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IDS iSYS automated intact procollagen-1-N-terminus pro-peptide assay: method evaluation and reference intervals in adults and children

Abstract

Background: We carried out a technical evaluation of the Immunodiagnostic Systems (IDS) automated intact pro-collagen-I N-terminus propeptide (PINP) assay on the iSYS platform, and established reference intervals for PINP in both adults and children.

Methods: Assay imprecision, recovery and interference were studied. Serum and plasma values were compared, and PINP stability was assessed. Using 828 specimens, IDS iSYS intact PINP and Roche E170 total PINP values were compared. Specimens from 597 adults and 485 children and adolescents were used to establish reference intervals for intact PINP.

Results: The method demonstrated good recovery and acceptable imprecision. The assay was unaffected by icterus and lipaemia, but haemolysis decreased measured PINP. Serum and plasma values were comparable. There was a non-linear relation between IDS intact and Roche total PINP values. Pre- and post-menopausal women had comparable PINP values, but there was a difference between women of different age groups. Serum PINP in men showed a decline in young age up to 45 years, but remained steady thereafter. Separate reference intervals were established for four age groups in women and for two age groups in men. Data for children were partitioned into four-year age groups, and these showed PINP to be high with no major gender differences until 12 years of age. Thereafter, values in females decreased in 13–16 years age groups and further in 17–20 years age groups, whereas PINP increased in boys of 13–16 years of age with a subsequent decline at 17–20 years.

Conclusions: The IDS iSYS PINP intact assay appears to be reliable. We have established gender- and age-related reference intervals for children and adults based on a relatively large healthy North European population.

Keywords: children; immunoassay; Immunodiagnostic Systems (IDS); procollagen-I N-terminus propeptide (PINP); reference interval; reference range.

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Introduction

Type I collagen is the most abundant collagen in connective tissues, but its overall synthesis is highest in the bone, where it forms most of the tissue's organic matrix. Its precursor, procollagen I, is synthesised by osteoblasts, and the terminus propeptides of the molecule are cleaved off extracellularly. Procollagen-I N-terminus propeptide (PINP) is 35 kDa, and is cleared by the hepatic endothelium. Its circulating concentration correlates with osteoblastic activity, which is normally coupled to osteoclastic bone resorption [1]. Although there is no convincing evidence that serum PINP concentrations can predict bone fracture [2], its measurement has been used to monitor the efficacy of anti-resorptive treatment in patients with osteoporosis [3]. Furthermore, PINP has also been recommended for use as a bone formation marker in observational studies and intervention trials.

As with type I collagen from which it is cleaved, PINP is a heterotrimer [4]. Intact PINP assays detect solely this form, whereas total PINP assays also detect monomers or possibly fragments of PINP, although these molecules have not been characterised well [5]. An automated total PINP immunoassay (Roche Diagnostics) has been available for some years [6], and in 2009, an automated chemiluminometric immunoassay for intact PINP was made available by Immunodiagnostic Systems Ltd (IDS) on their iSYS analyser. An evaluation of this assay and analyser

was undertaken recently by Koivula et al., who also evaluated the intact PINP assay results in patients with renal failure, and compared the values with those obtained by the Roche E170 total PINP assay [7]. In the current bicentre study, we have carried out additional independent evaluations of the iSYS intact PINP assay, and have established reference intervals for both children and adults.

Materials and methods

Subjects and specimen collections

Blood specimens were collected at two study centres: Liège, Belgium, and Oxford, UK. For establishing reference intervals, specimens were collected from healthy children and adults with no history of bone disease according to ethically-approved local protocols. No criterion related to food intake was used for the specimen collections.

In Liège, the specimens were collected during normal working hours between Oct 2010 and May 2011. Serum was separated within 1 h of collection, aliquots stored at −80°C, and parathyroid hormone (PTH), 25(OH)-vitamin D [25(OH)D] and creatinine were measured (v.i.). After exclusion of specimens with a PTH that was outside the reference interval (4–26 pg/mL; Diasorin 1-84 PTH assay), a 25(OH)D concentration of <75 nmol/L, or an estimated glomerular filtration rate (eGFR) of <60 mL/min/1.73 m^2, a total of 382 specimens from adults over 20 years of age, and 496 samples from children and adolescents up to the age of 20 years were identified. The adults' specimens were from 140 men (median age 57 years; range 21–94 years), 114 pre-menopausal (median age 36 years; range 21–49 years) and 128 menopausal women (median age 65.5 years; range 54–88 years).

In Oxford, blood specimens were collected from 08.30 to 15.00 h between September 2009 and March 2010, and serum and plasma were separated within 2 h of collection, aliquots stored at −20°C. The specimens were from 198 apparently healthy adults, comprising 106 men (median age 48 years; range 18–62 years), 72 pre-menopausal (median age 31.5 years; range 19–50 years) and 20 post-menopausal women (median age 57 years; range 51–69). Creatinine and 25(OH)D status were assessed retrospectively. All measurements of alkaline phosphatase and albumin-adjusted calcium gave normal values. Separately and solely for an assessment of the relationship between PINP and renal function, data on 167 consecutive specimens that had been collected routinely for both PINP and creatinine measurements from patients attending osteoporosis clinics were collected.

Assays

In Liège, 25(OH)D was measured by the use of the DiaSorin total 25(OH)D assay (DiaSorin, Stillwater, MN, USA) on a Liaison® analyser [coefficient of variation (CV) <12.8% at a 25(OH)D of >15 nmol/L]. Parathyroid hormone was measured by the DiaSorin two-site, chemiluminometric 1-84 assay kit, also on a Liaison® analyser (CV <10% at a PTH of >4.0 pmol/L). Creatinine was measured enzymatically by a Roche Modular analyser (Roche, Mannheim, Germany) (CV <6.1% at a creatinine of >80 nmol/L), and eGFR was calculated according to the abbreviated Modification of Diet in Renal Disease (MDRD) equation $[175\times(\text{Creat}/88.4)^{-1.154}\times(\text{Age})^{-0.203}\times(0.742 \text{ if female})]$. Calcium was also measured on a Roche Modular analyser by the o-cresophthalein complexone method (CV <2.1% at a calcium of >1.55 mmol/L).

In Oxford, 25(OH)D was measured by the use of the IDS competitive, chemiluminometric assay on the iSYS analyser [8] (CV <15% at a 25(OH)D of >23 nmol/L). Creatinine was measured by the use of a kinetic Jaffe method on a Siemens Advia® 2400 analyser (Siemens Healthcare Diagnostics Ltd, Frimley, UK) (CV <4.4% at a creatinine of >53 nmol/L), and eGFR was calculated according to the abbreviated MDRD equation. Calcium was measured by arsenazo III method, also on an Advia® 2400 analyser (total CV for adjusted calcium <5.8% at a calcium of >1.83 mmol/L and an albumin of >30 g/L).

PINP assays

IDS intact PINP kits were employed on iSYS analysers according to the manufacturer's protocol. The two-site chemiluminometric assay employed a two-point calibration in triplicate (top calibrant values around 135 μg/L), quality control materials in duplicate, and 20 μL of sample in singleton. Specimens that had PINP values above the assay range (2–230 μg/L) were diluted in specimens with low PINP concentrations. In each study centre, analyses of specimens for establishing reference intervals were carried out over several analytical batches and by the use of the method during routine analyses of clinical samples. Stored specimens were analyses within 2 months of collection.

Total PINP was measured electrochemiluminometrically by the use of a Roche E170 automated analyser (Roche Diagnostics, Burgess Hill, UK and Vivoord, Belgium). According to the manufacturer and as corroborated by other studies, the assay had a within- and between-calibration CVs of <3.7% and <2.9% [7].

For comparing the IDS iSYS and the Roche E170 PINP assay results, 828 serum specimens from healthy individuals in Liège and osteoporotic patients in Oxford were used. The specimens were stored frozen as described above, and were analysed by the two assays in at least three batches in each centre within 1 month of collection.

Recovery and interference

Recovery was assessed by diluting five serum specimens with PINP concentrations of 122–218 μg/L up to five-fold in five serum specimens that had PINP of 7.3–14.5 μg/L, and comparing the results with expected values. The effects of haemolysis, lipaemia and icterus were assessed by the addition of: 1) lysates of saline-washed red cells (final haemoglobin concentration range of 0.02–0.5 g/L); 2) Intralipid® (Fresenius Kabi Ltd, Runcorn, UK) (final triglyceride concentration range of 1.7–33.8 mmol/L); or 3) icteric plasma (final bilirubin concentration range of 42.5–340 μmol/L), and comparing the obtained values with those expected.

Imprecision

Within-batch imprecision was assessed by 10 repeated analyses of two specimens, and also by duplicate analysis of 51 clinical

specimens. Between-batch imprecision was studied by analysing three quality control specimens over 1 year using at least 10 independent calibrations and four different lots of reagents.

Matrix effect and PINP stability

Becton Dickinson blood sampling tubes with gel separator (SSTII for serum and PSTII for plasma) were used. Paired serum and lithium heparin plasma were analysed in 45 specimens in the same assay batch.

Stability of separated plasma specimens was assessed by analysing 18 plasma samples, leaving them at room temperature (RT; circa 20°C) for 3 days and subsequently at 4°C for 4 days before reanalysis. Separately, aliquots of five clotted and four heparinised blood specimens were centrifuged: 1) immediately, or after being kept at RT for 2) 1 day or 3) 3 days. After each centrifugation, separated serum and plasma aliquots were frozen at –20°C. Separate serum and plasma aliquots of the same specimens that had been kept at RT for 3 days were also 4) stored at 4°C for a further 4 days before being frozen at –20°C. All frozen, stored aliquots were thawed and analysed in one assay batch.

In order to assess the effects of short-term storage at –20°C, a further 23 serum specimens were analysed (PINP range 7.8–87.3 µg/L), and at least five of these samples were re-analysed in batches after storage at –20°C for various lengths of time. Separately, the effects of freezing and thawing of specimens on measured PINP was studied by repeat analysis in one assay batch of 10 specimens (PINP range 28.5–12.3 µg/L) after a further two and four freeze-thaw cycles.

Statistical analyses

Data were analysed using Analyse-It software (Analyse-It®, Leeds, UK), as an add-on for Microsoft Excel. For stability and matrix effect evaluations using same calibration, differences between paired values were assessed by Student's t-test. For assessment of long-term stability at –20°C using different calibrations, pairs of values were compared by repeated measures analysis of variance (ANOVA). PINP values obtained by different methods were compared by Passing and Bablok regression [9], and cumulative sum linearity test was performed to investigate deviation from linearity between two sets of data. Confidence intervals (CI) of 95% were used to assess whether slope and intercept values were significantly different from 1 and 0, respectively. Associations between PINP values and age or biochemical measures were assessed by linear regression. PINP values from adults in different age groups were compared by one-way ANOVA with Bonferroni correction for multiple comparisons. Differences between PINP values according to age, gender and also menopausal state in women were assessed by Mann-Whitney test. A probability of <5% was considered statistically significant.

Shapiro-Wilk test was performed to assess whether distributions of values used for establishing reference intervals were normal. Where a distribution was non-Gaussian, either a logarithmic or a square-root transformation was employed in order to normalise the distribution (assessed by Shapiro-Wilk W normality test). Values that were less than median –(3×IQR) or greater than median +(1.5×IQR) were deemed outliers, and were considered for removal from the transformed values based on ad hoc assessments of the data (IQR=inter-quartile range). For establishing reference intervals, Analyse-It® software's parametric method of calculation was used when transformation normalised the data. The 95% reference interval and 90% CIs of both the upper and lower limits were determined, and the calculated values were converted back to measured units. For non-normalised data or when the number of observations was small, a non-parametric procedure was employed. Values were ranked, and the 2.5 and 97.5 percentiles were obtained as the 0.025 (n+1) and 0.975 (n+1) ordered observations. Linear interpolation was used for non-integer ranked values.

Results

Recovery

The assay showed average recoveries of between 89% and 100% (SDs of 9.0%–17.4%). No trend in recoveries was observed at increasing dilution of up to five-fold.

Assay interference and matrix effects

No interference either from high triglycerides (recoveries of 97.4%–104.4%) or from high bilirubin concentrations (recoveries of 97.0%–103.7%) was observed. However, the addition of increasing amounts of haemolysate to serum specimens resulted in a significant and progressive decrease in measured PINP. Although an average PINP decrease of 1.5% at a haemoglobin concentration of 0.6 g/L was negligible, the assay under-estimated PINP by averages (SD) of 5% (8.5%), 11% (4.5%), 18% (8.3%) and 33% (10.3%) at haemoglobin concentrations of 0.5, 1.5, 2.5 and 5.0 g/L, respectively.

PINP concentrations in plasma were higher than those in serum due to a significant proportional bias (p=0.0091; plasma PINP=(1.06×serum PINP)+1.0). However, the mean difference between the two values was only 1.2 µg/L (95% CI 0.3–2.2 µg/L over a serum PINP range of 17.5–90.8 µg/L), making the difference in values between the two types of specimen not clinically significant.

Imprecision

The within-batch imprecision was 3.6% at a PINP concentration of 22.4 µg/L, and 4.7% at 123.3 µg/L. Between-batch imprecision at PINP concentrations of 18.0, 45.6 and 115.8 µg/L were 7.2%, 8.5% and 7.0%, respectively.

Stability

Heparinised plasma specimens left at RT for 3 days resulted in an average decrease in measured PINP of 5.5% (95% CI 3.3%–7.7%). Although this difference was significant (p=0.0001), it was within the assay's between-batch imprecision. No significant difference was observed between PINP values of serum or plasma that was separated and frozen immediately and either: 1) serum or plasma of the same blood that was left unseparated at RT for a period of up to 3 days (p=0.955), or 2) the same serum or plasma that was left at 4°C for a further 4 days (p=0.736 compared with controls).

The effects of storage of specimens at –20°C for various lengths of time have been described in Table 1. Storage of specimens at –20°C for up to 133 days did not have any significant effect on PINP results (p>0.056). The mean CVs of pairs of values obtained at the outset and after storage for up to 133 days were comparable with the between-batch CV of the assay, and there was no consistent trend in values over time. However, storage of serum (n=11) at –20°C for 2.5 years resulted in an increase in measured PINP of +41.3% (SD=34.8%; p<0.0001). Freezing and thawing of specimen did not have any significant effect on measured PINP. After three and five freeze-thaw cycles, PINP measured in the same analytical batch changed by averages of –1.2% (SD=3.5%; p=0.245) and –1.5% (SD=4.7%; p=0.167) compared with values after one freeze-thaw cycle.

Table 1 Stability of PINP in serum stored at –20°C.

Period stored at –20°C	n	Mean % change, SD	Mean CV, SD	p-Value
36 days	7	+6.7 (10.5)	5.5 (5.6)	0.077
64 days	6	+2.0 (4.1)	2.4 (1.8)	0.128
87 days	6	+7.1 (10.4)	6.7 (4.4)	0.084
110 days	5	–7.4 (5.7)	5.6 (4.4)	0.057
133 days	6	–6.3 (7.2)	5.6 (4.2)	0.340
2.5 years	11	+41.3 (34.8)	18.9 (6.9)	<0.0001

Stored specimens were assayed in separate batches at specified times. Mean percentage changes (and their SDs) relative to values obtained at the outset have been given. Mean CVs (and their SDs) for pairs of values, one obtained at the outset and the other at reanalysis after the specified periods, have also been given in order to allow comparisons with the between batch CVs of the assay (7.0%–8.5%).

Comparison with the Roche E170 total PINP assay

For comparisons between IDS iSYS and Roche E170 PINP assays, 828 pairs of values were used. In the case of eight pairs of results, E170 gave much higher values compared with iSYS. The obtained intact and total PINP concentrations of these eight specimens were as follow (intact/total): 28/122, 66/196, 72/298, 643/6447, 667/6397, 726/6097, 1406/7157 and 1341/5678 µg/L (range of ratios of total:intact=3.0–10.0). No reason for the discrepancies was obvious (five healthy children with normal C-terminus collagen type-I telopeptide values, two osteoporotic patients and one diabetic). For the remaining 820 specimens (iSYS PINP concentration range of 6.5–2159 µg/L), the iSYS assay values were significantly higher than those obtained by the E170 (p<0.0001). Figures 1 and 2 show the relationship between the values obtained by the two methods. The mean proportional and absolute biases of iSYS values compared with E170's were +5% (95% CI 4%–6%) and –1.4 µg/L (95% CI –0.8 to –1.9 µg/L), respectively. However, the relationship between the two sets of values was non-linear (p<0.0001; Figure 2's inset), and at the low- and high-end total PINP concentrations of <100 µg/L and >670 µg/L, iSYS intact PINP assay gave lower values than E170 total PINP assay (p<0.0001 at both these low and high concentration ranges). Overall, 19.3% of iSYS values were >15% higher, and 8.8% of iSYS values were >15% lower, than the corresponding E170 results.

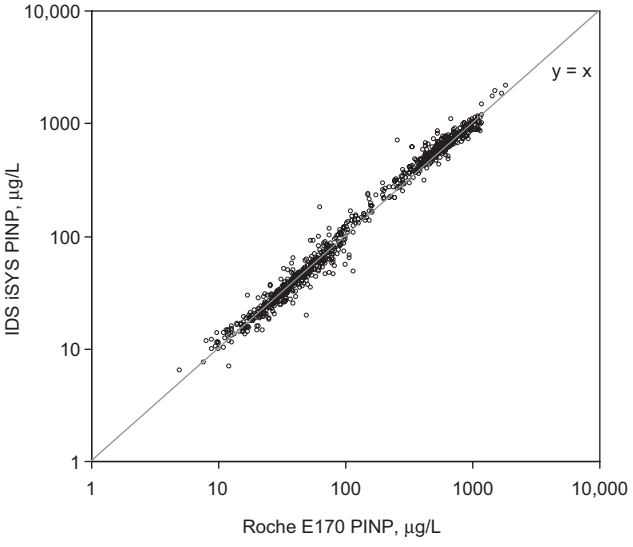

Figure 1 Relationship between IDS iSYS and Roche E170 PINP values obtained on 820 serum specimens (see text for the description of eight outliers that have not been shown).

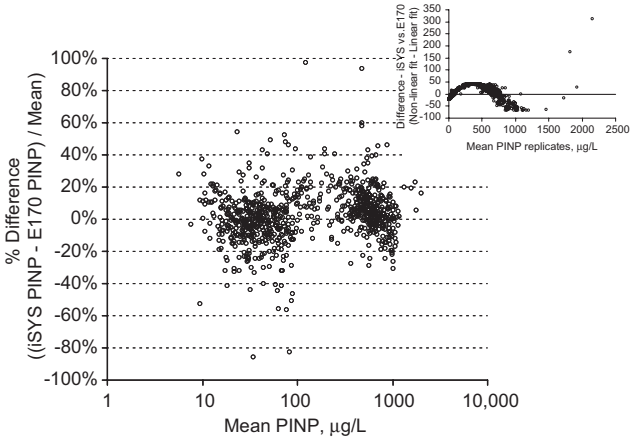

Figure 2 Bland and Altman plot of the percentage difference between IDS-iSYS PINP and Roche E170 values relative to the mean (n=820; see text for the description of eight outliers that have not been shown).
The inset depicts the deviation from the linear fit between the two sets of data according to mean PINP concentration.

Assessment of PINP reference intervals

Relationship between PINP and other parameters

Assessment of the relationship between IDS iSYS PINP and 25(OH)D in specimens from healthy volunteers in Oxford showed that there was no significant association between PINP and either 25(OH)D values [n=187; p=0.0821; r^2=0.02; lowest 25(OH)D=18.2 nmol/L; an average PINP increase of 0.7 µg/L for every 10 nmol/L increase in 25(OH)D], or albumin-adjusted calcium (p=0.556; r^2=0.00). Similarly, in healthy volunteers no significant association was found between iSYS PINP values and either creatinine (n=187; p=0.8276; r^2=0.00; highest creatinine=121 µmol/L) or eGFR (n=186; p=0.0728; r^2=0.02 with positive slope; 6 values <60 mL/min with mean (SD) PINP of 24.4 (11.1) µg/L; lowest eGFR=53 mL/min/1.73 m^2). Further investigation of the relationship between PINP and renal function using specimens from 167 patients attending osteoporosis clinics also showed no significant association between PINP and either creatinine (p=0.853; r^2=0.00) or eGFR [p=0.614; r^2=0.00; median (IQR) eGFR=75 (59–96) mL/min; lowest eGFR=13 mL/min]. Therefore, for establishing reference intervals for adults, the PINP data from all Oxford's healthy volunteers were pooled with those from Liège.

PINP reference intervals in adults

Frequency distribution of PINP values showed a negative skew (p<0.0001) for both genders. Logarithmic transformation of PINP values normalised the distribution for pre-menopausal females (skewness=−0.04; kurtosis=−0.18; p=0.612) and for males (skewness=−0.07; kurtosis=−0.16; p=0.465), whereas a square-root transformation was used for menopausal women's values (skewness=0.16; kurtosis=−0.65; p=0.122). Assessment of the relationship between women's serum PINP values and their age showed no association between the two (p=0.459; Figure 3A). Pre- and post-menopausal women's PINP values were not significantly different (p=0.117), and reference intervals for these two groups have been given in Table 2A. However, when PINP concentrations were divided according to age groups 18–35, 36–50, 51–65 and >65 years (Figure 3B), there was a significant difference between the four groups (p=0.009). Comparing PINP

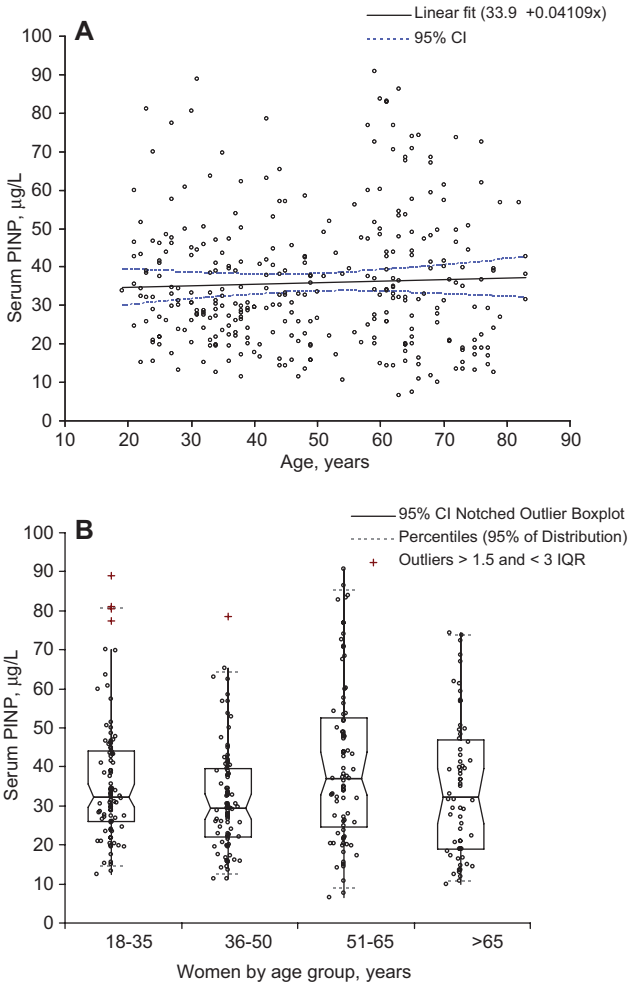

Figure 3 Serum PINP according to age in healthy females (A; n=331) and by age group (B).
A value of 255 µg/L for an 80-year-old female has not been shown. n=93, 87, 81 and 64 for women aged 18–35, 36–55, 51–65 and >65 years, respectively.

values from adjacent age groups showed that the only significant difference was between those from 36 to 50 years and 51–65 years age groups (p=0.006) (p>0.070 for the other two comparisons). Table 2A also describes the reference intervals for women according to the four age groups.

Values in men decreased significantly with age (p<0.0001; Figure 4A). In men aged <45 years, there was an average decrease in PINP of 4.6 µg/L (CI 1.8–7.3 µg/L) for each 5 years increase in age (p<0.0014), and separating men's PINP according to age (Figure 4B) showed that PINP values in 36–45 year olds (n=31) was lower than in those aged 18–35 years (n=46) (p<0.0001). No significant difference was found between PINP concentrations in age groups 46–55 and >55 (p=0.174), and each of these groups had significantly lower PINP concentrations compared with men aged 18–45 years (p<0.0001 for both comparisons). Therefore, reference limits were established for men aged 18–45 and >45 years (Table 2A).

Differences between PINP values from the two centres

Pre-menopausal women from Liège had significantly higher PINP concentrations than those from Oxford

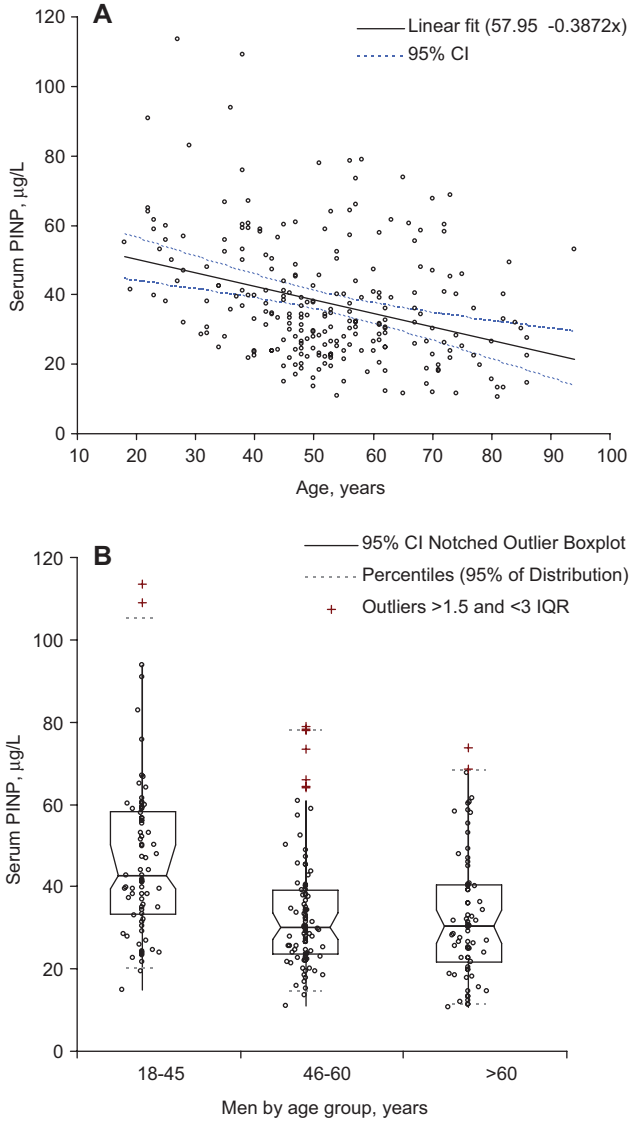

Figure 4 Serum PINP according to age in healthy males (A; n=244) and by age group (B).
n=77, 96 and 71 for men aged 18–40, 41–60 and >65 years, respectively.

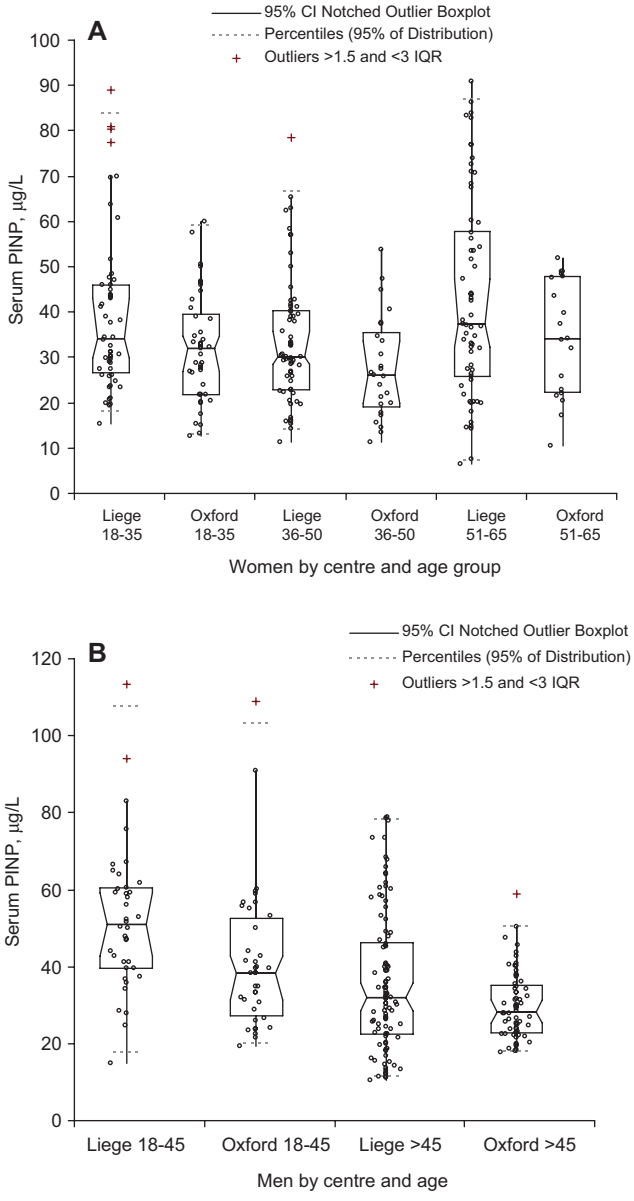

Figure 5 Distribution of iSYS PNP values according to age in women (A) and men (B) in the two study centres, Liege and Oxford.

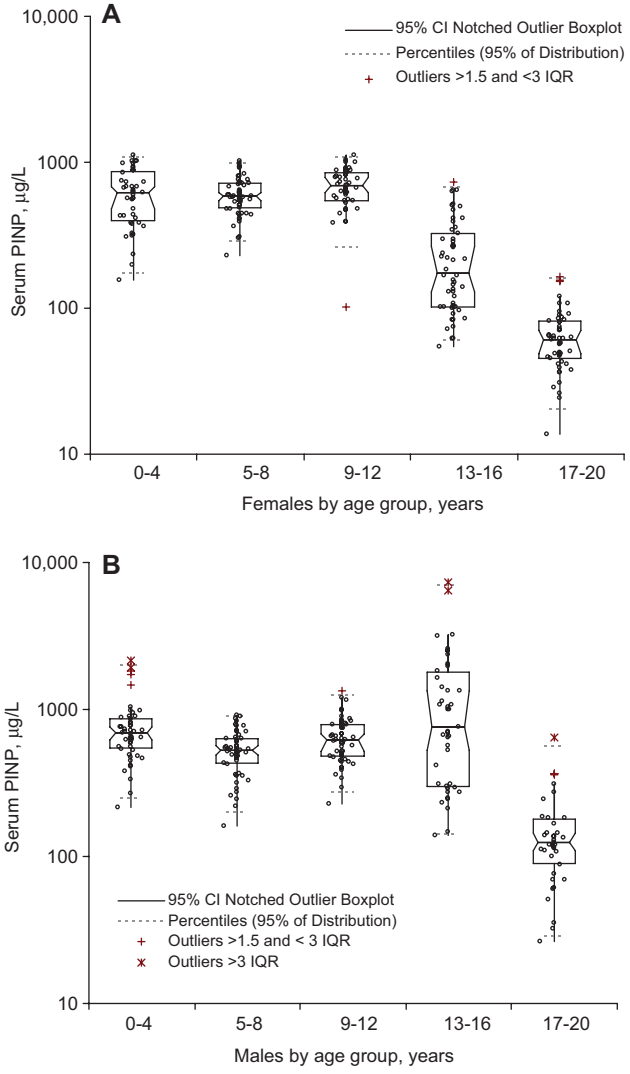

Figure 6 Distribution of iSYS PINP values according to age group in female (A) and male (B) children and young adults.

(p=0.035), but the differences in values were not significant for the pre-menopausal women of 18–35 years (p=0.086) and 36–50 years of age (p=0.057). The values for post-menopausal women from Liège and Oxford were not different (p=0.606). There was only one woman aged >65 years from Oxford, precluding comparison between the values in this age group from the two centres. Figure 5A compares women's PINP values from the two study centres according to age. Men from Oxford also had lower serum PINP compared with men from Liège (p=0.009). This difference was related to those aged 18–45 years (p=0.001), and there was no difference between men aged >45 years from the two centres (p=0.095) (Figure 5B).

PINP reference intervals in children and young adults

PINP values for males and females up to the age of 20 years were divided into 4-year age groups. This compromise allowed each age group to have at least 40 PINP values (except for n=38 for group of males aged 17–20 years; Table 1B). The distributions of the values are presented in Figure 6. Frequency distribution of PINP values showed a significant kurtosis and a negative skew in all age groups, except for females aged 0–4 and 9–12 years, and males 5–8 years. The upper limits of normal PINP for boys and girls were comparable up to, and including, the 9–12 year age group, but were higher for males in both the 13–16 and 17–20 year groups. After 12 years of age, PINP values decreased in females, with both groups' values showing a decrease in those 17–20 year age group. Table 1B describes the reference intervals for individuals aged up to 20 years.

Discussion

In this study, we evaluated the automated IDS intact PINP assay on the iSYS instrument. We found the assay to have good reproducibility, recovery and precision, and to be unaffected by icterus and lipaemia. However, haemolysis caused a significant under-estimation of PINP, and caution should be exercised in reporting PINP results on haemolysed specimens. Our recovery and precision data agree well with those reported by Koivula et al., who also evaluated the IDS iSYS intact PINP assay [7]. By the use of gel-filtration studies, pooled sera and a haemodialysed serum, these authors found the assay to be specific for intact PINP.

Our data on stability of the PINP molecule detected by the assay suggest that the results are not affected by the general, common delays encountered during specimen transport from primary care, and by further delays in laboratory prior to analysis. These findings confirm earlier reports by Lomeo et al. that PINP has good stability compared with some other bone markers [10]. The comparability of results obtained on serum and heparinised plasma also provides robustness with respect to sample type. The analytical range of the iSYS assay (up to circa. 230 µg/L) would allow the analysis of specimens from adults without a need for sample dilution, but makes it necessary to dilute most paediatric specimens before analysis. Repeated freezing and thawing of specimens did not have a significant influence on measured PINP values, and specimens stored at –20°C for up to 133 days gave PINP values that were not significantly different from

Table 2 The upper and lower limit (UL and LL) of reference intervals established for IDS iSYS PINP in adults (A) and children (B). The oldest man was 94 and the oldest woman 83 years of age.

A

Group	Age, years	n	Reference intervals			
			Lower limit	90% CI of lower limit	Upper limit	90% CI of upper limit
Pre-menopausal women	All	180	13.7	12.6–15.0	71.1	65.2–77.7
	18–35	93	14.7	13.0–16.6	74.6	66.1–84.1
	36–50	87	12.9	11.4–14.6	66.8	58.8–75.8
Post-menopausal women	All	145	8.2	6.2–10.4	82.6	86.0–89.5
	51–65	81	9.0	6.2–12.3	87.7	78.5–97.5
	>65	64	7.5	4.9–10.7	75.3	66.3–84.8
Men	18–45	77	19.4	17.0–22.1	95.4	83.8–108.7
	>45	167	12.8	11.7–14.1	71.9	65.3–79.1

B

Group	Age, years	Gender	n	Reference intervals			
				Median	Inter-quartile range	Lower limit (2.5th centile)	Upper limit (97.5th centile)
Children	0–4	F	44	619	468	174	1079
		M	45	650	246	299	1010
	5–8	F	56	584	220	307	985
		M	53	524	199	200	900
	9–12	F	48	691	291	386	1070
		M	53	625	308	323	1242
	13–16	F	54	175	225	59.3	672
		M	43	754	1492	142	6929
	17–20	F	51	62	24.9	25.2	160
		M	38	113	79.6	28.1	369

All PINP values are in μg/L. CI, confidence interval; n, number.

those measured at the outset. However, long-term storage of specimens at this temperature caused an increase in measured PINP concentrations. Whether this represents a breakdown of trimeric or high-molecular-weight structures that expose α1 chain or other structures that cross-react with the intact assay is not known [11].

Comparisons made between the iSYS intact and the Roche E170 total PINP assays showed a non-linear relationship between the two sets of values. At both the range of values seen in adults and very high concentrations seen in some children (<100 and >670 μg/L), the iSYS PINP values were generally lower than those obtained by the E170 assay, with an opposite trend for values in-between. These data, together with the very large, unexplained discrepancies observed in the case of eight specimens, suggest that 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. The advantage of the intact assay appears to be its specificity for PINP and its lack of cross-reactivity with the so-called monomeric or other forms [5, 12]. The identities of these molecules are unknown; they may be either pro-α1 chains or fragments of PINP, but they have also been reported to have a larger molecular weight than PINP [7]. The retention of some of these molecules in blood of patients with renal impairment can make the interpretation of some total PINP results difficult [5]. It has been suggested that the monomeric form detected by the total PINP assay may be a breakdown product of collagen, and in breast cancer patients, for example, PINP has been shown to be higher by the Roche E170 total PINP assay as compared with the IDS iSYS method [13]. Therefore, whether the detection of such PINP molecules by total PINP assays may offer some additional information in specific groups of patients, e.g. those with metastatic bone disease, remains unknown [14, 15].

In our study, we did not find a relationship between intact PINP and either creatinine, estimated GFR or

25(OH)D. Previously, no association between total PINP (Roche E170) and 25(OH)D had been found in an evaluation of PINP reference intervals [16, 17]. Although serum PINP concentrations were not different between pre- and post-menopausal women, differences were present between age-specific subgroups. Therefore, we defined separate reference intervals for four age groups in women, and for pre- and post-menopausal women as a whole.

Age-related changes for total PINP have been reported previously. Studies in women have shown a decline in total PINP (Roche) with age until the eighth decade of life, when an increase in concentrations have been reported [16, 18]. However, Glover et al. found that whereas PINP in women decreased between the ages 30 and 35 years, it was stable over the age range 35 and 45 years [19]. Other bone markers have shown an increase during post-menopausal years [20]. The decline in bone markers until middle-age has been suggested to be due to an increase in skeletal maturity [21], whereas their increase in old age may be attributed, at least in part, to a decrease in gonadal hormones, which play a crucial role in maintaining bone mass. Both the use of oral contraceptives and oestrogen replacements have been shown to lower serum concentrations of PINP and other bone markers [19, 21–24]. Recker et al. showed an increase in bone remodelling after menopause [25], and changes in bone markers during the menstrual cycle have also been documented [21]. One limitation of our study, therefore, is that we were unable to exclude women on exogenous oestrogens or establish reference intervals accordingly.

We observed a progressive decrease in PINP in men with increasing age up to 45 years. This decrease may be related to gonadal hormone levels, and Leder et al. found that suppressing androgens and oestrogens in men aged 22–44 years increased PINP and other bone markers [26]. However, others have reported only a weak relation between PINP and testosterone in young men, with a much stronger inverse association between PINP and oestrogens [27, 28]. Our cohort of men aged <45 years was too small to allow establishing reference intervals according to narrower age bands in this group, and a larger study population will be needed for this purpose. Our PINP data in older men are in agreement with those reported by Olmos et al., who found no change in total PINP (Roche) with age in men >50 years old [17]. Since there was no difference in PINP between subgroups of men aged >45 years, we defined reference intervals for ≤45 and >45 years age groups.

Our findings that young men and women from Oxford had lower PINP concentrations than those from Liège are similar to those of Glover et al., who reported a lower PINP in women from the UK than in those living in Belgium and France [21]. Although these differences are not very large, the data highlight the variability of circulating PINP concentrations in different populations, and the need to exercise caution when adopting reference intervals derived from a different population.

PINP values obtained in children were divided into 4-year age groups. This was a compromise, but allowed (with the exception of aged 17–20 males) evaluations of groups with at least 40 PINP values in each. We found the limits of normal PINP values to be not too dissimilar in boys and girls up to the age of 12 years. For 13–16 years age groups, PINP decreased in girls, and declined further at 17–20 years of age. By contrast, PINP increased for 13–16-year-old boys, before decreasing, but not quite reaching adult levels, at 17–20 years of age. These fluctuations are consistent with those previously observed with PINP and other bone-formation markers [29, 30]. As with other bone-markers, the magnitude of changes in PINP during growth in children, as well as the large inter-individual variations at each age, impose limitations on defining the limits of normality with a high degree of certainty in this group of individuals.

In summary, our findings have shown the IDS intact PINP assay to be reliable, precise, and unaffected by pre-analytical variables other than haemolysis. Using a relatively large cohort of adults and children, we have established reference intervals for use in these groups according to gender and age, and these intervals have been employed in our laboratories. Further work is required in particular to define reference intervals according to age in men <45 years of age, as well as those in children according to Tanner stage. To our knowledge these data are amongst the largest available for establishing reference intervals for PINP.

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Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.
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