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Article Title: Clinical usefulness of bone turnover marker levels in osteoporosis

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X Structured abstract

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X **References are in journal format. References in text and reference list correspond exactly.**

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***Abstract**

Abstract:
Current evidence continues to support the potential for bone turnover markers (BTM) to provide clinically useful information particularly for monitoring the efficacy of osteoporosis treatment. Many of the limitations identified earlier remain, principally in regard to the relationship between BTM and incident fractures. Important data are now available on reference interval values for CTX and PINP across a range of geographic regions and for individual clinical assays. An apparent lack of comparability between current clinical assays for CTX has become evident indicating the possible limitations of combining such data for meta-analyses. Harmonization of units for reporting serum/plasma CTX (ng/L) and PINP (µg/L) is recommended. The development of international collaborations continues with an important initiative to combine BTM results from clinical trials in osteoporosis in a meta-analysis and an assay harmonization program are likely to be beneficial. It is possible that knowledge derived from clinical studies can further enhance fracture risk estimation tools with inclusion of BTM together with other independent risk factors. Further data of the relationships between the clinical assays for CTX and PINP as well as physiological and pre-analytical factors contributing to variability in BTM concentrations are required.

*Response to Reviewers

Professor Philippe Gillery
Managing Guest Editor Clinica Chimica Acta Special Issue

Ms. Ref. No.: CCA-D-16-00181
Title: Clinical usefulness of bone turnover marker levels in osteoporosis Clinica Chimica Acta

Dear Professor Gillery

Dear Philippe,

We are pleased to resubmit this manuscript which has been revised according to the comments from the reviewers'.
We thank the reviewers for their constructive comments and believe that the manuscript has been significantly improved with their comments.

Reviewer #1: The authors have addressed most of my remarks. Some changes (eg. regarding tables) have not been made but I respect these choices. Nice paper indeed! Very minor points left:
- In some parts "levels" have not been changed to "concentrations" (eg. section 4, title and §3 line 4). Please check all text.
The text has been carefully reviewed and changes made to all but one use of the term 'levels' which we believe is preferable. Of course where 'level' is used in the title of a cited publication that is a change we cannot make.
- In section 5 (§ 3 and 4), many repetitive references are made to tables 3 and 4. This could be simplified.
- Tables 3-6 and related text (section 5): not clear whether values correspond to quartiles, mean+/- SD. Could it be explained in legends?
We suggest that these two points raised above are contradictory and for this reason we have not simplified the many references to tables 3 and 4. We believe that this section is highly valuable because it is the only review of these data, to our knowledge, in the scientific literature. As stated in the second point above, the published data of the references intervals are derived by a range of statistical methods. We do not have access to the original data and therefore do not have the opportunity to reanalyse the data in a uniform manner. However we do believe that the method for deriving the reference interval is clearly described. We believe that this point is best described in the text and not in a figure legend as it would make the legend much too long. Therefore we believe that the presentation as made in the original text with a full description of the derivation of the reference interval for each study in the text and with the summary in the table, with reference to the specific table in the text, is the best presentation of these data.
- Tables 4 and 6: make sure that µg/l is read, not ug.
The 'u' has been changed to 'µ'. We thank the reviewer for noticing this issue.

Reviewer #2: I support publication of the revised manuscript. The authors have adequately addressed the comments and requested changes made by each reviewer during the initial review process.

Reviewer #3: The authors revised the manuscript carefully according to the reviewers comments. Therefore, the revised one is ready for publication.

We hope that you now find the manuscript acceptable for publication.
With best regards

Howard Morris

Corresponding author

Highlights:

New data support the potential for bone turnover markers to inform on fracture risk and efficacy of osteoporosis treatment.

Reference intervals for CTX and PINP for geographic regions and individual assays are available.

Harmonization of units for reporting serum/plasma CTX (ng/L) and PINP (µg/L) is recommended.

Clinical usefulness of bone turnover marker concentrations in osteoporosis

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Abstract:
Current evidence continues to support the potential for bone turnover markers (BTM) to provide clinically useful information particularly for monitoring the efficacy of osteoporosis treatment. Many of the limitations identified earlier remain, principally in regard to the relationship between BTM and incident fractures. Important data are now available on reference interval values for CTX and PINP across a range of geographic regions and for individual clinical assays. An apparent lack of comparability between current clinical assays for CTX has become evident indicating the possible limitations of combining such data for meta-analyses. Harmonization of units for reporting serum/plasma CTX (ng/L) and PINP (µg/L) is recommended. The development of international collaborations continues with an important initiative to combine BTM results from clinical trials in osteoporosis in a meta-analysis and an assay harmonization program are likely to be beneficial. It is possible that knowledge derived from clinical studies can further enhance fracture risk estimation tools with inclusion of BTM together with other independent risk factors. Further data of the relationships between the clinical assays for CTX and PINP as well as physiological and pre-analytical factors contributing to variability in BTM concentrations are required.

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Keywords:
Bone turnover markers; osteoporosis; fracture risk; reference intervals; monitoring efficacy for treatment of osteoporosis; CTX; PINP.

1. Introduction:

Osteoporosis is the most prevalent metabolic bone disease and with an aging population its impact is expected to rise throughout the world. It is defined as a disease characterised by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and consequent increase in risk of fracture [1]. Low bone mass, measured as bone mineral density (BMD), is asymptomatic and its important outcome is fracture, a cause of morbidity and mortality [2]. Therefore, the clinical management focus in osteoporosis is to prevent or reduce the risk of fracture and follow the response to therapy. Its total cost burden, including pharmacological prevention, in the European Union was recently estimated to correspond to approximately 3.5% of the total spending on health care at €37 billion [3]. Similar relative cost burdens are experienced in other parts of the world with the steepest rises in number of fractures in the coming years expected to be reported from the high population countries of Asia, all largely dependent on the ageing of the population [4].

The first line of medical testing for diagnosis of osteoporosis and estimation of risk of fracture whether at clinical presentation or following initiation of treatment is measurement of BMD, most commonly using dual-energy x-ray absorptiometry (DXA) [5]. Algorithms to estimate fracture risk based on BMD and other clinical features such as FRAX® are commonly used in clinical practice to guide the treatment of individual patients [6]. Bone turnover markers (BTM) are not included in such algorithms.

BTM have a long history in research on metabolic bone diseases including osteoporosis and assays for a wide range have been developed. A review of this complete range is beyond the scope of this manuscript although others are available [7, 8]. BTM largely represent products of bone proteins, particularly type I collagen which undergoes considerable post-translational modification during synthesis of new bone and within the bone environment such that particular modifications increase the specificity for assessing bone formation or bone resorption. Other BTMs are products of bone cells, reflecting the number of particular cells within the bone environment at any time.

In 2010 the International Osteoporosis Foundation (IOF)–International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) Joint Working Group on Bone Marker Standards (WG-BMS) published an extensive review concluding that there were insufficient data to include bone turnover markers values in current clinical practice [9]. The Working Group recommended one bone formation marker (serum-procollagen type I N-propeptide (PINP))

and one bone resorption marker (serum C-terminal telopeptide of type I collagen, (CTX)) be used as reference markers, to be measured by standardised assays in observational and intervention studies in order to assess their clinical performance as well as provide data by which alternatives could be assessed thus enlarging the international experience of the application of these markers to clinical medicine. In 2012 the National Bone Health Alliance extended the literature review on this subject arriving at similar recommendations [10].

The IFCC-IOF Working Group for the Standardization of Bone Marker Assays was established in 2012 to standardize or harmonize serum/plasma CTX and PINP assays depending on feasibility. After initial discussions with representatives of clinicians, clinical laboratorians and the In Vitro Diagnostic industry, it was agreed that a strategy of harmonization of assays was preferable because of the current lack of data indicating their clinical usefulness. A project is underway to describe the relationship for CTX and PINP values generated by the various assays used by clinical laboratories for patients presenting to an osteoporosis clinic. In the first instance a statistical method will be used to harmonise values where the assays provide significantly different concentrations.

2. BTM concentrations for predicting fracture risk

The IOF-IFCC WG-BMS review by Vasikaran et al described 22 studies, in which the relationship between bone turnover markers and incident fractures was examined [9]. Eighteen of them showed that one or more markers were associated with risk of subsequent fracture with the concentration of bone resorption markers more consistently associated with fracture risk than bone formation markers. This was the case for studies in both men and women. Since that time three more studies have been published including a meta-analysis (Table 1). The meta-analysis examined the performance characteristics of two BTM, PINP and CTX, for fracture risk prediction in untreated individuals. The analysis included 6 prospective, cohort studies with the first incident fracture as the primary outcome. Only studies in middle-aged or older men and women were included. The expression of risk varied between the original studies, but all results were transformed into hazard ratio (HR) per standard deviation (SD) which is the gradient of risk (GR). The meta-analysis found a modest, but significant association between both PINP and CTX concentrations at baseline and fracture risk (see Table 1) [11]. This analysis combined results for CTX generated by the two clinical laboratory automated assay methods currently available. As presented below (see Section 6) these assays do not appear to provide comparable values for CTX. Similarly the

PINP data were generated by different assays and while the lack of comparability of these assays is less certain again the GR would likely be reduced by combining assay data which are not comparable. In the Australian Health In Men Study the association of bone turnover markers with hip fracture incidence in older men was examined. Total osteocalcin (tOC), undercarboxylated osteocalcin (ucOC) and CTX were associated with hip fractures in univariate analyses, but only tOC remained significantly associated with incident hip fractures in multivariate analyses adjusting for age and glucocorticoid use [12]. In contrast to the above, a Japanese study of the Taiji cohort of both men and women failed to demonstrate a significant association between a broad range of markers of bone formation and bone resorption and incident fracture risk. However, the study was insufficiently powered for a fracture endpoint as this cohort included relatively young subjects (mean age approximately 60 years) resulting in a low number of osteoporotic fractures (32) during the 10-year follow-up period [13].

These more recent findings support the previous interpretation in the Vasikaran review [9]. There are significant associations between bone turnover markers and incident fracture risk, though the association is modest. Most studies demonstrate a relation between bone turnover markers and fracture, yet there are limitations to the studies. These include the variable use of markers of bone formation (BAP, PINP, PICP, total osteocalcin, intact osteocalcin) and of bone resorption (ICTP, CTX, NTX-I, PYR, DPD, beta-CTX), differences in analytical assays and platforms, inconsistencies in expression of risk, as well as inconsistent predictive value for a specific marker in the individual studies reported. (See Table 1 for abbreviations of BTMs)

3. BTM concentrations for monitoring treatment

The IOF-IFCC WG-BMS review [9] also reported seven studies concerning the relationship between change in BTM and fracture risk reduction with drugs given for postmenopausal osteoporosis. These drugs included alendronate, risedronate, zoledronic acid, raloxifene, and strontium ranelate. One of the outcomes from such studies is to assess the extent to which a biological marker is a surrogate end-point for a clinical event, which is known as the ‘treatment effect explained’. In the case of clinical trials for osteoporosis treatment the clinical end-point is fracture and the surrogate biological markers are BTM. In these trials the treatment effect explained varied from 27-77% indicating that about half of the fracture risk

reduction with these drugs, which work through the inhibition of bone turnover, could be associated with the measured change in BTM during the first year of treatment.

There have now been two further studies that examine this question, one a follow-up analysis of zoledronic acid and the other a new analysis with bazedoxifene, a selective estrogen receptor modulator, similar to raloxifene (Table 2). They are both believed to reduce the risk of fracture by the reduction in bone turnover. Jacques and colleagues [14] reported on the relationship of changes in PINP and fracture risk reduction in the HORIZON trial. This was a study of 7736 postmenopausal women with osteoporosis who were randomized to receive zoledronic acid 5 mg intravenously once a year for three years, or placebo. All patients received calcium and vitamin D. A bone marker subset analysis included 1132 women in whom PINP was measured. This marker was chosen as the samples were not taken with the patients in the fasting state and PINP has proven to be informative in other studies, for example with raloxifene where the mean change in PINP at 12 months was 56% [15]. The change in PINP at one year explained 58% of the treatment effect on new vertebral fracture (statistically significant), and there was a significant association with non-vertebral fracture. This figure was similar to the 54% treatment effect explained change in total hip BMD over three years and vertebral fracture. The effect explained by PINP was independent of that explained by total hip BMD, so the results of these two tests are complimentary.

Bruyere and colleagues [16] reported on the relationship of changes in the BTM (CTX and OC) and fracture risk reduction in a phase 3 trial of bazedoxifene. This was a study of 5244 postmenopausal women with osteoporosis who were randomized to receive bazedoxifene 20 mg or 40 mg daily, or raloxifene 60 mg daily, or placebo for three years. All patients received calcium and vitamin D. The median reductions in response to 20 mg daily were CTX (46%), OC (37%) and for 40 mg daily were CTX (49%), OC (39%) [17]. The change in CTX at one year explained 16% and change in OC 6% of the treatment effect on new vertebral fracture (statistically significant). There was no overall reduction of non-vertebral fractures in this study so any relationship with marker change could not be tested. These figures were similar to the figures of 14% treatment effect explained by the change in total hip BMD and 5% for lumbar spine BMD over three years and vertebral fracture.

Once again the conclusions made in the original report [9] are at least partially supported by these new analyses. The treatment effect explained by BTM is at least as great as BMD. The

finding of significant positive associations between the reduction in BTM and the reduction in fracture risk support the use of BTM in monitoring treatment. The limitation noted in the original report that studies were often small subsets of the main trial was true for the zoledronic acid study but not for the bazedoxifene study, which is the largest study to date. The studies were also criticized for not obtaining samples under optimal conditions. This again was not true of these two studies as the patients from the bazedoxifene study were in the fasting state for the blood draw, a critical requirement for serum CTX.

4. The effect of renal impairment on BTM concentrations

Bone health is very frequently altered in Chronic Kidney Disease (CKD) and these patients are at increased risk of fractures whether they are dialyzed [18] or not [19]. Indeed, these patients often are characterized by either increased or decreased bone turnover, linked to over- or under-secretion of parathyroid hormone (PTH). The gold standard to evaluate bone turnover is bone biopsy. Unfortunately, use of bone biopsies to determine bone turnover is hampered by the invasive nature of the procedure and the difficulty for correct interpretation of the results, limiting its use to a few specialized centres [20]. In clinical practice repeated bone biopsies are problematic for the follow-up of the patients or to assess effect of a treatment. Hence, BTM are essential in clinical practice to evaluate bone turnover. In 2009 the international recommendations in nephrology, Kidney Disease: Improving Global Outcomes (KDIGO) guidelines [21] recommended the measurement of PTH and the bone turnover marker Bone Specific Alkaline Phosphatase (BAP) in the assessment of metabolic bone disease of CKD (CKD-MBD). BAP was selected because serum concentrations are unaffected by renal function since it is cleared by the liver and with a molecular weight above 50,000 D it is unlikely to be filtered at the kidney. BAP does suffer from some analytical and clinical issues, which have been discussed elsewhere [22].

PINP has been recommended as the bone formation marker by IOF and IFCC for clinical research studies in osteoporosis [9]. It consists of three subunit chains of type 1 procollagen (2 pro-α1 chains and 1pro-α2 chain) that are non-covalently linked and is produced in equimolar amounts with collagen deposited in bone tissue [23]. Once in the circulation, PINP is rapidly bound and internalized by liver endothelial cells through their scavenger receptors [24]. In human serum, PINP is present in two major forms, an intact trimeric form and a monomeric form. This latter form tends to be elevated in CKD patients. PINP determination can be performed either with automated (Roche Elecsys/Cobas and IDS iSYS) or manual (Orion

Diagnostica) methods but the "Total" PINP assay (Roche Elecsys/Cobas) recognizes both the trimeric form and the monomers whereas the "Intact" PINP assays (IDS iSYS and Orion Diagnostica) recognize the trimeric form only. In CKD patients, it has been shown that patients with a glomerular filtration rate (GFR) below 30 ml/min/1.73 m^2 have PINP concentrations that are overestimated by the "Total" assay due to the cross-reactivity with the monomeric form [25]. Assays specific for "Intact" PINP are recommended for use with CKD patients.

While IOF and IFCC recommend serum CTX as the bone resorption biomarker for clinical research studies in osteoporosis it is not recommended in CKD-MBD by the KDIGO guidelines since serum PTH or BAP are more effective at predicting clinical outcomes or bone histology [21, 26]. Serum CTX concentrations in patients undergoing haemodialysis are some five times higher than those of the normal population due to its accumulation with decreased renal function and frequent secondary hyperparathyroidism [26]. Tartrate resistant acid phosphatase 5B (TRAP-5B) may be a suitable alternative for the monitoring of the bone resorption in CKD patients as it presents very interesting features: its serum concentrations are not influenced by kidney function and it is a non-collagen bone resorption marker with serum concentrations significantly correlating with histological indices of osteoclast number, bone formation rate and mineral apposition rate in uremic patients [27]. By the same token, it is not a good marker of change in bone resorption following treatment with cathepsin K inhibitors, which reduce bone resorption without reducing osteoclast numbers. TRAP-5B has recently become available on the automated IDS iSYS platform which may increase its potential as a routine marker for clinical laboratories increasing the data on this marker since such information is scarce [26].

Fibroblast Growth Factor 23 (FGF23) is produced by osteocytes and is increased in CKD patients. High concentrations of FGF23 are associated with improved indices of skeletal mineralization in dialyzed pediatric patients with high turnover renal osteodystrophy [28]. Thus, FGF-23 measurements may indicate skeletal mineralization status, at least in this population [29]. However, since concentrations of FGF23 are extremely high in CKD patients compared to healthy individuals, it would appear unlikely that subtle changes in FGF23 concentrations will be clinically significant. These high concentrations add to the difficulty of measuring FGF23 with current manual assays. It is unclear whether such highly diluted specimens provide values that reflect the true value in serum or whether matrix effects

confound these results. New studies, with better analytical tools, are needed to prove the usefulness of FGF23 to reflect bone mineralization in CKD patients.

Sclerostin, also produced in the osteocytes, is an inhibitor of the Wnt signalling pathway thus decreasing bone formation [30]. Sclerostin is an independent predictor of bone loss in CKD patients on dialysis [31]. High concentrations of sclerostin have surprisingly been found in dialysis patients with higher bone volume and density and it is unclear whether sclerostin has a true protective effect or if these high values arise as a secondary phenomenon [32]. Sclerostin accumulates in CKD which adds further complexity for interpretation of results [33]. Even more problematic is the lack of concordance between the different assay kits confounding the interpretation of serum levels [34]. With a new anti-sclerostin agent becoming available, interest in this analyte will likely grow but robust analytical methods are required to provide true measurements suitable for clinical interpretation.

5. Interpretation of bone turnover markers concentrations – the role of reference intervals

BTM reference intervals are useful for interpreting the results from osteoporosis patients but by themselves they are of limited value for fracture prediction in untreated, individual patients. The measurement of very high BTM values (> 3 standard deviations above the mean of the reference values) during initial assessment of patients with osteoporosis is suggestive of other metabolic disease including malignancy [9]. The need to establish reference intervals from healthy premenopausal women aged 30-45 years when concentrations are at a nadir has been emphasised [9, 35]. Ideally the subjects used for these studies should have normal BMD at the spine [9]. Expert opinion also suggests that the mean of the premenopausal reference interval can be used as a treatment target for anti-resorptive therapy [9, 35].

It is considered necessary to establish reference intervals for different geographic areas and ethnicities [9]. Furthermore due to differences that currently exist between results from the different commercial clinical assays, current reference intervals need to be method specific; reference intervals from different methods cannot be used interchangeably. The following data providing reference interval data for CTX and PINP from various countries and assays are summarized in Tables 3-6.

de Papp et al studied healthy premenopausal women from across the US including users and non-users of the oral contraceptive pill [OCP]. Serum samples were collected in the morning

after an overnight fast. CTX values were log transformed to obtain a normal distribution and the geometric mean ±2 SD was used to determine the overall mid 95% range for CTX (Table 3). Data from Italian healthy premenopausal, non-OCP using women aged 20-49 years were examined for the central 95% distribution for PINP and CTX [37]. Serum samples were collected between 7.30 am and 8.30 am after an overnight fast. BTMs were considerably higher in women aged 20–25 years and decreased progressively until 45–50 years of age. The reference intervals in women aged 45–50 years are presented in Tables 3 and 4. Healthy French premenopausal, non-OCP using women provided serum samples after an overnight fast before 10 am. The 2.5^{th} to 97.5^{th} percentile distribution for CTX and PINP are shown in Tables 3 and 4 [38]. Reference intervals for English premenopausal, non-OCP using women were established from serum samples collected between 8 am and 10 am after an overnight fast. Data for serum CTX and PINP were log transformed and 95% reference interval was calculated as mean±1.96 SD (Tables 3 and 4) [39].

French, Belgium, US and UK healthy premenopausal women including OCP non-users and users provided serum samples collected between 8 and 10 am after an overnight fast [40]. CTX and PINP values were log transformed to achieve normal distributions (Tables 3 and 4). Healthy premenopausal Saudi Arabian, non-OCP using women provided serum samples collected between 9:00 and 11:00 am after an overnight fast [41**]**. The central 95% calculated for each BTM (Tables 3 and 4). A cross-sectional registry study examined premenopausal healthy European Caucasian women not on OCP from France and Denmark [42]. Serum samples were collected after an overnight fast between 08:00 and 09:30 am. BTM data were log transformed to obtain a normal distribution and the reference intervals were determined as mean±1.96 SD for normalized values (Tables 3 and 4). An Australian study that included premenopausal women from the Geelong Osteoporosis Study examined reference intervals by decades of age [43]. Serum samples were collected after an overnight fast between 07:30 and 11:45 am and stored at -80^{0}c for >10 years. Optimal age-related reference intervals were determined for each BTM based on the central 90% of the distribution (Tables 3 and 4). Harmonized reference intervals for use in Australia have been developed for automated Roche assays for CTX and PINP based on published studies listed above with most weighting given for the Australian data [44, 45].

Serum samples were collected from healthy premenopausal Spanish, non-OCP using women between 8 and 10 am after an overnight fast [46]. A quantile regression was used to estimate the 5^{th}, 50^{th} and 95^{th} percentiles. The reference intervals are provided in Tables 3 and 4 for the automated Roche assay and Tables 5 and 6 for the automated IDS iSYS assay. The German Study of Health in Pomerania examined healthy premenopausal women after excluding those with any predetermined illness, OCP use or serum 25-hydroxyvitamin D concentration less than 25 nmol/L. Blood sampling was performed between 8.00 am and 8.00 pm from the mostly non-fasting subjects [47]. Reference intervals were defined as the central 95% range between the 2.5th and 97.5th percentiles (Tables 5 and 6). Note this study included mostly non fasting subjects and sampling was performed throughout the day. Morovat et al studied apparently healthy premenopausal women as part of a larger study in two centres [48]. No mention is made of OCP use. Serum samples were collected during working hours in Belgium and between 8.30 am and 3.00 pm in UK. PINP was measured by automated IDS-iSYS assay. PINP values were log transformed to obtain a normal distribution and the 95% reference interval determined and calculated values were converted back to measured units (Tables 5 and 6).

The largest variation between the reference intervals appear to be between the Roche and IDS-iSYS assays for CTX although data for the IDS-iSYS assay are limited. The variation across geographic regions appears to be minor except for those from Saudi Arabia. Possibly data from other regions are largely derived from Caucasian populations and therefore there remain limited data from other ethnic groups as discussed previously [9].

6. Comparability of PINP and CTX values generated by current clinical assays

As discussed previously currently there are three clinical assays available for PINP and for CTX in blood. EDTA plasma has been stated as the preferred specimen type for the assay of CTX and is identified as such when specific reference is made. PINP is less affected by specimen type. The relationships between results produced by these different clinical assays for CTX and PINP have been examined. Note that CTX is variously reported in units of ng/L or ng/mL; in this review all results are converted to ng/L. P1NP is reported in µg/L in most studies.

Koivula et al examined the relationships between the PINP results produced by two assays, the automated Roche Elecsys 2010 assay which measures total PINP and the

radioimmunoassay for intact PINP (Orion Diagnostica UniQ PINP) [49]. The subjects were: 34 apparently healthy blood donors (26 men, 8 women; ages between 19 and 62 years), 39 patients with chronic renal failure and 173 bedridden geriatric (age >65 years) in-patients. The serum samples were kept frozen at -20^{0}C till analysis. The Passing-Bablok regression data are given in Tables 7 and 8. They concluded that PINP concentrations were similar in healthy blood donors but different in haemodialysis or bedridden geriatric patients with the Roche assay giving significantly higher results. In the most extensive study of P1NP methods, Morovat et al compared automated Roche E170 Total PINP and IDS iSYS Intact PINP in 828 serum specimens from healthy individuals and osteoporotic patients [50]. This study is notable for including a significant number of healthy children (>45% of the whole cohort), which had the effect of extending the range of P1NP values in the comparison. The relationship between the two assays was non-linear. Overall the iSYS results were significantly higher than those obtained by the Roche E170 but at total PINP concentrations of < 100 μg/L and > 670 μg/L, the iSYS assay gave lower values than the E170 assay. Cavalier et al compared the automated Roche Elecsys Total PINP and IDS iSYS Intact PINP assays in two populations; 157 patients in stage 3–5 CKD and 125 patients in stage 5D patients [51]. They concluded that the two assays produce the most discrepant results when eGFR decreases below 30 mL/min/1.73 m^2 although discrepancy is apparent even for eGFR values between 30 and 60 mL/min/1.73 m^2 (Table 8).

Wheater et al examined the relationships between the results produced by two automated systems, Roche Elecsys 2010 and IDS iSYS, for PINP and CTX in blood from 127 subjects: 72 self-reported healthy volunteers (28 males, 28 females < 50 years and 5 males, 11 females > 50 years) with no known bone disease and 55 rheumatoid arthritis (RA) patients (1 male, 4 females < 50 years and 10 males, 40 females > 50 years) [52]. All patients had an estimated glomerular filtration rate (eGFR) > 30 mL/min/1.73 m^2. Serum samples were stored at -80^{0}c immediately after venepuncture and used for both assays. The Passing-Bablok regression data are shown in Tables 7 and 9. Whereas the P1NP assays appeared to give equivalent results, these authors found significant proportional and systematic biases between the CTX assays. Chubb et al measured plasma CTX by all three commercial assays on 169 adult patients (119 females and 50 males, median age 65 years [inter-quartile range 57–75.75] years) attending hospitals for routine investigation of metabolic bone disease including osteoporosis [53]. EDTA plasma was frozen at -20^{0}c before analysis after storage at 4^{0}C for up to 7 days. They also found significant proportional and systematic bias when the IDS iSYS assay was

compared to both the IDS ELISA and Roche methods. The Passing Bablok regression parameters are given in Table 9 and 10. In contrast, in a conference abstract, Cavalier et al. reported no systematic bias and lower proportional bias (the slope of the regression line was 1.12) between the Roche and IDS iSYS automated assays for CTX [54] (Table 9). Huvelle et al compared CTX results by the IDS iSYS assay and the IDS ELISA on 97 serum samples collected from patients presenting to hospital for bone and mineral metabolism work-up (females 78; males 19; mean age: 67 years) [55]. Their regression data are shown in Table 10. They concluded that their limited study suggested the two assays could be used interchangeably.

In summary, the results of two studies suggest that all PINP assays give similar results in healthy subjects with eGFR >30 mL/min/1.73m^2 [49, 52]. However, based on the largest comparison study of the IDS iSYS and Roche E170 assays, Morovat et al have concluded: "although there is a broad, general agreement between the intact and total PINP assays, there are some variations between the two results, and the differences can be large, unpredictable and clinically significant" [50]. Clearly the total PINP assay gives significantly higher values than the intact PINP assays in patients where there is an accumulation of the monomer; e.g. renal failure patients with eGFR <30 mL/min/1.73m^2, and in patients who are bedridden long-term [49, 51].

For CTX assays, Wheater et al and Chubb et al found significant proportional and systematic inter-method biases [52, 53], whereas Cavalier et al and Huvelle et al did not [54, 55]. Two reference interval studies for CTX, each carried out using more than one assay support the presence of significant inter-assay biases for CTX [42, 46]. The basis for these differences in outcomes between studies is unclear although variation between plasma or serum specimens may contribute. Such effects may hamper efforts to achieve harmonisation of results between assays.

7. Conclusions

The current status in this field continues to support the potential for BTM to provide clinically useful information although many of the limitations identified earlier remain, particularly in regard to the relationship between BTM and incident fractures. Significant progress has been made on the usefulness of BTM for monitoring the efficacy of osteoporosis treatment. Important data are now available on reference interval values for CTX and PINP across a

range of geographic regions and for individual assays. Perhaps most importantly the apparent lack of comparability between current clinical assays for CTX has become evident indicating the possible limitations of combining such data for meta-analyses. In order to overcome the limitations and to gain additional knowledge of the value of bone turnover marker measurements for predicting fracture risk, we reiterate the suggestions of the IOF-IFCC Bone Marker Standards Working Group [9] and NBHA [10] that future clinical studies should focus on using standardized analytical methods of reference analytes. Further study of the relationships between the clinical assays for CTX and PINP as well as factors, including physiological and pre-analytical issue, contributing to variability in BTM concentrations is required.

It is encouraging that the development of international collaborations continues. One is an initiative to bring all data from clinical trials in osteoporosis together in an individual meta-analysis. The Foundation of the National Institutes of Health in the US are obtaining all BTM results from the clinical trials in osteoporosis and planning such an analysis. (http://www.fnih.org/what-we-do/current-research-programs/biomarkers-consortium-bone-quality-project) This should overcome the criticisms of inconsistent statistical methodology and small sample size. It is possible that this knowledge can contribute to further enhance fracture risk estimation tools such as FRAX with inclusion of bone turnover markers together with other independent risk factors.

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Table(s)

Table 1. Studies of bone turnover markers to predict fractures in men and women not on treatment for osteoporosis

Study	Population and setting	Age (years)	Expression of risk	Length of follow-up	Fracture type	Outcome
Johansson 2014 [11]	Meta-analysis, 6 prospective cohort studies, middle-aged or older men (2 studies) and women (4 studies)	>50	HR for fracture per SD in BTM (GR)	From 2 to 6.5 years	Different between studies: Hip, non-vertebral, osteoporotic	HR per SD (95% CI). Different settings for adjustment. Fracture combined (hip, non-spine, osteoporotic, any, low-trauma) PINP HR=1.23 (1.09-1.39) CTX HR=1.18 (1.08-1.29) HR=1.19 (1.05-1.34) (if women only) HR=1.17 (1.04-1.31) (if adjusted for age) HR=1.12 (0.97-1.29) (if adjusted for BMD) Hip fracture CTX HR=1.23 (1.04-1.47) HR=1.17 (0.95-1.44) (If women only)
Yoshimura 2011 [13]	307 middle-aged and elderly Japanese recruited by age- and gender –stratification in the Taiji cohort (147 men and 160 women), 32 with fractures	40-79	HR per SD	10 years	Osteoporotic (spine, pelvis, ribs, distal radius, forearm, humerus and hip)	HR per SD. However, HR are not shown in article, as no significant associations were found s-OC, s-tOC, s-BAP,s- PICP, s-PINP, s-ICTP, s-beta-CTX, s-NTX, u-PYR, u-DPD
Chubb 2015 [12]	4,028 community-dwelling older men from Perth, Australia enrolled in the population-based Health In Men Study (HIMS), 114 with hip fractures, 3,896 in control group	70-89	OR per SD in BTM	From 8 to 11 years	Hip fractures	OR per SD (95% CI) Log10(tOC) 1.20 (1.00-1.42) (after adjustment for age and GC use) Log10(PINP and Log10(CTX-I) not significantly associated with incident hip fracture after adjustment for age and GC use (P>0.17)

ICTP: C-terminal cross-linking telopeptide of type I collagen generated by matrix metalloproteinase; BAP: bone-specific alkaline phosphatase; beta-CTX: beta-isomerized C-terminal cross-linking telopeptide of type I collagen; BTM: bone turnover marker; CI: confidence interval; CTX: C-terminal cross-linking telopeptide of type I collagen; DPD: deoxypyridinoline cross-links of collagen; GC: glucocorticoid; GR: gradient of risk; HR: hazard ratio; NTX: N-terminal cross-linking telopeptide of type I collagen; OC: intact osteocalcin; OR: odds ratio; PICP: C-terminal propeptide of type I collagen; PINP: N-terminal propeptide of type I collagen; PYR: pyridinoline cross-links of collagen; SD: standard deviation; tOC: total osteocalcin

Table 2 Studies of bone turnover markers following initiation of osteoporosis treatment

Treatment	Trial	Author	N	BTM	Months	Change, %	Duration, yr	Fracture	Treatment Effect Explained		
Zoledronic Acid	HORIZON	Jacques 2012 [14]	1132	PINP	12	56	3	Vertebral	58%		
Bazedoxifene (all) 20 mg daily 40 mg daily	International	Bruyere 2012 [16]	5244	CTX OC	12	 CTX (46), OC (37) CTX (49), OC (39)	3	Vertebral	CTX, 18% (3-41) OC, 14% (0-46) CTX, 20% (4-44) OC, 4% (0-21) CTX, 25% (3-68) OC, 29% (0-85)		

BTM abbreviations are as described for Table 1.

Table 3: Reference intervals for CTX in pre-menopausal women measured by the automated Roche assay

Region	Age range (n)	Reference Interval (ng/L)	Mean/median ng/L	Reference
USA	28-45 (237)	94 to 659	280	De Papp et al [36]*
Italy	45-50 (82)	70–610	250	Adami et al [37]
France	35-45 (157)	105 – 589	N/A	Claudon et al [38]
England	35-45 (153)	100 - 620	270	Glover et al [39]
France, Belgium, US and UK	30-39 (637)	114 - 628	317	Glover et al [40]*
Saudi Arabia	35-45 (765)	163 - 274	217	Ardawi et al [41]
France, Denmark	35-39 (188)	111 - 791	297	Eastell et al [42]
Australia	30-39 (215) 40-49 (209)	100-700 100-600	N/A N/A	Jenkins et al [43]
Australia	20-49 30-39	150–800 100-700	N/A	Vasikaran et al [44]
Spain	35-45 (164)	137 - 484	255	Guanabens et al [46]

*Included OCP users

Table 4: Reference intervals for PINP in pre-menopausal women measured by the automated Roche assay

Region	Age range (n)	Reference Interval (ug/L)	Mean/median ug/L	Reference
Italy	45-50 (82)	14.6–63.5	34.7	Adami et al [37]
France	35-45 (157)	17.9–60.4	N/A	Claudon et al [38]
England	35-45 (153)	16.2 – 60.9	33.1	Glover et al [39]
France, Belgium, US and UK	30-39 (637)	16.3 – 78.2	38.7	Glover et al [40]*
Saudi Arabia	35-45 (765)	22.3 – 42.9	32.5	Ardawi et al [41]
France, Denmark	35-39 (188)	17.3 – 83.4	38.0	Eastell et al [42]
Australia	30-39 (215) 40-49 (209)	15-80 15-60	N/A N/A	Jenkins et al [43]
Australia	25-49 25 - 34	15–70 15–90	N/A	Vasikaran et al [44]
Spain	35-45 (164)	22.7 – 63.1		Guanabens et al [46]

*Included OCP users

Table 5: Reference intervals for CTX in pre-menopausal women measured by the automated IDS assay

Region	Age range (n)	Reference Interval (ng/L)	Mean/median ng/L	Reference
Spain	35-45 (164)	109 - 544	249	Guanabens et al [46]
Germany	30-54 (382)	50 - 670	230	Michelsen et al [47]*

*Sample collected from 8 am to 8 pm, non-fasting

Table 6: Reference intervals for PINP in pre-menopausal women measured by the automated IDS assay

Region	Age range (n)	Reference Interval (ug/L)	Mean/median ug/L	Reference
Spain	35-45 (164)	21.8 – 65.5	36.6	Guanabens et al [46]
Belgium and UK	18-50 (180)	13.7-71.1	N/A	Morovat et al [48]*

*Samples collected during the day, non-fasting. OCP use not specified

Table 7

Regression equations describing the relationships of PINP values in healthy subjects generated by current clinical assays

Method 1 (x)	Method 2 (y)	n	Slope (95% CI)	Intercept (95% CI) (µg/L)	Reference
Orion	Roche	34	0.94 (0.80 – 1.15)	-3.6 (-18.4 – 3.6)	Koivula et al [49]
Roche	iSYS	127	0.98 (0.94 – 1.03)	- 1.42 −2.86 – - 0.08	Wheater et al [52]
Roche	iSYS	820	1.05 (1.04-1.06)	-1.4 (-1.9 – -0.8)	Morovat et al [50]

Table 8
Regression equations describing the relationships of PINP values in renal failure and bed-bound patients

Method 1 (x)	Method 2 (y)	n	Slope (95% CI)	Intercept (95% CI) (µg/L)	Reference
Orion	Roche	39	5.74 (4.56–8.57)	- 95.6 -240.9 – -31.9)	Koivula et al [49] (Haemodialysis patients)
Orion	Roche	173	1.57 (1.43 – 1.73)	-12.0 (-19.0 – -5.7)	Koivula et al [49] (Elderly bed-bound patients)
Roche	iSYS	81	0.74 (0.67 – 0.81)	+ 3.7 (1.2 – 5.8)	Cavalier et al [51] (eGFR 30-60 mL/min/ 1.73 m^2)

Table 9
Regression equations describing the relationships of CTX values from two automated assays

Method 1 (x)	Method 2 (y)	n	Slope (95% CI)	Intercept (95% CI) (ng/L)	Reference
Roche	iSYS	127	1.29 (1.24 - 1.34)	–24 (-34.08 − -12.81)	Wheater et al [52]
Roche	iSYS	156	1.61 (1.545 - 1.664)	-109 (-129.4 – -91.5)	Chubb et al [53]*
Roche	iSYS	98	1.12 (N/A)	-23 (N/A)	Cavalier et al [54]

* Note EDTA plasma specimens were used for these analyses, N/A not available

Table 10
Regression equations describing the relationships of CTX values from the IDS automated assay and the IDS ELISA

Method 1 (x)	Method 2 (y)	n	Slope (95% CI)	Intercept (95% CI) (ng/L)	Reference
ELISA	iSYS	156	1.266 (1.192 - 1.337)	-108.6 (-132.9 – -78.8)	Chubb et al [53]*
ELISA	iSYS	93	0.94 (0.81-1.10)	-5.91 (-54.47-42.69)	Huvelle et al [55]

* Note EDTA plasma specimens were used for these analyses