Evaluation of a Fully Automated Serum Assay for Total N-Terminal Propeptide of Type I Collagen in Postmenopausal Osteoporosis

Patrick Garnero,1,2* Philippe Vergnaud,1 and Nicholas Hoyle3

BACKGROUND: Biochemical markers of bone turnover can provide prognostic information about the risk of fracture and may be useful for monitoring efficacy of antiresorptive and anabolic therapy in osteoporosis. We evaluated the performance of a fully automated assay for serum total N-terminal propeptide of type I collagen (P1NP), a marker of bone formation.

METHODS: Serum P1NP was measured on the Elecsys 2010 automated analyzer (Roche) in 230 healthy premenopausal women, age 30–49 years, 179 postmenopausal women with osteoporosis participating in the previously published 1 year randomized Parathyroid Hormone and Alendronate for Osteoporosis study of full-length parathyroid hormone (PTH 1-84, >100 μg/day subcutaneously; n = 119) or oral alendronate 10 mg/day (n = 60), and 64 healthy men, age 40 to 65 years.

RESULTS: The within-run and between-run (total) imprecision (CVs) were ≤1.7% (n = 20) and 4.4% (n = 15), respectively. The median within-person variability of results (3 measurements over 3 months in 15 postmenopausal women) was 7.2%, resulting in a least significant change (LSC) value of 20%. Serum P1NP concentrations were 74% (P < 0.0001) higher in postmenopausal women than in premenopausal controls. After 3 months of treatment, 83% and 88% of patients treated with PTH 1-84 and alendronate, respectively, demonstrated changes of serum P1NP that exceeded the LSC.

CONCLUSION: The automated assay for serum total P1NP is precise and sensitive enough to detect changes that exceed the LSC in a majority of postmenopausal women after 3 months of treatment with PTH 1-84 or alendronate. Because of its convenience and high throughput, this bone formation marker may be useful for the monitoring of patients with osteoporosis.

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In women with postmenopausal osteoporosis, bone markers have been suggested to be useful to monitor the efficacy of treatments and to predict fracture risk (1). Because most of the current effective therapies are antiresorptive, major efforts have been concentrated on identifying bone resorption markers (1). More recently anabolic drugs have been developed, including intermittent injections of parathyroid hormone (PTH4-1-84 or teriparatide; PTH 1-34) (2–4). Sensitive bone formation markers may be helpful to assess efficacy of these anabolic drugs.

Two of the earliest bone formation markers were osteocalcin and bone alkaline phosphatase (bone ALP). These 2 markers have some limitations. Osteocalcin accounts for only a minor fraction of bone proteins. Circulating osteocalcin comprises different immunoreactive forms, including the intact molecule and various fragments (5). The heterogeneous origin of circulating osteocalcin may complicate the clinical interpretation of osteocalcin data (5,6). Because osteocalcin gene expression is directly regulated by 1,25 OH D3 and corticosteroids, serum osteocalcin data in patients treated with these 2 hormones should be interpreted cautiously (1). Other concerns are that serum osteocalcin is poorly sensitive in Paget disease (7), is of limited value in patients with renal failure because osteocalcin is cleared by the kidney (8), and is poorly stable when samples are stored at room temperature. Assays for bone ALP exhibit a high degree of cross-reactivity with liver ALP. Thus, the concentration of ALP may be affected by liver diseases (9,10).

Type I collagen, which constitutes 90% of bone proteins, is synthesized as type I procollagen. During the extracellular processing of type I procollagen, there is cleavage of the amino terminal [N-terminal propeptide of type I collagen (P1NP)] and carboxy terminal propeptide (P1CP). These propeptides circulate in blood, where they are markers of bone formation.

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Nonstandard abbreviations: PTH, parathyroid hormone; ALP, alkaline phosphatase; P1NP, N-terminal propeptide of type I collagen; P1CP, carboxy terminal propeptide; PaTH, Parathyroid Hormone and Alendronate for Osteoporosis study; BMD, bone mineral density; CTX, carboxy-terminal telopeptide of type I collagen; LSC, least significant change.
contrast to serum P1CP, which is a single protein, P1NP circulates as different forms, including the intact authentic trimeric P1NP, a monomer, and several fragments (11, 12). The trimeric structure is unstable at 37 °C and can be transformed to the stable monomeric form (12). The 1st P1NP assays used antibodies raised against synthetic peptides from the α1 chain of P1NP and recognized trimeric and monomeric P1NP and fragments. For P1CP these assays yielded disappointing results in patients with osteoporosis (13, 14). The currently available manual RIA, which recognizes only the intact trimeric form of P1NP (15), has been shown to be sensitive for monitoring changes of bone formation in various situations, including after treatment with PTH (2–4, 16).

Important variables for the routine clinical use of biochemical markers include low intraindividual variability, robustness with regard to sample stability, and high throughput. For resorption markers based on type I collagen, intrapatient variability has been reported to be relatively high, especially for measurements in urine (17, 18). The intrapatient variability of serum C-terminal crosslinked telopeptide of type I collagen, currently one of the most useful biochemical marker of bone resorption in osteoporosis, has been reported to be lower than for urinary markers, but its concentrations is significantly affected by food intake (19). Conversely, concentrations of bone formation markers, especially P1NP, are virtually unaffected by food intake, and the intrapatient variability is low (19).

One way to reduce laboratory variability and to increase throughput is to use fully automated analyzers that are suitable for routine use in clinical chemistry laboratories. The aim of this study was to evaluate the performances of an automated analyzer for serum P1NP that detects the intact molecule in both its trimeric and monomeric forms, but not its fragments.

Materials and Methods

STUDY PARTICIPANTS
Total P1NP concentrations were measured on fasting serum samples collected between 7.30 and 10.00 am in the following populations. All samples were stored at a temperature below −70 °C before assay. Measurements were performed in a specialized central laboratory (Synarc, Lyon, France).

Healthy premenopausal women. We recruited 230 women (mean (SD) age, 40.3 (4.9); range 30–49 years) participating in a blood donor program in Lyon, France. All women were menstruating regularly, and none had disease or treatment that could interfere with bone metabolism.

Postmenopausal women with osteoporosis. The Parathyroid Hormone and Alendronate for Osteoporosis study described earlier (4) recruited 238 postmenopausal women aged 55 to 85 years with osteoporosis (4). They had a bone mineral density (BMD) T-score by dual x-ray absorptiometry of ≤−2.5 at the hip or spine, or a T-score ≤−2.0 and at least 1 of the following risk factors: age ≥65 years, history of postmenopausal fracture, or maternal history of hip fracture. Women who had been treated with bisphosphonates for >12 months or for >4 weeks during the previous 12 months or who had disease or took medications known to affect bone metabolism were excluded.

Healthy men. Sixty-four men (mean age 55 years, range 40–65 years) participating in a blood donor program in Lyon, France, were investigated. None of these men had disease or treatment known to influence calcium metabolism.

Effects of PTH 1-84 and alendronate. The 238 postmenopausal women with osteoporosis in the PaTH study (4) were randomly assigned to PTH (1-84) (100 μg daily, subcutaneous injections; NPS Pharmaceuticals, n = 119); alendronate (10 mg oral daily; Fosamax, Merck, n = 60) or PTH plus alendronate (n = 59). All women also received 400 IU/day of vitamin D and 500 mg/day of calcium. The average changes of biochemical markers after 1, 3, and 12 months in these 3 groups have previously been published (4). In the current posthoc analysis of the same study, we analyzed the individual changes of bone markers in the 2 monotherapy groups only and only after 3 months at the time of the plateau effect on markers for both PTH and alendronate (4). Compliance to treatment was defined as ≥80% of injection (PTH) or pills (alendronate) taken.

COMPARISON OF AUTOMATED ASSAY FOR TOTAL P1NP AND MANUAL RIA FOR INTACT P1NP
Serum total P1NP by automated assay and intact P1NP by manual RIA were measured in 59 healthy pre- and postmenopausal women who were participants in a blood donor program in Lyon, France, and were not undergoing treatment known to affect bone metabolism.

INTRAINDIVIDUAL VARIABILITY
Serum total P1NP was measured in 15 healthy untreated postmenopausal women, age 56–72 years. Fasting serum samples were obtained in all of these women at baseline, day 30, and day 90.

The studies were approved by the local ethics committees, and informed written consent was obtained from all participants.
SERUM PINP ASSAYS

MANUAL RIA FOR SERUM INTACT PINP
Serum intact PINP was measured by a competitive RIA (Uniq™ PINP RIA Orion Diagnostica) using a polyclonal antibody raised against the α1 homotrimer variant of human PINP and intact human PINP as a calibrator (15). This assay, which recognizes PINP only in its trimeric form, uses 100 μL of serum for duplicate measurements, and the total procedure time is 3 h. Intra-assay variation (3 samples, 20 replicates per run) ranged from 3.7% to 5.0%. Interassay variation (4 samples, 20 different runs) ranged from 4.1% to 7.6%. Analytical sensitivity, the concentration that was 2SD above the zero calibrator value, was determined to be 1 μg/L.

AUTOMATED ASSAY FOR SERUM TOTAL PINP
Measurements were performed on the Elecsys 2010 automated analyzer (Roche Diagnostics GmbH), a 2-site assay using monoclonal antibodies raised against intact human PINP purified from pooled 2nd-trimester human amniotic fluid. This assay detects both intact mono- and trimeric forms (total PINP) as previously described (12). A biotinylated antibody is incubated with 20 μL of serum, then a 2nd antibody labeled with ruthenium is added together with streptavidin-coated microparticles. A sandwich complex is formed and binds to the microparticles via biotin-streptavidin interaction. These microparticles are magnetically captured onto the surface of an electrode. Application of a voltage on this electrode then induces a chemiluminescent emission detected by a photomultiplier and compared to a calibration curve, which is generated by 2-point calibration. The total duration of this automatic process is 18 minutes. The automated PINP assay has been available within Europe for several years on the Roche Elecsys analyzer with a limited license to be used only for diagnostic purposes but not for research, clinical studies, or trials. In the summer of 2007, Roche Diagnostics acquired all of the rights to use the assay and assay technology (electrochemiluminescence) for these other applications as well.

SERUM CTX ASSAY
Serum concentrations of the carboxy-terminal telopeptide of type I collagen (CTX) were determined on the Elecsys 2010 analyzer (Serum Crosslaps) (20). Intra- and interassay CVs were below 4.1% and 5.7%, respectively, and the analytical sensitivity was 0.01 μg/L.

STATISTICAL ANALYSES
Because PINP concentrations did not show a gaussian distribution, data were log transformed before analysis. Comparison of PINP concentrations measured by RIA and automated assay were analyzed using the Bland-Altman bias plot method (21). The least significant change (LSC) at a significance level of P <0.05 was calculated by a two tailed test according the formula (17):

\[
LSC = 1.96 \times \sqrt{2 \times CV_i},
\]

where CVi is the intraindividual variability assessed over a 3-month period. We analyzed the percentage of patients with changes of total PINP and CTX that exceeded the LSC threshold both for PTH and alendronate therapy. Difference of changes of serum PINP between compliant and noncompliant patients was assessed by the nonparametric Mann-Whitney test.

All analyses were performed using the statistical analysis software (SAS).

Results

ANALYTICAL PERFORMANCE OF THE AUTOMATED ASSAY FOR SERUM TOTAL PINP

Precision and sensitivity. The intraassay variations assessed by 20 measurements of 4 samples (mean PINP values: 35.1, 64.1, 108.3, and 147.1 μg/L), were 1.17%, 1.70%, 1.00%, and 1.27%, respectively. The interassay variations evaluated by repeated measurements (n = 15) of 6 samples (mean values: 27.6, 62.7, 105.0, 153.7, 231.3, and 285.5 μg/L) were 2.70%, 2.91%, 2.53%, 2.72%, 2.00%, and 4.41%, respectively. The lower limit of detection, calculated as the concentration lying 3 SD above that of the lowest calibrator, was determined to be <5 μg/L. The lower limit of quantification, defined as the lowest concentration of PINP in human serum that can be measured with accuracy and a precision error of <20%, was determined to be 9.2 μg/L.

Dilution test. Three samples were diluted 1:2, 1:4, 1:8, and 1:16 with assay buffer. As shown in Table 1, the dilution recovery ranged from 89% to 118% (mean: 99%) when 1 value below the lower limit of quantification was excluded.

Recovery test. Known amounts of PINP calibrator were added to 3 serum samples. The recovery ranged from 94% to 103% (Table 2).

Sample stability. As shown in Table 3A, serum PINP was stable for up to 5 days of incubation at both room temperature and 4 °C. After 5 days, the median change compared to day 0 was −0.10% (P = 0.67) and +0.15% (P = 0.57) at room temperature and 4 °C, respectively. Stability of serum samples was also investigated after long term storage at temperature below −18 °C and below −70 °C. There was no significant loss of PINP after 24 months of storage at below −18 °C (median change: −1.12%,

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from −15% to +12%, *P* = 0.55) (Table 3B). Concentrations measured in serum after 19 months at −70 °C were also not significantly different than concentrations measured at day 0 (data not shown). Serum P1NP values measured after 4 freeze-thaw cycles in 3 samples (mean initial concentrations: 63.2, 157.7, and 235 g/L) were very similar to concentrations measured at day 0 with differences of 0.6%, 0.4%, and 0.3%, respectively.

### COMPARISON OF THE AUTOMATED ASSAY FOR SERUM TOTAL P1NP ASSAY WITH THE MANUAL RIA FOR INTACT P1NP

Both the manual RIA and the automated analyzer were used to measure serum P1NP concentrations in 59 healthy women. Fig. 1 shows the Bland-Altman bias plot of the comparison between automated and RIA methods. Serum automated P1NP values were on average 9.8 μg/L higher (*P* < 0.0001) than those obtained by RIA and there was a trend of increased difference between the 2 methods with increased mean PINP concentrations.

### INTRAINDIVIDUAL VARIATION OF AUTOMATED SERUM TOTAL P1NP

Intraindividual variability of serum automated total P1NP was assessed in 15 untreated postmenopausal women during a 3-month period. At baseline, mean (SD) serum P1NP concentration was 60.5 (22.9) μg/L. The median intraindividual CV (CVi) was 7.2% (25; 75th percentiles: 5.3; 13.9%). Based on this CVi, the LSC was determined to be 20.0%.

### CHANGES WITH SEX, AGE, AND MENOPAUSE OF SERUM TOTAL P1NP LEVELS

In healthy premenopausal women there was a significant association between age and serum total P1NP (*r* = −0.25, *P* < 0.001) (Fig. 2A). When the analyses was restricted to women age 35 years and older, values did not vary with age and remained stable up to 49 years (*P* = 0.25). The mean (SD) value of serum total P1NP was significantly higher in the 38 women age 30 to 34 years than in the 192 women 35 years and older [40.1 (12.9) μg/L vs 31.1 (12.1) μg/L, *P* < 0.0001]. The reference range based on the 95% CI of the back log-transformed data in premenopausal women aged 35 years and older was 13.8 to 60.9 μg/L. In the 238 postmenopausal women with osteoporosis participating in the PaTH study (4), the mean (SD) values [54.1 (30.2) μg/L] before initiating therapy were 74% higher (*P* < 0.001) than in healthy premenopausal women age 35 years and older, and 32.5% had values above the upper limit of the reference interval (60.9 μg/L). In healthy men, there was no significant change of total P1NP from age 40 to 65 years (Fig. 2B), with mean (SD) concentrations of 38.1 (18.4) μg/L. The lower and upper reference limits in men based on the 95% CI of the back log-transformed data were 13.9 and 85.5 μg/L, respectively.

### INDIVIDUAL CHANGES OF SERUM P1NP WITH PTH AND ALENDRONATE

As shown on Fig. 3, when all patients were considered independent of their compliance to treatment, 83% and 88% of women had changes of serum P1NP that...
Table 3. Stability of the automated assay for serum total P1NP.

**A. P1NP stability at room temperature after various incubation times.**

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**B. P1NP stability in frozen specimens.**

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*a Storage at a temperature below –18 °C.
exceeded the LSC after PTH (≥+20%) or alendronate (≤−20%) treatment, respectively (Fig. 3). There were 71% and 82% patients compliant to PTH 1-84 and alendronate, respectively. The increase of P1NP after 3 months of PTH 1-84 was significantly higher ($P = 0.005$) in compliant [median (25; 75 pct): 206% (57.1; 329)] than noncompliant [63% (7.7; 154)] patients. The decrease of serum P1NP was larger in patients compliant to alendronate [median (25; 75 pct): $−59.8\% (−72.5; −45.7)$] than noncompliant individ-
In fasting individuals, the intrapatient variability of serum CTX has been reported to range from 9.4% (17) to 19.1% (16), resulting in LSCs of 26% and 53%, respectively. Based on these previously reported LSCs, the corresponding percentages of patients demonstrating a variation of serum CTX that exceeded the LSC after 3 months in the PaTH study were 70% and 52% with PTH and 90% and 75% with alendronate when cutoff values of 26% and 53% were used to calculate the LSC, respectively.

Discussion

In this report we summarize the evaluation of an automated assay for serum total P1NP. We found that this assay exhibits a high analytical reproducibility, is robust with respect to sample storage conditions, and can document changes that exceed the LSC in a majority of individuals receiving PTH 1-84 or alendronate for 3 months. The assay for serum total P1NP is rapid (18 minutes) and the intra- and interassay variations are lower than 2% and 5%, respectively. These characteristics compare favorably with those of the manual RIA for intact P1NP. A previous study using the manual RIA reported stability of intact P1NP up to 48 h at room temperature (22). We confirmed the high stability of P1NP at room temperature (up to 5 days), which may be further increased by using the current automated assay to detect both the trimeric and monomeric forms because the monomeric form results from thermal conversion of trimeric intact P1NP (12).

In healthy women, values obtained with the automated method were significantly higher than those observed with the RIA, and the difference tended to increase with larger P1NP concentrations. The increased absolute values are likely attributable to the fact that the auto-

Fig. 3. Individual percentage changes of serum total P1NP measured by the automated assay in postmenopausal women with osteoporosis after 3 months of treatment.

Treatment was with subcutaneous PTH 1-84 (100 μg/day, n = 119, closed circles) or oral alendronate (10 mg/day; n = 60, closed triangles). The horizontal plain lines and associated percentage values are the median of the percentage change in each treatment group. The grey box represent the variation of P1NP within the least significant change range of –20 and +20%.
mated assay measures the monomeric form of intact P1NP in addition to the trimeric structure, which is the only form detected by the manual RIA. In vivo, native P1NP is initially produced as a single trimeric form. At physiological temperature, however, there is a transition of the trimeric to the monomeric form, for which a half-life of 10 h has been estimated (12). Thus, the manual RIA that detects only the trimeric form of P1NP may underestimate the rate of type I collagen synthesis. The reasons for the increasing difference between methods with larger P1NP values are unclear. Because the dynamic range of the RIA is narrower than that of the automated assay, there may be a ceiling effect at high concentrations with RIA. It is also possible that the production and/or clearance rates of the monomeric and trimeric forms of P1NP, which are differently detected by the 2 assays, may vary with bone remodeling.

The intraindividual variation over a 3-month period of serum automated P1NP values in postmenopausal women was 7.2%, a result that is very similar to the 7.4% reported for manual RIA of intact P1NP in 11 healthy postmenopausal women during a 6-month period (17) and the 10.6% reported for the same automated assay in 20 premenopausal women during a period of 10 consecutive days (17).

As previously reported in other studies of bone formation markers including P1NP (23–25), we found that serum automated total P1NP concentrations decreased from age 30 to 35 years and then remained stable up to 50 years in healthy premenopausal women. The higher values in younger women probably reflect the fact that skeletal maturity has not yet been achieved, especially at the cortical sites, a time of increased bone turnover. The reference values based on premenopausal women 35 years and older were very similar to those recently reported by Glover et al., who used the same assay in 196 UK women 35–45 years old (25). In postmenopausal women with osteoporosis, concentrations of total P1NP were 74% higher than in premenopausal women. This finding is in agreement with previous observations obtained with various markers of bone formation and bone resorption (23, 26). In healthy men there was no significant change of serum total P1NP from age 40 to age 65 years, as previously reported for intact P1NP by manual RIA in larger samples of healthy men from France (27) and the UK (28). Larger populations including younger and older men would be helpful to fully characterize the age-related changes of serum total P1NP in men.

The goal of treatment in osteoporosis is to reduce the occurrence of fragility fractures, but their incidence is low, and therefore the absence of events during the 1st year(s) of therapy does not necessarily indicate that treatment has been effective or ineffective. Measurement of BMD by dual x-ray absorptiometry is a surrogate marker of treatment efficacy that has been widely used in clinical trials. Given a precision error of 1% to 1.5% of BMD at the spine and hip, the individual change must be >3% to 5% to be seen as significant, a value that is generally obtained after 1 or preferably 2 years of treatment. Another limitation of BMD is that changes appearing during treatment with antiresorptive therapies (29) or PTH (30) explain only a small part of antifracture efficacy. Conversely, the early decrease of bone markers, including P1NP, has been shown to predict the efficacy of alendronate (31) and the selective estrogen receptor modulator raloxifene (32) in reducing the risk of spine fracture. Although the association between changes of bone turnover markers with PTH and fracture risk has not yet been reported, this treatment induces rapid (within 1 month) and marked increases of serum P1NP (4, 16) that are associated with changes of spinal BMD after 12 to 18 months (16, 33).

To translate data obtained in population studies to routine clinical use it is essential to demonstrate that a biological response can be easily demonstrated in a single individual. The use of LSC to define a biological response is well established in clinical chemistry (34), although this method may not necessarily translate to optimal clinical efficacy. In the PaTH study (4), it was found that after only 3 months of therapy, more than 80% of patients treated with PTH or alendronate demonstrated changes in total P1NP that were greater than the LSC. This proportion was actually higher than that observed when serum CTX (currently one of the most sensitive biochemical markers of bone resorption in postmenopausal osteoporosis) was used to document a biological response to PTH. Our data, obtained in a clinical setting through procedures conducted according to a strict sampling protocol, probably underestimate the LSC found in clinical practice because the sample may not be obtained at the same time of the day at all visits and during the same fasting status. Such discrepancies, however, are likely to be more problematic for biochemical markers of bone resorption than P1NP, which is virtually unaffected by these 2 preanalytical factors (19).

In summary, the automated assay for serum total P1NP is convenient to use, precise, and robust to sampling conditions. Total P1NP enabled detection of the increase of bone turnover in postmenopausal women and was sensitive to significant bone turnover changes induced by PTH 1-84 and alendronate. This assay should be useful for the routine management of patients with osteoporosis and other metabolic bone diseases.
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