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Analytical considerations and plans to standardize or harmonize assays for the reference bone turnover markers PINP and β -CTX in blood

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Abstract

Procollagen type I N-propeptide (PINP) and the C-terminal telopeptide of type I collagen (β -CTX) in blood have been designated as reference bone turnover markers in osteoporosis by the International Osteoporosis Foundation (IOF) and International Federation of Clinical Chemistry and Laboratory Medicine (IFCC). The IFCC Committee on Bone Metabolism (C-BM) has

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examined current commercial assays and performed a multicentre study to examine the agreement between assays for PINP and β -CTX in serum and plasma. The results of these studies will inform our work towards the harmonization of PINP assays and the standardization of β -CTX assays in blood, with the development of common calibrators and reference measurement procedures in collaboration with the reagent manufacturing industry. Successful achievement of these goals will help develop universally acceptable practice guidelines for the management of osteoporosis with the inclusion of common reference intervals and treatment targets for PINP and β -CTX.

Keywords

Procollagen type I N-propeptide; PINP; C-terminal telopeptide of type I collagen; β -CTX; bone turnover markers; bone formation; bone resorption; standardization; harmonization

Introduction

Osteoporosis is a disease characterized by low bone mass and micro-architectural deterioration of bone tissue, leading to an increased risk of fracture with associated morbidity and mortality [1]. It constitutes a major global public health burden with increasing prevalence and morbidity worldwide [2]. Methods for the identification of at-risk patients and the diagnosis of osteoporosis are widely recognized, and effective treatments are available to reduce the risk of fractures [3–5]. Bone turnover markers in blood and urine are useful tools in monitoring treatment effects and may be useful for improving treatment adherence, and therefore, outcomes [6–8]. The IOF-IFCC Joint Working Group on Bone Marker Standards (WG-BMS) recommended PINP and β -CTX be used as blood reference markers for bone formation and bone resorption, respectively, in osteoporosis [9]. However, it was also recognized that due to inter-method variation, the standardization or harmonization of commercial assays would need to be achieved in order for these assays to be widely used and interchangeably employed in clinical practice as well as in research studies [9]. This narrative review describes the current status of assays for PINP and β -CTX in blood, as well as the plans for and progress towards the achievement of harmonization or standardization of commercial assays for PINP and β -CTX undertaken by IFCC C-BM.

Structures of PINP and β -CTX molecules

The organic compartment of the bone is constituted mainly by osteoblast-produced collagen, the main structural protein being type I collagen. Collagens are characterized by repeating sequences of glycine-proline-hydroxyproline in domains of triple-helical conformation, with type I collagen having two α 1 and one α 2 chains. Intracellularly, the translated polypeptide, i.e., the pre-pro- α -chain contains a signal sequence and amino (N)- and carboxyl (C)-terminal propeptide extensions [10]. Once secreted, the N- and C-terminal propeptides are cleaved by specific peptidases, and these enter the circulation [11].

During bone resorption, osteoclasts resorb bone by the secretion of acid and proteases and digest mature type I collagen into fragments which may contain pyridinium cross-links or the N- and C-telopeptide domains [12]. These fragments enter the circulation and are excreted in urine (Figure 1).

The two bone markers designated as reference markers in osteoporosis as mentioned above are the N-terminal propeptide (PINP), which is cleaved and released during bone formation, and the C-telopeptide (β -CTX), which is released during bone resorption [9].

Current commercial assays and their descriptions and performance characteristics

PINP assays

PINP was first isolated from amniotic fluid and was referred to as fetal antigen 2, prior to its identification as a homomer of $\alpha 1$ chains of PINP [13]. Polyclonal antibodies formed the basis for the first PINP enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) [14–17]. The anti-PINP antibodies recognize two molecular forms of PINP in this ELISA, but only the high molecular weight form is recognized by the ^{125}I -labelled anti-PINP antibodies in this RIA [18]. Pertaining to assay technology, it has since been recognized that the thermal stability of soluble collagen is low, and the high molecular weight form of PINP are the intact $\alpha 1$ chains in a trimeric moiety and the low molecular weight form the monomer [19]. Assays that measure the trimeric form are named intact PINP assays and those that measure trimeric and monomeric forms are termed total PINP assays.

Presently there are four commercially available assays for the measurement of serum PINP. Table 1 summarizes the key characteristics of the four commercially available assays for serum PINP and table 2 shows some analytical performance data of these methods.

In 1995, Orion Diagnostica developed a manual radioimmunoassay (RIA) for the intact N-terminal propeptide of type I procollagen and it was validated by Tahtela et al. in 1997 [20]. The Orion Diagnostica UniQ™ PINP RIA (Espoo, Finland) was approved by the US Food and Drug Administration (FDA) in 2005. A manual total PINP ELISA assay was developed by Usen Life Science Inc., China in 1996 and subsequently validated by Orum et al [15]. The measuring range, and hence the values, reported by the latter assay are an order of magnitude lower than other commercial assays. There are no published comparison studies for this assay with other commercial assays. To our knowledge, this assay has not been used in any publications of clinical trials

The automated intact PINP chemiluminescence immunoassay (CLIA) assay was launched in 2009 by IDS for the iSYS instrument (IDS, Boldon, UK) and validated in 2010 by Koivula et al. [21]. The automated total PINP electrochemiluminescence immunoassay (ECLIA) was developed by Roche Diagnostics, Mannheim, Germany and validated by Garnero et al. in 2008 [22].

β -CTX assays

All β -CTX assay methods employ the CrossLaps antibodies (Osteometer BioTech A/S, Denmark), which recognize an 8 glu-lys-ala-his-asp-gly-gly-arg amino acid sequence (EKAHD- β -GGR) in the C-terminal telopeptide region of the $\alpha 1$ chain [23,24]. Among other reasons, including a large diurnal variation, a need for urine volume correction by

creatinine, an inability for quantification in very dilute urine samples, the cumbersome nature of 24-hour urine collection, and other preanalytical factors the use and development of assays for β -CTX in blood have been favored [9]. EDTA plasma is the preferred matrix for β -CTX measurement in blood due to better stability [25]. A fasted sample is necessary and early morning sampling is preferred to minimize the effects of circadian variation [25]

The first β -CTX assay in blood was a competitive polyclonal antibody ELISA [26]. Subsequently, a one-step ELISA using two monoclonal antibodies was developed [27] and then recalibrated using a synthetic cross-linked polypeptide containing two copies of the EKAHD- β -GGR sequence [28]. The manual ELISA assay has been available commercially as CrossLaps® [29]. The CrossLaps® ELISA is FDA approved and is currently marketed by IDS. Both IDS (UK) and Roche Diagnostics, (Germany) have adapted the one-step ELISA to develop automated assays for β -CTX. Table 3 summarizes the key characteristics of the commercially available assays for CTX in blood.

The Roche ECLIA assay was evaluated by Garnero et al. in 2001 [30] and the CLIA serum β -CTX assay on the IDS-iSYS analyser was assessed by Seres et al. in 2010 [31]. Performance specifications of the three commercial assays are detailed in Table 4.

Comparison studies of current commercial assays for PINP and β -CTX in blood

PINP

IFCC C-BM conducted a multi-centre study of β -CTX and PINP in serum and EDTA plasma with the participation of four laboratories in Europe. The results for PINP have been published elsewhere [32] and the results for β -CTX are currently being analyzed for publication. Herein, we have summarized the published results. PINP was measured by three commercial assays in both serum and EDTA plasma samples taken from 796 osteoporosis clinic patients with eGFR > 30 ml/min/1.73 m²). Each assay gave similar results for serum and EDTA plasma, leading us to conclude that both matrices were acceptable and the results from either matrix could be used interchangeably for each assay [32].

The automated methods for PINP (Roche Cobas and IDS iSYS) gave similar results (see Table 5) [32]. The Bland-Altman plots and the V-shaped models showed that 87.4% and 86.1% of the samples were within the V-shape limits for serum and plasma, respectively. The median difference observed between the two methods was 0.25 μ g/L, which was not significant [32]. In contrast, a significant proportional bias was observed between the Orion RIA and the two automated assays [32]. The Passing-Bablok regression for Roche Elecsys against the Orion RIA was Elecsys = 1.22 \times Orion-0.0 in serum and Elecsys = 1.24 \times Orion RIA-0.2 in plasma. The Bland-Altman plots showed a significant proportional bias and the V-shaped model showed 61.1% and 60.3% of serum and plasma samples, respectively, were within limits. The Passing-Bablok regression for the IDS iSYS method against the Orion RIA was iSYS=1.35 \times Orion RIA-3.2 in serum and iSYS = 1.45 \times Orion RIA-3.7 in plasma. The Bland-Altman plots showed a significant proportional bias, and the V-shaped model showed 57.4% of serum samples and 49.1% of plasma samples to be within limits. Results

from other studies of the three commercially available assays broadly reflect our findings [33,34].

Jørgensen et al. in a large study of 2308 individuals with eGFR > 30 mL/min/1.73m² (1250 males and 1058 females, age range: 24–76 years) measured serum PINP using IDS-iSYS and Roche Cobas analyzers [33]. They found an R² of 0.8573 (p < 0.001) for PINP measured by the two assays. The mean difference was -3 µg/L, which was statistically significant. The variability of the difference increased as the mean values increased. The reference intervals for PINP (calculated as a central 95%) did not differ greatly between the two assays for men or women [33].

Koivula et al. examined the relationships between the Roche Elecsys PINP assay and the Orion RIA in the serum of 34 apparently healthy blood donors, 39 patients with chronic renal failure, and 173 bedridden elderly in-patients > 65 years [34]. The PINP results by the two assays were similar for the healthy blood donors, but the Elecsys assay gave significantly higher results in haemodialysis patients and bedridden elderly patients [34]. The Passing-Bablok regression equations are shown in Table 5.

Morovat et al. compared serum PINP results by Roche Elecsys and IDS iSYS in 828 healthy subjects, including children and osteoporotic patients [35]. The relationship between the two assays was non-linear. In addition, the iSYS results were significantly higher than those obtained by Elecsys, except at low and high PINP concentrations of < 100 µg/L and > 670 µg/L, respectively, where iSYS gave lower values than Elecsys. Cavalier et al. compared serum PINP by Roche Elecsys and IDS iSYS in two CKD populations: 157 patients in stage 3–5 CKD and 125 patients in stage 5D [36]. The authors found that the two assays produced the most discrepant results when eGFR was < 30 mL/min/1.73 m². However, some discrepancy was apparent even for eGFR values between 30 and 60 mL/min/1.73 m², which raises the question whether the two assays can be harmonized only in subjects with eGFR >60 mL/min/1.73 m².

Wheater et al. examined serum PINP results from Roche Elecsys and IDS iSYS in 127 subjects with eGFR > 30 mL/min, 72 self-reported healthy volunteers (56 < 50 years and 16 > 50 years) with no known bone disease, and 55 rheumatoid arthritis (RA) patients (5 < 50 years and 50 > 50 years) [37]. The median difference for PINP values between the two systems was 2.0 µg/L (95% CI 1.3, 2.8 µg/L), which was statistically significant [37].

Overall, these results suggest that the two automated PINP assays (Roche Diagnostics and IDS iSYS) provide values that were very similar in healthy subjects with eGFR >30 mL/min/1.73 m², but that a significant proportional bias (albeit with good correlation) exists between the Orion RIA and the two automated assays. Further work to standardize or harmonize the three commercial assays for PINP is required. Since the molecular structures of the PINP molecule and the different peptides/fragments recognized by the intact and total PINP assays have not been clearly ascertained, the synthesis of a reference standard and the development of a reference measurement procedure required for the standardization of these assays will be problematic. Therefore, the IFCC/IOF Joint Committee on Bone Metabolism is of the view that the harmonization of PINP assays is a more realistic goal [38]. Universal

harmonization of PINP assays needs to be achieved in order for international multicentre trials to be conducted using any commercially available assays, and will be undertaken in collaboration with the commercial reagent manufacturers. This is also a requirement for the development of clinical guidelines with uniform reference intervals and treatment targets. Given the results of the studies summarized above, the harmonization (if not standardization) of commercially available assays should be possible by the use of common calibrators and the development of a reference method for PINP for subjects with a GFR > 60 mL/min/1.73 m² [38]. The drawback of this approach is that only international multicenter studies with a reasonable kidney function can benefit. Any new commercial assay developed in the future should be traceable to the new developed calibrators and reference method.

β-CTX

As stated above, the results for β-CTX from the IFCC C-BM multi-centre study of four laboratories are yet to be published. Previous studies have examined the relationship between commercial assays for β-CTX in blood.

Jørgensen et al. in their study of 2308 individuals with eGFR > 30 mL/min/1.73 m² (1250 males and 1058 females, age range: 24–76 years) measured serum β-CTX using IDS-iSYS and Roche Cobas analyzers. They found an R² of 0.8924 (p < .001) for β-measured by the two assays [33]. The mean difference was 13 ng/L, which was statistically significant. In addition, they found a complex systematic bias between the methods with Cobas giving a higher value at low concentrations and iSYS giving a higher value at higher concentrations. The upper reference limit for serum β-CTX (calculated from the central 95%) measured with the iSYS assay was higher than that by the Cobas assay in both men and women. The authors concluded that the complex correlation between the two methods meant that no simple conversion factor could be applied between the methods, thus making the potential for harmonization more complicated [33].

Chubb et al. examined β-CTX measured in EDTA plasma from 161 patients [119 females and 42 males, median age: 65 (inter-quartile range 57–76) years] with metabolic bone disease by the two automated assays (Roche Elecsys and IDS iSYS) as well as the manual ELISA assay marketed by IDS [39]. They found significant proportional and systematic biases when the iSYS assay was compared to both the ELISA and Elecsys methods. They also confirmed the findings of Jørgensen et al. that the iSYS assay gave lower β-CTX values in the lower range and higher values in the higher range compared to the Elecsys assay [39].

Wheater et al. in their study of 127 subjects also found a significant negative systematic bias and positive proportional bias when results from the iSYS were compared to the Elecsys assay [37]. The median difference for the β-CTX values between the two systems was -30 ng/L (95% CI -45, -21 ng/L), which was statistically significant. However, this single estimate of bias was inadequate to describe the variation in bias across the full range due to the complex relationship between the values given by the two assays [37].

In summary, the results of the above studies show poor agreement in results given by the current commercial assays for β-CTX in blood. Since the structure of the β-CTX molecule (EKAHD-β-GGR), the measurand, is well characterised, the synthesis of a reference

standard preparation is possible. The development of a reference measurement procedure would allow for the standardization of commercial assays for β -CTX in blood. It is the goal of the IFCC/IOF Joint Committee on Bone Metabolism to achieve standardization of all commercial assays for β -CTX in blood.

Next steps

IFCC C-BM in collaboration with commercial reagent manufacturers plan to prepare commutable international reference materials and develop common measurement procedures for PINP and β -CTX in blood. Once harmonization or standardization, as appropriate, is achieved, regulatory authorization of these modified assays will be sought. At a clinical level, common reference intervals and universally-acceptable decision limits and treatment targets for β -CTX and PINP will be developed in collaboration with IOF. Further studies are also needed to determine fracture prediction strength of the reference bone turnover markers.

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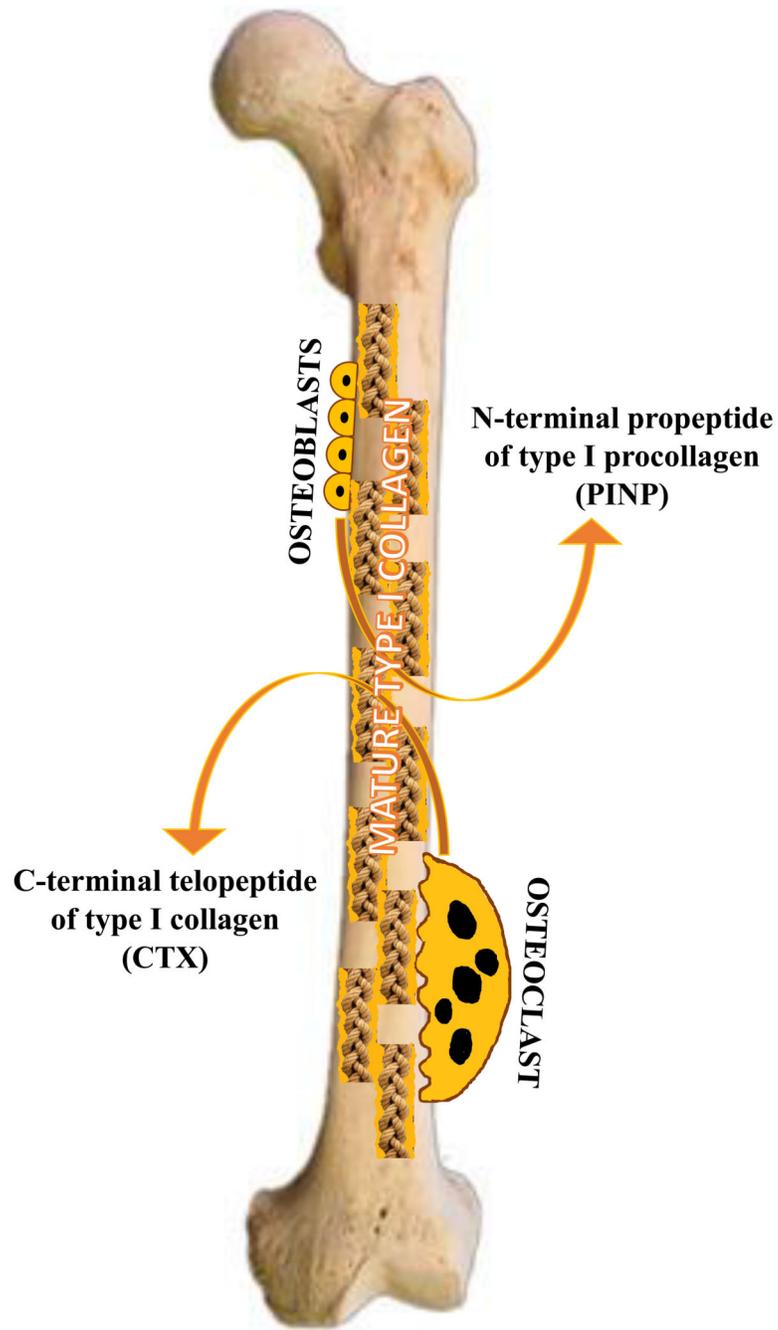


Figure 1. Schematic diagram illustrating the secretion of procollagen type I N-propeptide (PINP) and the C-terminal telopeptide of type I collagen (CTX) into the circulation.

Table 1

Key characteristics of four commercially available assays for serum N-terminal propeptide of type I collagen (PINP).

Manufacturer	Methodology	Measurand	Analytcs
Orion Diagnostica, Finland	Radioimmunoassay	Intact PINP	Manual
Immunodiagnosics Systems (IDS), UK	Chemiluminescence immunoassay	Intact PINP	Automated
Roche Diagnostics, Germany	Electrochemiluminescence immunoassay	Total PINP	Automated
Uscn, Life Science, China	Enzyme-linked immunosorbent assay	Total PINP	Manual

Table 2

Performance specifications of the four commercially available assays for serum N-terminal propeptide of type I collagen (PINP) [15, 20–22].

PINP assay	Measuring range	Lower limit of detection	Intra-assay CV	Inter-assay CV
Orion Diagnostica, Finland	5 – 250 µg/L	2.3 µg/L	2.3 – 3.5%	2.7 – 6.1%
iSYS (IDS), UK	2 – 230 µg/L	2.0 µg/L	2.6 – 3.0%	4.2 – 5.3%
Roche Diagnostics, Germany	5 – 1200 µg/L	5.0 µg/L	1.4 – 2.3%	2.1 – 4.5%
Uscn, Life Science, China	0.78 – 6.2 µg/L	0.041 µg/L	2.9 – 4.9%	4.6 – 5.3%

Table 3

Key characteristics of commercially available assays for C-terminal telopeptide of type I collagen (β -CTX) in blood.

Manufacturer	Methodology	Measurand	Analytics
IDS, UK.	Chemiluminescence immunoassay	β -CTX	Automated
Roche Diagnostics, Germany	Electrochemiluminescence immunoassay	β -CTX	Automated
IDS, UK	Enzyme-linked immunosorbent assay	β -CTX	Manual

Table 4

Performance specifications of the two automated assays for serum C-terminal telopeptide of type I collagen (CTX) [references 27–29].

β-CTX assay	Measuring range	Lower limit of detection	Intra-assay CV	Inter-assay CV
iSYS, IDS, UK	50-6000 ng/L	20 ng/L	2.7-3.7%	2.5-5.2%
Roche Diagnostics, Germany	10-6000 ng/L	10 ng/L	1.2-4.1%	<5.7%
CrossLaps®, IDS, UK	20-3380 ng/mL	20 ng/L	<2.5%	2.2-5.5%

Table 5

The Passing-Bablok regression equations for the Roche assay and IDS iSYS assay for PINP (references 32, 34–37)

Subjects	Regression equation	Slope 95% CI	Intercept 95% CI
Osteoporosis patients ³²	Cobas = 0.91 iSYS + 2.6	0.90, 0.92	2.2, 3.1
Healthy blood donors ³⁴	Elecsys = 0.94 iSYS – 3.6	0.80, 1.15	– 18.4, 3.6
Healthy subjects, patients with osteoporosis ³⁵	iSYS = 1.05 Cobas – 1.4	1.04, 1.06	– 1.9, – 0.8
Healthy volunteers, patients with rheumatoid arthritis ³⁷	iSYS = 0.98 Elecsys – 1.42	0.94, 1.03	– 2.86, 0.08
Haemodialysis patients ³⁴	Elecsys = 5.74 iSYS – 95.6	4.56, 8.57	– 240.9, – 31.9
Patients with CKD ³⁶	iSYS = 0.74 Cobas + 3.7	0.67, 0.81	1.2, 5.8
Bedridden elderly patients ³⁴	Elecsys = 1.57 iSYS – 12.0	1.43, 1.73	– 19.0, – 5.7

Table 6

The Passing-Bablok regression equations for the Roche automated assay and IDS iSYS automated assay for β -CTX (references 37 and 39)

Subjects	Regression equation	Slope 95% CI	Intercept 95% CI
Healthy volunteers, patients with rheumatoid arthritis ³⁷	iSYS = 1.29 Elecsys -24	1.24, 1.34	-34, -13
Patients with bone disease ³⁹	iSYS = 1.61 Elecsys -109	1.55, 1.66	-129, -92