
-80.0
-100.0
% Difference between methods (µg/L)
0.00
0.20
0.40
0.60
0.80
1.00
Mean [β-CTX] of both methods (µg/L)
B

831

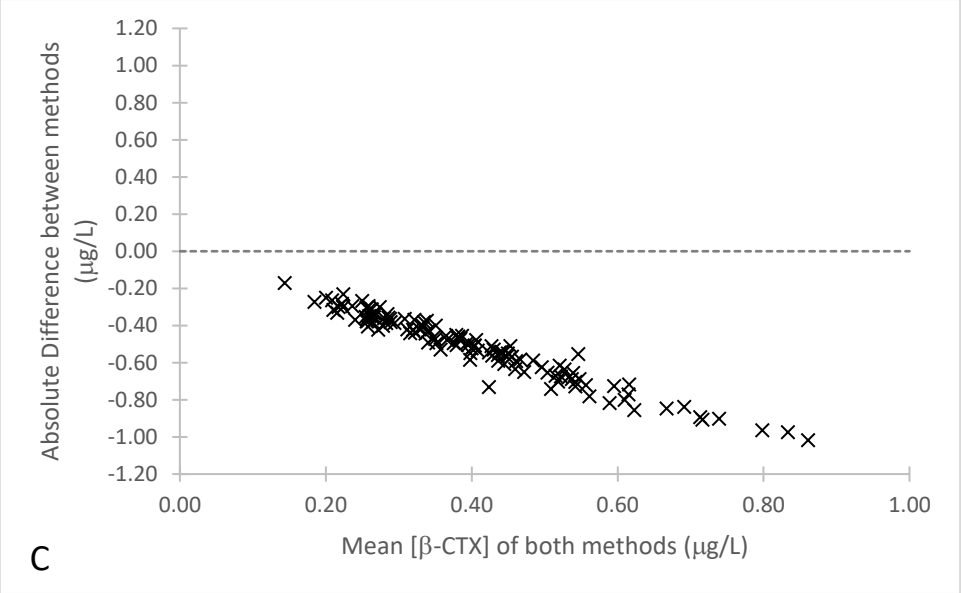


Figure 10. Data analysis without correcting for HCT. (A) Direct comparison (linear regression) between plasma and VAMS measurements. Red dotted line indicates identity, black dotted line denotes linear trend-line for data points (equation at bottom right corner). Bland-Altman plots for all samples showing (B) percentage difference between conventional sampling and VAMS sampling. Green dotted line denotes average bias between the two methods, grey dotted line represents zero bias and red dotted lines indicate ±1.96 SD limits. (C) absolute difference between both sampling methods.

CHAPTER 4

Discussion/ Conclusions

Any potential new weapon in the armoury of researchers and clinicians battling against the increase in the prevalence of bone and mineral metabolism disorders associated with the global ageing population should be investigated. Here I have evaluated whether β-CTX and total P1NP, the most routinely used biomarkers for the monitoring of bone turnover, can be assessed from samples collected using Mitra™ Volumetric Absorptive Micro Sampling devices.

Whilst the assessment of β-CTX and total P1NP concentrations from microsamples described herein did not render results acceptable for implementation in routine use, these methods do show potential. What has been highlighted is the significance of haemolysis of the microsample on the interference in the assays for both β-CTX and total P1NP, and therefore the importance of correct handling and preparation of the samples collected in this way. Had the MoD collection centre had access to the appropriate equipment (agitator) and had been able to maintain hydration of the microsamples therefore minimising clotting, I am confident the results would have been more comparable to conventional plasma samples and this collection method could be implemented in routine use. Further work is required to demonstrate this with sufficient samples to be statistically significant.

The analyser sampling errors encountered during total P1NP analysis could be eliminated by either increasing the number of VAMS tips used so a larger volume of diluent could be utilised, or collecting two sets of VAMS samples from the subjects, one each for β-CTX and total P1NP. However, participants reported that taking 6 VAMS samples was uncomfortable, suggesting there would be a reluctance to increase the number of finger-pricks.

The importance of correcting for HCT has been highlighted above. To improve the HCT assessment, alternative methods for HCT determination include potassium (K^+) elucidation, as K^+ is primarily located within red blood cells and concentrations are tightly controlled physiologically (Capiau et al., 2013, De Kesel et al., 2014), or using a different surrogate molecule such as haemoglobin (Hb). Noncontact diffuse reflectance spectroscopy could also be employed (Capiau et al., 2016) to estimate HCT.

To make VAMS a truly at-home sampling method, the samples would need to be allowed to dry, as the methods described herein require laboratory equipment to accomplish. In order for this to be achieved, further investigations into eliminating the irreversible interaction between the erythrocyte cell walls and the polymer of the sampling device need to be performed, perhaps by examining different anticoagulants (such as K_3-EDTA, lithium-heparin or sodium citrate) impregnated onto the tips prior to use. Investigation into any interferences from these anticoagulants would be required. It is worth noting one of the major limitations of the work presented herein, is that the preliminary work and extraction optimisation was conducted on whole blood samples containing K_3-EDTA, so not reflective of the "real-world" scenario. Having K_3-EDTA present would have prevented coagulation and potentially also the irreversible interaction between the erythrocytes and the polymer of the Mitra™ tips observed in the ADAPT study. Alternatively, further work could be carried out to explore methods for keeping the VAMS tip hydrated during transport to the laboratory.

If the further work suggested here is carried out, then VAMS may well be another armament in the arsenal of clinicians and researchers in the future.

REFERENCES

ARBIOL-ROCA, A., IMPERIALI, C. E., MONTSERRAT, M. M., CERRO, A. S., BOSCH DE BASEA, A. C., NAVARRO, L. S., DOT BACH, D. & POLITI, J. V. 2018. Reference intervals for a complete blood count on an automated haematology analyser Sysmex XN in healthy adults from the southern metropolitan area of Barcelona. *EJIFCC,* 29**,** 48-54.

ATKINS, P. & DE PAULA, J. 2006. Biochemical Spectroscopy. *Physical Chemistry for the Life Sciences.* Oxford, UK: Oxford University Press.

BIOMARKERS DEFINITIONS WORKING GROUP. 2001. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Ther,* 69**,** 89-95.

BLAND, J. M. & ALTMAN, D. G. 1986. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet,* 1**,** 307-10.

BONDE, M., GARNERO, P., FLEDELIUS, C., QVIST, P., DELMAS, P. D. & CHRISTIANSEN, C. 1997. Measurement of bone degradation products in serum using antibodies reactive with an isomerized form of an 8 amino acid sequence of the C-telopeptide of type I collagen. *J Bone Miner Res,* 12**,** 1028-34.

BONDE, M., QVIST, P., FLEDELIUS, C., RIIS, B. J. & CHRISTIANSEN, C. 1994. Immunoassay for quantifying type I collagen degradation products in urine evaluated. *Clin Chem,* 40**,** 2022-5.

BONDE, M., QVIST, P., FLEDELIUS, C., RIIS, B. J. & CHRISTIANSEN, C. 1995. Applications of an enzyme immunoassay for a new marker of bone resorption (CrossLaps): follow-up on hormone replacement therapy and osteoporosis risk assessment. *J Clin Endocrinol Metab,* 80**,** 864-8.

BRANDT, J., KROGH, T. N., JENSEN, C. H., FREDERIKSEN, J. K. & TEISNER, B. 1999. Thermal instability of the trimeric structure of the N-terminal propeptide of human procollagen type I in relation to assay technology. *Clin Chem,* 45**,** 47-53.

BRIGGS, C., LONGAIR, I., KUMAR, P., SINGH, D. & MACHIN, S. J. 2012. Performance evaluation of the Sysmex haematology XN modular system. *J Clin Pathol,* 65**,** 1024-30.

BURGESON, R. E. 1988. New collagens, new concepts. *Annu Rev Cell Biol,* 4**,** 551-77.

CAPIAU, S., STOVE, V. V., LAMBERT, W. E. & STOVE, C. P. 2013. Prediction of the hematocrit of dried blood spots via potassium measurement on a routine clinical chemistry analyzer. *Anal Chem,* 85**,** 404-10.

CAPIAU, S., WILK, L. S., AALDERS, M. C. & STOVE, C. P. 2016. A Novel, Nondestructive, Dried Blood Spot-Based Hematocrit Prediction Method Using Noncontact Diffuse Reflectance Spectroscopy. *Anal Chem,* 88**,** 6538-46.

CHRISTGAU, S., ROSENQUIST, C., ALEXANDERSEN, P., BJARNASON, N. H., RAVN, P., FLEDELIUS, C., HERLING, C., QVIST, P. & CHRISTIANSEN, C. 1998. Clinical evaluation of the Serum CrossLaps One Step ELISA, a new assay measuring the serum concentration of bone-derived degradation products of type I collagen C-telopeptides. *Clin Chem,* 44**,** 2290-300.

CLARKE, B. 2008. Normal bone anatomy and physiology. *Clin J Am Soc Nephrol,* 3 Suppl 3**,** S131-9.

DATA INNOVATIONS. Total Allowable Error Tables - beta-CTX. [Online]. Available: http://www.datainnovations.com/allowable-total-error-table?field_category_type_tid=All&title=C-Telopeptide+type+I+procollagen

DATA INNOVATIONS. *Total Allowable Error Tables - HCT* [Online]. Available: http://www.datainnovations.com/allowable-total-error-table?field_category_type_tid=All&title=Hematocrit

DE KESEL, P. M., CAPIAU, S., STOVE, V. V., LAMBERT, W. E. & STOVE, C. P. 2014. Potassium-based algorithm allows correction for the hematocrit bias in quantitative analysis of caffeine and its major metabolite in dried blood spots. *Anal Bioanal Chem,* 406**,** 6749-55.

DENNIFF, P., PARRY, S., DOPSON, W. & SPOONER, N. 2015. Quantitative bioanalysis of paracetamol in rats using volumetric absorptive microsampling (VAMS). *J Pharm Biomed Anal,* 108**,** 61-9.

DENNIFF, P. & SPOONER, N. 2010. The effect of hematocrit on assay bias when using DBS samples for the quantitative bioanalysis of drugs. *Bioanalysis,* 2**,** 1385-95.

DENNIFF, P. & SPOONER, N. 2014. Volumetric Absorptive Microsampling: A Dried Sample Collection Technique for Quantitative Bioanalysis. *Analytical Chemistry,* 86**,** 8489-8495.

DIGIROLAMO, D. J., CLEMENS, T. L. & KOUSTENI, S. 2012. The skeleton as an endocrine organ. *Nat Rev Rheumatol,* 8**,** 674-83.

DÉGLON, J., THOMAS, A., MANGIN, P. & STAUB, C. 2012. Direct analysis of dried blood spots coupled with mass spectrometry: concepts and biomedical applications. *Anal Bioanal Chem,* 402**,** 2485-98.

EASTELL, R., CALVO, M. S., BURRITT, M. F., OFFORD, K. P., RUSSELL, R. G. & RIGGS, B. L. 1992. Abnormalities in circadian patterns of bone resorption and renal calcium conservation in type I osteoporosis. *J Clin Endocrinol Metab,* 74**,** 487-94.

ETTINGER, B., SIDNEY, S., CUMMINGS, S. R., LIBANATI, C., BIKLE, D. D., TEKAWA, I. S., TOLAN, K. & STEIGER, P. 1997. Racial differences in bone density between young adult black and white subjects persist after adjustment for anthropometric, lifestyle, and biochemical differences. *J Clin Endocrinol Metab,* 82**,** 429-34.

FLEDELIUS, C., JOHNSEN, A. H., CLOOS, P. A., BONDE, M. & QVIST, P. 1997. Characterization of urinary degradation products derived from type I collagen. Identification of a beta-isomerized Asp-Gly sequence within the C-terminal telopeptide (alpha1) region. *J Biol Chem,* 272**,** 9755-63.

FUJIMURA, R., ASHIZAWA, N., WATANABE, M., MUKAI, N., AMAGAI, H., FUKUBAYASHI, T., HAYASHI, K., TOKUYAMA, K. & SUZUKI, M. 1997. Effect of resistance exercise training on bone formation and resorption in young male subjects assessed by biomarkers of bone metabolism. *J Bone Miner Res,* 12**,** 656-62.

GELSE, K., PÖSCHL, E. & AIGNER, T. 2003. Collagens--structure, function, and biosynthesis. *Adv Drug Deliv Rev,* 55**,** 1531-46.

GINEYTS, E., CLOOS, P. A., BOREL, O., GRIMAUD, L., DELMAS, P. D. & GARNERO, P. 2000. Racemization and isomerization of type I collagen C-telopeptides in human bone and soft tissues: assessment of tissue turnover. *Biochem J,* 345 Pt 3**,** 481-5.

GREENSPAN, S. L., DRESNER-POLLAK, R., PARKER, R. A., LONDON, D. & FERGUSON, L. 1997. Diurnal variation of bone mineral turnover in elderly men and women. *Calcif Tissue Int,* 60**,** 419-23.

HANNON, R., BLUMSOHN, A., NAYLOR, K. & EASTELL, R. 1998. Response of biochemical markers of bone turnover to hormone replacement therapy: impact of biological variability. *J Bone Miner Res,* 13**,** 1124-33.

JENKINS, N., BLACK, M., PAUL, E., PASCO, J. A., KOTOWICZ, M. A. & SCHNEIDER, H. G. 2013. Age-related reference intervals for bone turnover markers from an Australian reference population. *Bone,* 55**,** 271-6.

JENSEN, C. H., HANSEN, M., BRANDT, J., RASMUSSEN, H. B., JENSEN, P. B. & TEISNER, B. 1998. Quantification of the N-terminal propeptide of human procollagen type I (PINP): comparison of ELISA and RIA with respect to different molecular forms. *Clin Chim Acta,* 269**,** 31-41.

JENSEN, L. T., OLESEN, H. P., RISTELI, J. & LORENZEN, I. 1990. External thoracic duct-venous shunt in conscious pigs for long term studies of connective tissue metabolites in lymph. *Lab Anim Sci,* 40**,** 620-4.

JUDD, A., PARRY, J., HICKMAN, M., MCDONALD, T., JORDAN, L., LEWIS, K., CONTRERAS, M., DUSHEIKO, G., FOSTER, G., GILL, N., KEMP, K., MAIN, J., MURRAY-LYON, I. & NELSON, M.

2003. Evaluation of a modified commercial assay in detecting antibody to hepatitis C virus in oral fluids and dried blood spots. *J Med Virol,* 71**,** 49-55.

KELLY, P. J., POCOCK, N. A., SAMBROOK, P. N. & EISMAN, J. A. 1989. Age and menopause-related changes in indices of bone turnover. *J Clin Endocrinol Metab,* 69**,** 1160-5.

KHOSLA, S., MELTON, L. J., ATKINSON, E. J. & O'FALLON, W. M. 2001. Relationship of serum sex steroid levels to longitudinal changes in bone density in young versus elderly men. *J Clin Endocrinol Metab,* 86**,** 3555-61.

KHOSLA, S., MELTON, L. J., ATKINSON, E. J., O'FALLON, W. M., KLEE, G. G. & RIGGS, B. L. 1998. Relationship of serum sex steroid levels and bone turnover markers with bone mineral density in men and women: a key role for bioavailable estrogen. *J Clin Endocrinol Metab,* 83**,** 2266-74.

KIP, A. E., KIERS, K. C., ROSING, H., SCHELLENS, J. H., BEIJNEN, J. H. & DORLO, T. P. 2017. Volumetric absorptive microsampling (VAMS) as an alternative to conventional dried blood spots in the quantification of miltefosine in dried blood samples. *J Pharm Biomed Anal,* 135**,** 160-166.

KUWANA, T., SUGITA, O. & YAKATA, M. 1988. Reference limits of bone and liver alkaline phosphatase isoenzymes in the serum of healthy subjects according to age and sex as determined by wheat germ lectin affinity electrophoresis. *Clin Chim Acta,* 173**,** 273-80.

LEHMANN, S., DELABY, C., VIALARET, J., DUCOS, J. & HIRTZ, C. 2013. Current and future use of "dried blood spot" analyses in clinical chemistry. *Clin Chem Lab Med,* 51**,** 1897-909.

MAUTALEN, C. A. 1970. Circadian rhythm of urinary total and free hydroxyproline excretion and its relation to creatinine excretion. *J Lab Clin Med,* 75**,** 11-8.

MEIER, D. E., LUCKEY, M. M., WALLENSTEIN, S., LAPINSKI, R. H. & CATHERWOOD, B. 1992. Racial differences in pre- and postmenopausal bone homeostasis: association with bone density. *J Bone Miner Res,* 7**,** 1181-9.

MERA, P., FERRON, M. & MOSIALOU, I. 2018. Regulation of Energy Metabolism by Bone-Derived Hormones. *Cold Spring Harb Perspect Med,* 8.

MERA, P., LAUE, K., FERRON, M., CONFAVREUX, C., WEI, J., GALÁN-DÍEZ, M., LACAMPAGNE, A., MITCHELL, S. J., MATTISON, J. A., CHEN, Y., BACCHETTA, J., SZULC, P., KITSIS, R. N., DE CABO, R., FRIEDMAN, R. A., TORSITANO, C., MCGRAW, T. E., PUCHOWICZ, M., KURLAND, I. & KARSENTY, G. 2017. Osteocalcin Signaling in Myofibers Is Necessary and Sufficient for Optimum Adaptation to Exercise. *Cell Metab,* 25**,** 218.

MERCOLINI, L., PROTTI, M., CATAPANO, M. C., RUDGE, J. & SBERNA, A. E. 2016. LC-MS/MS and volumetric absorptive microsampling for quantitative bioanalysis of cathinone analogues in dried urine, plasma and oral fluid samples. *J Pharm Biomed Anal,* 123**,** 186-94.

MORA, S., PITUKCHEEWANONT, P., KAUFMAN, F. R., NELSON, J. C. & GILSANZ, V. 1999. Biochemical markers of bone turnover and the volume and the density of bone in children at different stages of sexual development. *J Bone Miner Res,* 14**,** 1664-71.

MORA, S., PRINSTER, C., BELLINI, A., WEBER, G., PROVERBIO, M. C., PUZZOVIO, M., BIANCHI, C. & CHIUMELLO, G. 1997. Bone turnover in neonates: changes of urinary excretion rate of collagen type I cross-linked peptides during the first days of life and influence of gestational age. *Bone,* 20**,** 563-6.

O'MARA, M., HUDSON-CURTIS, B., OLSON, K., YUEH, Y., DUNN, J. & SPOONER, N. 2011. The effect of hematocrit and punch location on assay bias during quantitative bioanalysis of dried blood spot samples. *Bioanalysis,* 3**,** 2335-47.

OLDKNOW, K. J., MACRAE, V. E. & FARQUHARSON, C. 2015. Endocrine role of bone: recent and emerging perspectives beyond osteocalcin. *J Endocrinol,* 225**,** R1-19.

ORUM, O., HANSEN, M., JENSEN, C. H., SØRENSEN, H. A., JENSEN, L. B., HØRSLEV-PETERSEN, K. & TEISNER, B. 1996. Procollagen type I N-terminal propeptide (PINP) as an indicator of type I collagen metabolism: ELISA development, reference interval, and hypovitaminosis D induced hyperparathyroidism. *Bone,* 19**,** 157-63.

OSEI-BIMPONG, A., MCLEAN, R., BHONDA, E. & LEWIS, S. M. 2012. The use of the white cell count and haemoglobin in combination as an effective screen to predict the normality of the full blood count. *Int J Lab Hematol,* 34**,** 91-7.

PARKER, S. L., ROBERTS, J. A., LIPMAN, J. & WALLIS, S. C. 2015. Quantitative bioanalytical validation of fosfomycin in human whole blood with volumetric absorptive microsampling. *Bioanalysis,* 7**,** 2585-95.

PROCKOP, D. J., SIERON, A. L. & LI, S. W. 1998. Procollagen N-proteinase and procollagen C-proteinase. Two unusual metalloproteinases that are essential for procollagen processing probably have important roles in development and cell signaling. *Matrix Biol,* 16**,** 399-408.

RAVN, P., CLEMMESEN, B., RIIS, B. J. & CHRISTIANSEN, C. 1996. The effect on bone mass and bone markers of different doses of ibandronate: a new bisphosphonate for prevention and treatment of postmenopausal osteoporosis: a 1-year, randomized, double-blind, placebo-controlled dose-finding study. *Bone,* 19**,** 527-33.

RESCH, H., PIETSCHMANN, P., KUDLACEK, S., WOLOSZCZUK, W., KREXNER, E., BERNECKER, P. & WILLVONSEDER, R. 1994. Influence of sex and age on biochemical bone metabolism parameters. *Miner Electrolyte Metab,* 20**,** 117-21.

RESNICK, L., VEREN, K., SALAHUDDIN, S. Z., TONDREAU, S. & MARKHAM, P. D. 1986. Stability and inactivation of HTLV-III/LAV under clinical and laboratory environments. *JAMA,* 255**,** 1887-91.

RICHTER, M. 2008. Electrochemiluminescence. *In:* LIGLER, F., TAITT, CR (ed.) *Optical Biosensors Today and Tomorrow.* "Second Edition" ed.

ROCHE DIAGNOSTICS. 2019. Elecsys Total P1NP Method Sheet. 02/2019 ed.

ROCHE DIAGNOSTICS. 2020. Elecsys Beta-CrossLaps/Serum Method Sheet. 03/2020 ed.

ROSEN, H. N., MOSES, A. C., GARBER, J., ILOPUTAIFE, I. D., ROSS, D. S., LEE, S. L. & GREENSPAN, S. L. 2000. Serum CTX: a new marker of bone resorption that shows treatment effect more often than other markers because of low coefficient of variability and large changes with bisphosphonate therapy. *Calcif Tissue Int,* 66**,** 100-3.

ROSENQUIST, C., FLEDELIUS, C., CHRISTGAU, S., PEDERSEN, B. J., BONDE, M., QVIST, P. & CHRISTIANSEN, C. 1998. Serum CrossLaps One Step ELISA. First application of monoclonal antibodies for measurement in serum of bone-related degradation products from C-terminal telopeptides of type I collagen. *Clin Chem,* 44**,** 2281-9.

SCHLEMMER, A. & HASSAGER, C. 1999. Acute fasting diminishes the circadian rhythm of biochemical markers of bone resorption. *Eur J Endocrinol,* 140**,** 332-7.

SEIBEL, M. J. 2005. Biochemical markers of bone turnover: part I: biochemistry and variability. *Clin Biochem Rev,* 26**,** 97-122.

SEO, J. Y., LEE, S. T. & KIM, S. H. 2015. Performance evaluation of the new hematology analyzer Sysmex XN-series. *Int J Lab Hematol,* 37**,** 155-64.

SHERMAN, V. R., YANG, W. & MEYERS, M. A. 2015. The materials science of collagen. *J Mech Behav Biomed Mater,* 52**,** 22-50.

SLEMENDA, C. W., PEACOCK, M., HUI, S., ZHOU, L. & JOHNSTON, C. C. 1997. Reduced rates of skeletal remodeling are associated with increased bone mineral density during the development of peak skeletal mass. *J Bone Miner Res,* 12**,** 676-82.

SPOONER, N., DENNIFF, P., MICHIELSEN, L., DE VRIES, R., JI, Q. C., ARNOLD, M. E., WOODS, K., WOOLF, E. J., XU, Y., BOUTET, V., ZANE, P., KUSHON, S. & RUDGE, J. B. 2015a. A device for dried blood microsampling in quantitative bioanalysis: overcoming the issues associated blood hematocrit. *Bioanalysis,* 7**,** 653-9.

SPOONER, N., DENNIFF, P., MICHIELSEN, L., DE VRIES, R., JI, Q. C., ARNOLD, M. E., WOODS, K., WOOLF, E. J., XU, Y., BOUTET, V., ZANE, P., KUSHON, S. & RUDGE, J. B. 2015b. A device for dried blood microsampling in quantitative bioanalysis: overcoming the issues associated with blood hematocrit. *Bioanalysis,* 7**,** 653-659.

TE KOPPELE, J. 1998. *EP 0829724A1*.

TEISNER, B., RASMUSSEN, H. B., HØJRUP, P., YDE-ANDERSEN, E. & SKJØDT, K. 1992. Fetal antigen 2: an amniotic protein identified as the aminopropeptide of the alpha 1 chain of human procollagen type I. *APMIS,* 100**,** 1106-14.

THIRUP, P. 2003. Haematocrit: within-subject and seasonal variation. *Sports Med,* 33**,** 231-43.

TSUKAHARA, H., MIURA, M., HORI, C., HIRAOKA, M., NOSAKA, K., HATA, K., KONISHI, Y. & SUDO, M. 1996. Urinary excretion of pyridinium cross-links of collagen in infancy. *Metabolism,* 45**,** 510-4.

TSUKAHARA, H., WATANABE, Y., HIRANO, S., TSUBOKURA, H., KIMURA, K. & MAYUMI, M. 1999. Assessment of bone turnover in term and preterm newborns at birth: measurement of urinary collagen crosslink excretion. *Early Hum Dev,* 53**,** 185-91.

UK, D. O. H. 2011. Governance arrangements for research ethics committees: a harmonised edition. *Department of Health UK*.

WICHERS, M., SCHMIDT, E., BIDLINGMAIER, F. & KLINGMÜLLER, D. 1999. Diurnal rhythm of CrossLaps in human serum. *Clin Chem,* 45**,** 1858-60.

WOITGE, H. W., FRIEDMANN, B., SUTTNER, S., FARAHMAND, I., MÜLLER, M., SCHMIDT-GAYK, H., BAERTSCH, P., ZIEGLER, R. & SEIBEL, M. J. 1998. Changes in bone turnover induced by aerobic and anaerobic exercise in young males. *J Bone Miner Res,* 13**,** 1797-804.