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Interlaboratory Variation of Biochemical Markers of Bone Turnover

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Background: **Biochemical markers of bone metabolism are used to assess skeletal turnover, but the variability of marker assays is still an issue of practical concern. We describe the results of an international proficiency testing program for biochemical bone markers among clinical laboratories.**

Methods: **Two serum and two urine pools (normal and increased marker concentrations) were sent on dry ice to 79 laboratories for analysis within 2 weeks of receipt.**

Results: **Data were submitted by 73 laboratories. The within-method interlaboratory CVs (CV_{IL}s) were as follows: serum bone-specific alkaline phosphatase (n = 47 laboratories), 16–48%; serum osteocalcin (n = 31), 16–42%; urinary free deoxypyridinoline (n = 30), 6.4–12%; urinary total deoxypyridinoline and pyridinoline (n = 29), 27–28%; urinary N-terminal cross-linked telopeptide of type I collagen (n = 10), 39%; serum C-terminal cross-linked telopeptide of type I collagen (ICTP; n = 8), 22–27%; urinary hydroxyproline (n = 13), 12%. Analytical results showed both systematic and nonsystematic deviations. In identical samples, results obtained for the same marker by the same method differed up to 7.3-fold. In urine-based assays, correction for urinary creatinine slightly increased CVs.**

Conclusion: **Even with identical assays and methods, results for most biochemical markers of bone turnover differ markedly among laboratories.**

Within the last decade, biochemical markers of bone metabolism have become a widely used tool for the evaluation of patients with bone diseases. In particular, bone markers are increasingly used for the therapeutic monitoring of patients with metabolic bone diseases *(1, 2)*. However, whether in large trials or for individual patients, these measurements can be useful only if they provide consistent and reproducible results.

For many years, variability of bone markers has been an issue of both practical and scientific concern. Several studies have investigated preanalytical factors that influence bone marker measurements, such as diet; sample handling; diurnal, menstrual, and seasonal rhythms; age; gender; and growth *(3–14)*. Other reports have focused on the analytical performance of bone marker assays within a standardized laboratory setting *(15, 16)*. To date, however, no information is available on the routine interlaboratory variation of bone marker measurements. We therefore implemented an international proficiency testing program for biochemical markers of bone metabolism among clinical laboratories in Europe.

Materials and Methods

The trial was announced via mail, advertisements in laboratory journals, and via the Internet. In these advertisements, laboratories offering biochemical markers of bone turnover as a routine clinical service were asked to participate in a pilot proficiency testing program. Of the 113 laboratories responding, 79 commercial or public institutions from five European countries (Germany, United Kingdom, Switzerland, The Netherlands, and Luxembourg) were eligible for the trial. Requirements for eligibility were (*a*) more than one biochemical marker of bone metabolism offered as a routine service and (*b*) more than 20 tests performed per week and per assay.

Testing materials were two serum and urine pools obtained on the same day from several healthy volunteers (age range, 20–55 years). Serum pool A ("low") consisted of sera from nine individuals with concentrations of serum bone-specific alkaline phosphatase (sBAP)[3] in the

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[3] Nonstandard abbreviations: sBAP, serum bone-specific alkaline phosphatase; EIA, enzyme immunoassay; uDPD, urinary deoxypyridinoline; uPYD, urinary pyridinoline; CLIA, chemiluminescence immunoassay; NTx, N-terminal cross-linked telopeptide of type I; CTx, C-terminal cross-linked telopeptide of type I; ICTP, C-terminal cross-linked telopeptide of type I, cathepsin labile; sOC, serum osteocalcin; LIA, luminescence immunoassay; and MMR, maximum/minimum ratio.

lower range of the premenopausal mean. Serum pool B ("high") was derived from eight subjects with sBAP concentrations above the premenopausal mean. sBAP was measured in our laboratory by a commercial enzyme immunoassay (EIA; Alkphase-B™; Metra Biosystems) *(17)*.

Urine pool A (high) consisted of urine obtained from three subjects with a total urinary deoxypyridinoline (uDPD) concentration above the premenopausal mean. Conversely, urine pool B (low) was derived from two subjects with total uDPD concentrations below the premenopausal mean. total uDPD was determined in our laboratory by ion-paired reversed-phase HPLC as described previously *(18)*.

Blood for the serum pools was collected in Vacutainer Tubes without additive, allowed to clot at room temperature for 30 min, and centrifuged at 4 °C. The serum was then pooled, mixed for 10 min in an ice-water bath, divided into 1-mL aliquots, and frozen at −80 °C until shipment. Urine was collected without additive, pooled, mixed for 10 min in an ice-water bath, centrifuged to remove solid particles, divided into 5-mL aliquots, and frozen at −30 °C until shipment. Storage under these conditions has been shown to prevent degradation or signal change for at least 6 months in all markers studied *(3)*. The storage time for all aliquots at our laboratory (i.e., the time between collection and shipment) was 20–22 days.

Each laboratory received one aliquot from each of the four pools on dry ice. Reception of materials in the frozen state was confirmed by phone and mail in each case. In one case, samples arrived thawed and were therefore resent within 2 days. Laboratories were advised to either analyze the samples immediately upon receipt or to keep them stored at −30 °C or lower until analysis. Once thawed, aliquots had to be kept at 4 °C, with urine samples protected from direct ultraviolet light. Although laboratories selected the number and types of assays according to their individual resources, analysis of both aliquots from the appropriate material (serum or urine) was mandatory for each test included. In case of multiple assays and/or markers, analyses had to be performed on the same day without refreezing and thawing of the testing material. Otherwise, laboratories were advised to follow their own standard operating procedures and the manufacturer's technical guide. Analysis of the testing materials had to be completed by the respective laboratories within 2 weeks of receipt. Urinary results were to be reported both as absolute and as values normalized for creatinine.

Results were reported on a standardized data sheet. Data were analyzed by a standardized program using Youden plots and descriptive statistics *(19)*. The latter included calculation of means, SDs, medians, CVs, and the 16th and 84th percentiles. In the Youden plots, all participants' results from samples A and B were plotted against each other. Both the y and x axes were chosen arbitrarily so that the data of interest and the method-dependent differences between them became visible. Values clustering in the center of the plot indicated low systematic and nonsystematic differences. Values scattered along the diagonal line of the plot indicated systematic differences, whereas values off this line were suggestive of nonsystematic differences in results. The plot for osteocalcin (Fig. 1, bottom left panel) is a good example of systematic differences between analytical procedures because three different clusters of values are seen scattered along the diagonal line of the plot. Because of a priori unknown reasons (e.g., differences in calibration), the use of one set of reagents produced a systematic bias with lower values for both sample A and sample B compared with another set of reagents. Differences in variability between similar assays were tested by two-tailed t-tests.

Results

Seventy-three (92%) of the participating laboratories completed the trial in time, reporting a total of 170 results (Table 1). Ninety-two results (54%) concerned bone resorption markers, of which almost two-thirds (n = 59) were on uDPD (35% of all analyses). Of these, 29 were simultaneous determinations of urinary total pyridinoline (uPYD) and uDPD by HPLC techniques. Of these, two

Table 1. Markers and assays reported by the participating laboratories.

Marker/Method	Total number of results reported	Number of manufacturers
sBAP	47	6
IRMA	22	1
EIA	16	1
Kinetic assay (LP[a])	6	2
Electrophoresis	3	2
sOC	31	6
RIA (5 different assays)	18	3[b]
LIA	8	2
EIA	5	1
Total uPYD and uDPD	29	
HPLC	29	Two commercial reagent sets and various in-house assays
Urinary free DPD	30	3
EIA	15	1
CLIA	12	1
RIA	3	1
Telopeptide assays	20	4
EIA (uNTx)	10	1
RIA (sICTP)	8	1
RIA (uCTx)	1	1
EIA (uCTx)	1	1
Urinary hydroxyproline	13	3
HPLC	7	1
Colorimetric	5	2

[a] LP, lectin precipitation.

[b] One measurement was performed by RIA without providing a specific manufacturer.

laboratories used a commercially available HPLC reagent set, whereas the remaining results were obtained with various in-house assays using different techniques and calibrators. Because results on total uPYD and uDPD were highly correlated (within-run correlation, $r = 0.95$), data on total uDPD were taken for most statistical evaluations. Thirty laboratories used immunoassays for free uDPD; one-half of those were obtained by a direct EIA (Pyrilinks-D™; Metra Biosystems), whereas 12 laboratories used an automated chemiluminescence immunoassay (CLIA; ACS:180 DPD™; Bayer). Results obtained by assays for the N-terminal cross-linked telopeptide of type I (NTx), C-terminal cross-linked telopeptide of type I (CTx), and C-terminal telopeptide of type I collagen, cathepsin labile (ICTP) contributed only 12% of all analyses reported (n = 20). Among these, the assay for urinary CTx (uCTx) was the least utilized (n = 2) and, therefore, was not evaluated statistically. Thirteen laboratories measured urinary hydroxyproline by either HPLC (reagent set provided by one manufacturer) or by colorimetric assay (7.6% of all analyses).

Among the bone formation markers, assays for sBAP were used more frequently than those for serum osteocalcin (sOC), contributing 27% of all analyses (sOC contributed 18%). For the measurement of sBAP, four different methods from a total of six manufacturers were used. However, 38 of 47 analyses (81%) were performed with only two immunoassays: Alkphase-B and Ostase™ (Beckman Inc.). In contrast, the test panel for sOC was more heterogeneous: three different immunoassay procedures [RIA, EIA, and luminescence immunoassay (LIA)] from a total of six manufacturers were used by the various laboratories. The RIA procedure for sOC included five assays obtained from three different commercial sources. Within this group, one source provided three different RIA procedures.

A summary of the interlaboratory CVs (CV_{IL}s) specified for the type of marker, the methods used, and the aliquot analyzed is given in Table 2. The CV_{IL} varied between 15% and 71% for the formation markers and between 6.4% and 39% for the resorption markers. On average, the CV_{IL} of urinary markers did not differ from that of the serum markers.

The immunoassays for sBAP exhibited mean CV_{IL}s of

Table 2. Minimum and maximum values and CV_{IL}s reported for identical bone markers and methods.

Marker	Assay	Units	Sample pool	Min[a]	Max	MMR	Group mean for sample[b]	SD[b]	CV_{IL}, %
BAP	IRMA (n = 22)	μg/L	A	4.8	15.8	3.3	9.7	2.4	25
			B	21.0	39.9	1.9	29.0	4.6	16
BAP	EIA (n = 16)	U/L	A	13.0	24.0	1.8	21.0	3.3	17
			B	27.5	63.6	2.3	46.0	8.7	20
BAP	LP (n = 6)	U/L	A	15	111	7.3	34.5	16.7	48
			B	79	149	1.9	118	25.4	22
OC	LIA (n = 8)	μg/L	A	1.8	3.4	1.9	2.9	0.6	21
			B	10.2	16.0	1.6	13.0	1.9	16
OC	EIA (n = 5)	μg/L	A	2.3	5.8	2.5	4.5	1.4	31
			B	14.9	24.6	1.7	17.3	4.1	24
OC	all RIAs (n = 18)	μg/L	A	0.1	13.0	130[c]	3.7[c]	2.8[c]	68[c]
			B	2.6	79.6	30[c]	20.7[c]	17.1[c]	71[c]
OC, RIA subgroups	1 (n = 6)	μg/L	A			2.5	2.65	0.42	16
			B			1.8	15	2.76	18
	2 (n = 6)	μg/L	A			2.7	8.97	3.81	42
			B			3.2	51.5	21.7	43
Total DPD	HPLC (n = 29)	nmol	A	148	710	4.8	450.4	121.0	27
			B	49	197	4.0	139.0	37.0	28
Free DPD	EIA (n = 15)	nmol	A	134	190	1.4	154.2	18.0	12
			B	61	73	1.2	67.3	4.3	6.4
Free DPD	CLIA (n = 12)	nmol	A	147	177	1.2	160.6	10.0	6.4
			B	69	100	1.5	79.1	8.8	11
NTx	EIA (n = 10)	nmol	A	1410	8056	5.6	4500	1750	39
			B	410	1564	3.8	820	320	39
ICTP	RIA (n = 5)	μg/L	A	11.1	14	1.3	88.0	24.2	27
			B	7.5	9.9	1.3	156.0	33.5	22
Hydroxyproline	HPLC (n = 7)	μmol/L	A	645	936	1.4	843	101	12
			B	186	232	1.2	111	13	12

[a] Min, minimum; Max, maximum; LP, lectin precipitation.
[b] The concentrations for sample pools A and B represent the group mean ± SD obtained from all values reported for the specific marker and method.
[c] Results from all RIAs included.

21% (IRMA) and 18% (EIA), whereas the lectin precipitation assay had a mean CV_{IL} of 30%. As shown in the top panels in Fig. 1, both immunoassays showed a tendency for systematic differences in results. Compared with the IRMA, systematic influences seemed to be more pronounced in the EIA. However, the difference in variability between the two immunoassays was statistically not significant.

Interlaboratory variability of sOC values was strongly dependent on the type of assay used. For results from all five RIAs, overall CV_{IL}s of 68% and 71% were calculated for sample pools A and B, respectively (Table 2). However, when the results were stratified into subgroups defined by manufacturer, subgroup 1 showed CV_{IL}s of 16% and 18%, whereas subgroup 2 had CV_{IL}s of 42% and 43%, respectively (sample pools A and B; Table 2). Subgroup 2 showed a strong tendency for systematic differences, suggesting three different clusters (Fig. 1, bottom left panel). Because of the small sample size, no CV_{IL}s were calculated for the two other RIAs. For comparison, the CV_{IL}s were 31% and 24% for the EIA (one manufacturer) and 21% and 16% for the LIA (two manufacturers), respectively (Table 2).

Nonnormalized analysis of total uDPD and total uPYD by HPLC yielded CV_{IL}s of 27% and 28% for sample pools A and B, respectively, with both substantial systematic and nonsystematic deviation among laboratories (Fig. 2, top left panel). Notably, except for two laboratories, all HPLC assays were run with different calibrators and techniques (in-house assays). Both immunoassays for

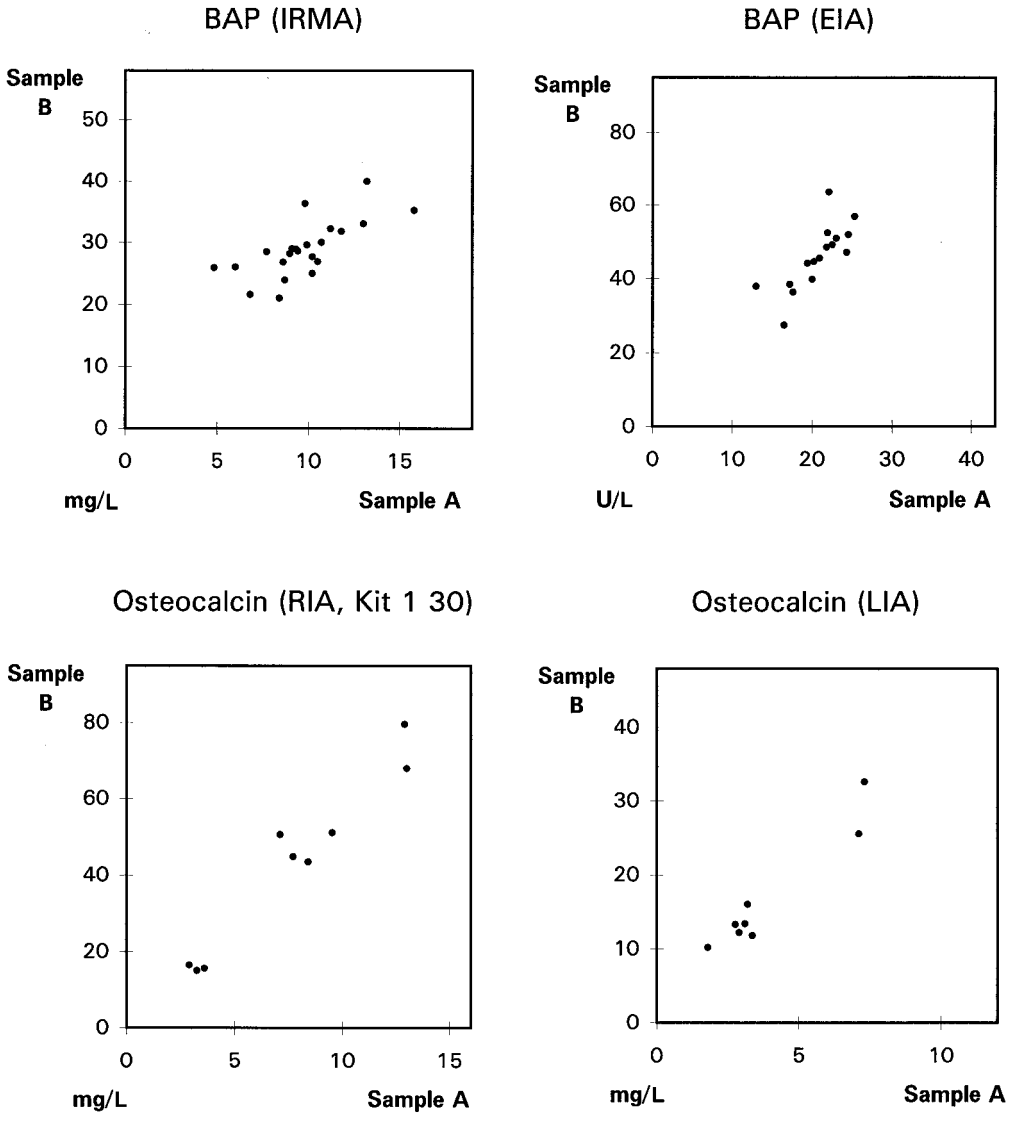

Fig. 1. Youden plots for BAP by IRMA (*top left panel*) and EIA (*top right panel*) and for sOC by RIA (*bottom left panel*) and by LIA (*bottom right panel*).

Each *point* represents the test results for serum samples A (*x axis*) and B (*y axis*), as reported by individual laboratories (see *Materials and Methods*). As evident from the analysis and later confirmed, three different RIAs from various sources were used by the nine laboratories shown in the *bottom left panel*. All tests were reported as RIAs without further specification.

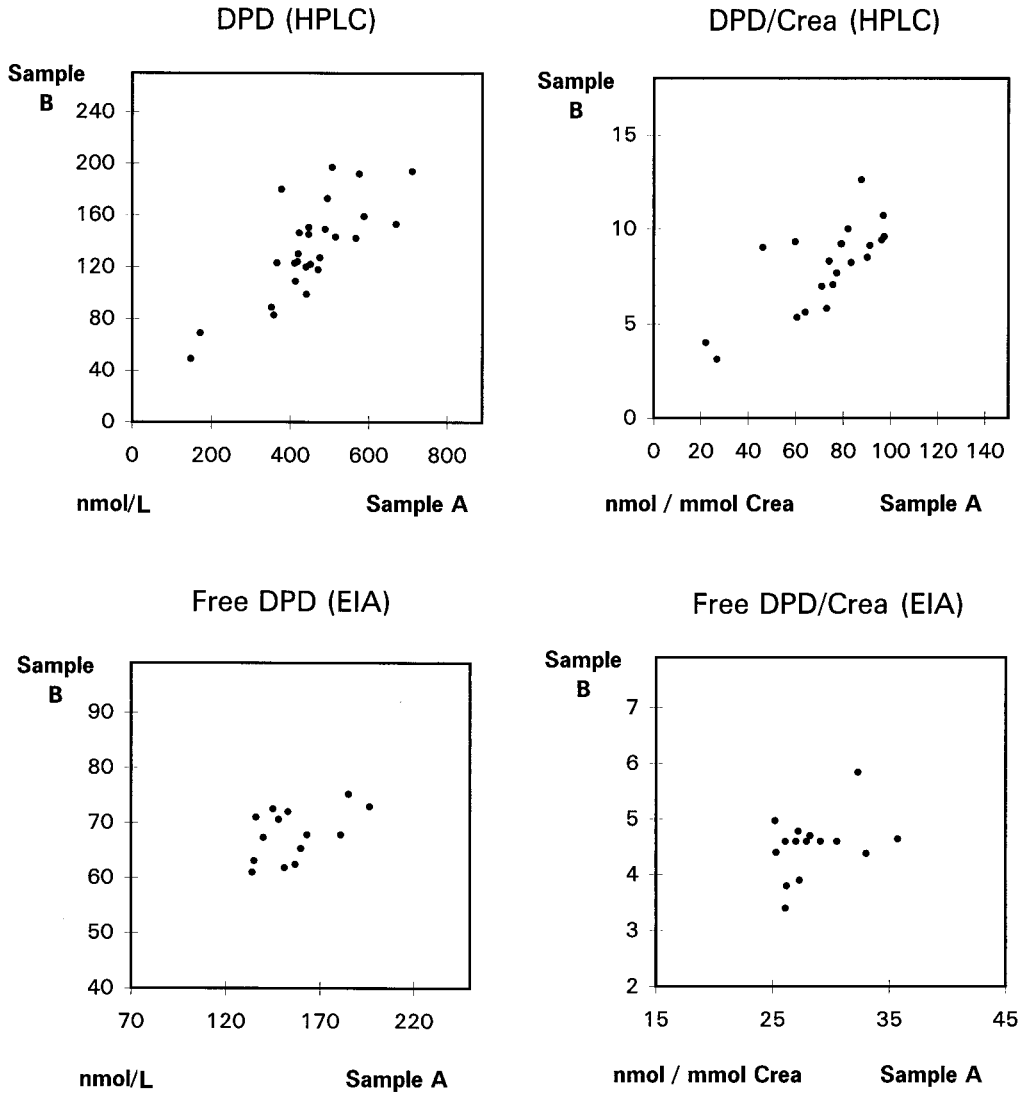

Fig. 2. Youden plots for urinary total DPD by HPLC (*top panels*) and for urinary free DPD by EIA (*bottom panels*).
Left panels, absolute values; *right panels*, values normalized for urinary creatinine (*crea*).

urinary free DPD revealed a mean CV_{IL} <10% (Table 2), with little deviation of the nonnormalized values (Fig. 2, bottom panels, and Fig. 3, top panels). In contrast, a much higher CV_{IL} was observed for uNTx (Table 2 and Fig. 3, bottom left panel). No difference was seen in the CV_{IL} of any marker or method when results were stratified by country of origin.

Minimum and maximum reported values and the calculated ratio between these numbers are shown in Table 2. The highest maximum/minimum ratio (MMR) was observed for sBAP by lectin precipitation (MMR = 7.3) and for the uNTx by EIA (MMR = 5.6). When all four RIAs for sOC were calculated together, the MMRs were 130 for sample pool A and 30 for sample pool B. However, when the MMRs for subgroups 1 and 2 were calculated separately, they were comparable to other immunoassays (Table 2). MMRs did not differ between serum- and urine-based assays, and there was no difference in results when data were analyzed according to country of origin.

In most urine-based immunoassays, creatinine normalization led to a slightly higher CV_{IL} for results obtained from identical samples (Table 3 and Figs. 2 and 3, right-hand panels).

Discussion

Preanalytical and analytical variability in the measurement of bone turnover markers is a major and sometimes neglected issue in the clinical and research use of these markers *(20)*.

Interlaboratory variation is a source of analytical variability that becomes relevant when results for the same analyte are provided from different laboratories. This situation frequently occurs in routine practice and is, among other reasons, the rational for proficiency testing

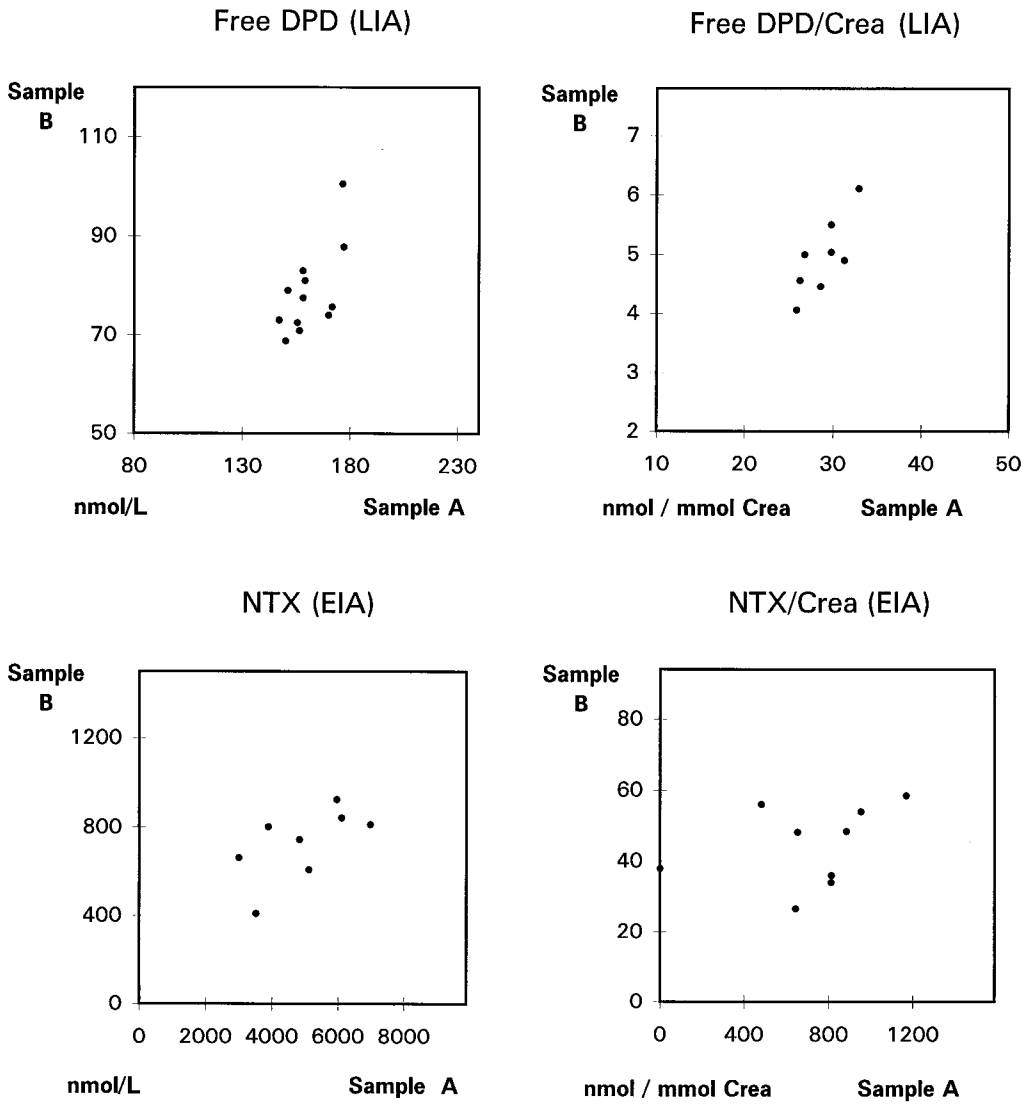

Fig. 3. Youden plots for urinary free DPD by LIA (*top panels*) and for urinary NTx by EIA (*bottom panels*).
Left panels, absolute values; *right panels*, values normalized for urinary creatinine (*crea*).

programs. The present study is the first of such programs for biochemical markers of bone metabolism performed among laboratories in different European countries. With the exception of the HPLC methods for total uPYD and total uDPD, all laboratories used commercially available and presumably standardized assays. Despite this fact, unacceptable interlaboratory variation was noted for almost all markers. Our results demonstrate that, at present, results for most markers of bone turnover cannot be compared among laboratories without previous cross-calibration. Because this type of variability is at least in part controllable, standardization of results should be

Table 3. Effect of creatinine normalization on CV_{IL}.

Marker	Assay	n	CV_{IL} (A), %	CV_{IL} (B), %	Mean CV_{IL}, %
Total DPD	HPLC	29	27	27	27
Total DPD/creatinine	HPLC	29	28	28	28
Free DPD	EIA	15	12	6.4	9.2
Free DPD/creatinine	EIA	15	11	12	12
Free DPD	CLIA	12	6.4	11	8.8
Free DPD/creatinine	CLIA	12	8.7	13	11
NTx	EIA	10	39	39	39
NTx/creatinine	EIA	10	47	25	36

improved and routine proficiency testing programs should be introduced for these markers.

Nonspecific variability is determined by analytical and preanalytical factors, and it strongly affects the practical usefulness of any given analyte. Among the preanalytical factors influencing bone marker concentrations, specimen characteristics and sample handling *(3–5, 20, 21)*, as well as subject-related effects such as age *(6, 10)*, gender *(10, 11)*, diurnal or day-to-day variation *(7, 8, 12)*, diet *(3)*, growth *(22)*, and renal and hepatic function *(23, 24)* all have been shown to be of importance. In addition, analytical performance, i.e., assay precision and accuracy, quality control, and standardization, will affect results. For most bone turnover markers, substantial overall variability has been noted, and its relevance to clinical situations such as therapeutic monitoring has been pointed out *(20, 25)*. To be clinically meaningful, changes in bone markers induced by therapeutic interventions need to exceed a predefined range of nonspecific variability. For antiresorptive treatments (e.g., hormone replacement therapy, bisphosphonates, and estrogen receptor modulators) and bone resorption markers, these cutoff values are usually between −20% (free uDPD) and −70% (uCTx). For bone formation markers (sBAP and sOC), the respective values are −20% to −40% (expressed as the percentage of decrease from the pretherapeutic baseline values). Changes below these marker-specific thresholds should be considered either ambiguous or nonspecific *(20)*.

With regard to the study design, it should be noted that no reference methods are available for any of the tests included in this trial. Therefore, results from individual laboratories can be compared only to the mean value of all results reported for a given marker. Our study therefore provides information on the distribution of values within, and relative to, the study cohort (expressed as CV_{IL}). Clearly, no information can be obtained regarding the accuracy of the measurements.

The CV_{IL} of any test is influenced by its analytical performance. Most assays used in the present study have intra- and interassay CVs of 5–15%. Given the cutoff values mentioned above and the analytical performance of most assays used in this study, a CV_{IL} <20% should be required to meet practical needs. However, among the bone formation markers included in this study, only the EIA for sBAP and one RIA for sOC (Table 2, RIA subgroup 1) had a CV_{IL} <20% for both samples. All other assays for bone formation markers showed higher interlaboratory variability, with individual CV_{IL}s up to 48%.

With regard to bone resorption markers, only the EIA and the CLIA for urinary free DPD and the HPLC assay for urinary hydroxyproline had a CV_{IL} <20% for both sample pools tested. Again, all other assays for bone resorption markers showed CV_{IL}s up to 39%, which far exceeded the intra- and interassay variability. In the case of HPLC measurements for total uPYD and uDPD, one should be aware of the fact that most of the results reported in this study were obtained from nonstandardized in-house assays. These HPLC assays not only use different techniques (e.g., manual or automated methods, isocratic or gradient elution), but also different calibrators from several sources. Therefore, although standardization of HPLC assays is certainly needed, the higher variability in the HPLC assays at present is to be expected. Notably, the total CV_{IL} of the nonstandardized HPLC methods was below or in the range of the CV_{IL} for most commercial, and presumably fully standardized, assays used in this trial. In addition, there was no genuine difference in variability between urine- and serum-based assays, nor was there a difference among countries.

Results from most immunoassays for bone turnover markers, therefore, cannot be exchanged between laboratories without careful cross-calibration. This fact is also evidenced by the unfavorable MMRs for many of the assays (Table 2). In comparison, when evaluated in large proficiency testing programs, common endocrine tests such as the assays for thyroid-stimulating hormone have a CV_{IL} of 5–10% *(19, 26)*.

When the total interlaboratory variation was calculated for all five RIAs used for the measurement of sOC, CV_{IL}s of 68% and 71% were obtained for the two serum pools. These values are no surprise because the antibodies used in the various assays are directed against different epitopes, and therefore, different analytes are being measured. Accordingly, previous studies have shown that absolute values from different immunoassays for sOC cannot be compared directly *(27, 28)*. However, consistent information may be obtained when the results are expressed as a percentage of osteocalcin in healthy individuals *(29)*. Our study, however, indicates that even identical and presumably standardized assays provide largely divergent results when performed in different laboratories. This situation becomes more complicated when a manufacturer offers different assays or methods for the same marker. In our study, this was the case for sOC, where three assays with different epitope specificities were provided by one manufacturer. Interestingly, although all three assays were used by the participating laboratories, all results were labeled "serum osteocalcin", and none of the laboratories reported the test name or the epitope specificity. Therefore, the high CV_{IL} for the various RIAs for sOC may well be relevant to the clinical situation because laboratories often do not specify the type of RIA used and thus the analyte reported. Consequently, whenever there are different assays for the same marker, laboratories need to report the manufacturer and the specific assay together with the analytical result.

In conclusion, our results show that many assays for bone turnover markers, independent of the matrix used, have not achieved satisfactory standardization. Results from one laboratory are not likely to be comparable with results from another laboratory, even when the same method and the same sample are being used. Obviously, several

factors contribute to the overall interlaboratory variation of assay results. In addition to batch-to-batch and intraassay variation, minor variations in technique or the instability of reagents and calibrators may play a role *(30, 31)*. To improve this situation, it is imperative to include bone turnover markers in routine laboratory proficiency programs. Furthermore, reference methods and international standards need to be developed, and reference laboratories should be appointed to define target values for standardization trials. Moreover, lot-to-lot-variation needs to be minimized by the manufacturers and stringent proof of product standardization should be required.

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