rum samples and different calibration slopes for human plasma and mouse/rat plasma. They observed an overestimation of ~20% for serum ADMA concentrations determined by the ELISA compared with LC-MS. Our results indicate a higher discrepancy between the 2 methods, possibly because our comparison used plasma samples, which have a different matrix effect on the ELISA results. The matrix dependence of the ELISA may be the cause of the calibration offsets. The calibration must be performed with the supplied calibration samples, which consist of ADMA in buffer solution. The systematically observed errors in the calibration may arise because these calibration samples are very different from plasma samples.

Overall, the ELISA appears to overestimate ADMA concentrations in plasma by a factor of nearly 2. In the group of healthy individuals, the mean (SD) ADMA concentration determined with the ELISA was 0.720 (0.111) μmol/L. Schulze et al. (10) found serum ADMA concentrations of 0.72 (0.16) μmol/L in healthy volunteers. The corresponding values for comparable groups described in recent studies focusing on analytical technology (8, 11–13) were 0.355, 0.42, 0.39, and 0.343 μmol/L.

The precision of the ELISA was good. We obtained a relative SD of 6.1% when we measured a sample from the healthy group 10 times. This is comparable to the value of 7.5% reported by Schulze et al. (10) and slightly above the value of 4.7% reported for the LC-MS assay (8).

The workloads for the laboratory personnel are comparable for the ELISA and the LC-MS assay. In the case of the ELISA, 20 μL of each sample must be pipetted into the microtiter plate well and acylated for 1.25 h. The ELISA process then takes place overnight in a refrigerator. The actual measurement of samples takes only seconds. Processing of a complete 12 microtiter plate begun in the afternoon is completed the next morning. The sample preparation for the LC-MS assay consists of pipetting the samples, addition of the internal standard, protein precipitation, centrifugation, evaporation of the samples under reduced pressure, and redissolution of the residue. The overall time required for preparing 40 samples is ~3 h, with ~1.5 h of hands-on time. The subsequent LC-MS determination time is 24 h for 40 samples with the system described by Martens-Lobenhoffer et al. (8).

In conclusion, the ADMA ELISA is suitable for clinical investigations in which groups of samples are compared and the endpoint is the shift of the ADMA concentration in response to an intervention. Differentiation between sample groups measured with the ELISA is less reliable than with LC-MS because the CV is higher and the matrix dependence can cloud or mimic differences. Overestimation of ADMA concentrations also makes comparison data in the literature very difficult. Expenses for consumable items are clearly higher for the ELISA than for the LC-MS assay, a situation that is aggravated by the necessity of measuring Arg (3) separately because this important analyte is not measured by the ELISA, whereas in the LC-MS assay it can be measured in the same run as ADMA with no additional costs. On the other hand, higher sample throughput is possible with the ELISA.

Short-Term Urine Deoxypyridinoline Biological Variability in the First 5 Years after Menopause, Marco Di Stefano,1 Federica Formoso,1 Cristina Tamone,1 Giuseppe Aimo,2 Giulio Mengozzi,2* Simona Bergui,1 and Giovanni Carlo Isaia1 (1 Department of Internal Medicine, University of Turin, Turin, Italy; 2 Clinical Chemistry Laboratory, San Giovanni Battista Hospital of Turin, Turin, Italy; * address correspondence to this author at: Clinical Chemistry Laboratory, San Giovanni Battista Hospital of Turin, Corso Bramante, 88, 10126 Turin, Italy; fax 39-011-676052, e-mail gmengozzi@molinette.piemonte.it)

There is evidence that bone turnover in women is more rapid during the first years after menopause than in subsequent years. The assessment of deoxypyridinoline (DPD) cross-links in a fasting urine sample is considered a specific index of bone resorption by osteoclasts and also can be used for monitoring the response to pharmacologic antiresorption treatment. The interpretation of results,
however, is hampered by biological and other preanalytical variability (1–4).

Specific degradation products of the bone matrix, such as DPD and pyridinoline (PYD), closely reflect the rate of bone metabolism. Vesper et al. (1) reported mean within-day variabilities of 67% for DPD (range, 53%–75%) and 71% for PYD (57%–78%). The mean between-day variability was 16% for both PYD and DPD (ranges, 5%–24% and 12%–21% for DPD and PYD, respectively). The mean between-person variabilities across different studies were 34% for DPD (8%–98%) and 26% for PYD (12%–63%) in healthy premenopausal women and 40% (27%–54%) and 36% (22%–61%), respectively, in postmenopausal women. Specimen instability and errors in creatinine measurements were additional sources of variability (1). Some authors have reported that the variability can be reduced by collecting urine for 24 h (or longer) instead of using single voids and by expressing the results as ratios to creatinine (5, 6).

The usefulness of urinary markers of bone turnover in monitoring therapy depends on the within-person variability of these markers compared with their changes in response to treatment (7). Thus, the biological and laboratory variabilities of DPD cross-links are important considerations for clinical evaluation.

We evaluated the biological variability of DPD cross-link concentrations in fasting morning urine specimens collected during a 2-week period from women in their first years of menopause. We examined 64 postmenopausal women (mean age, 53 years; range, 49–57 years) between 1 and 5 years after menopause (mean time after menopause, 39 months; range, 11–58 months). Written informed consent was obtained from all participants in the study.

Each participant had a lumbar bone mineral density t-score (dual-energy X-ray absorptiometry technique) in the range of −1 to −2 SD and a body mass index between 21 and 30 kg/m². None of the participants smoked or was affected by diseases known to cause secondary osteoporosis, nor had they been treated previously with therapy acting on bone calcium and phosphorus metabolism. Before starting the study, we measured each woman’s serum total and ionized calcium, total alkaline phosphatase, and 25-hydroxyvitamin D concentrations.

During the 2-week follow-up period, we measured DPD in fasting first morning urine samples at baseline and thereafter repeated this measurement 3 times each day at the same times and under the same conditions at 7-day intervals. Intra- and interperson variability was reduced by collecting specimens at a specific time of the day, i.e., the first morning void before 1000 in the morning, to avoid any possible influence of diurnal variation and by maintaining similar patient status at each specimen collection, taking into account circumstances such as intake of medications and dietary supplements.

The DPD assay was performed with a solid-phase chemiluminescent enzyme-labeled immunoassay [Pyrilinks-D on a multianalyte automated analyzer (Immulite 2000; manufactured by DPC and purchased from Medical Systems SpA)]. Data are expressed as the ratio to creatinine concentration [modified Jaffe colorimetric method performed on a Hitachi 917 analyzer (Modular Analytics, Roche Diagnostics)]. The within- and between-run imprecision for DPD cross-link measurements at 25 nmol/L were 4.5% and 8.7%, respectively. The intra- and interseries variations in urine creatinine assay were 1.3% and 1.7%, respectively. An equilibrium RIA procedure was used to determine 25-hydroxyvitamin D concentrations after rapid extraction of this and other hydroxylated metabolites from serum with acetonitrile (DiaSorin Inc.). The reference interval given by the manufacturer is 9.0–37.6 μg/L with a detection limit of 2.5 μg/L.

Variations in DPD values around the means in all 3 series of measurements did not follow a gaussian distribution (P <0.2, Kolmogorov–Smirnov test); thus, logarithmic transformation was applied (8, 9). The within-subject biological variation (CVI), based on ANOVA, was calculated with the following formula:

\[ CV_I = \frac{100}{\text{M}} \left( S^2_{I + A} - S^2_{A} \right)^{1/2} \]

in which \( S^2_{I + A} \) is the experimental variation in the results of the 3 determinations for each patient; \( S^2_{A} \) is the analytical variation, including both DPD and creatinine imprecision; and M is the mean DPD concentration of the 3 values obtained for each patient studied.

The between-person biological variation (CVG) was calculated with the following formula:

\[ CV_G = \frac{100}{\text{M}} \left( S^2_I - S^2_{I + A} \right)^{1/2} \]

in which \( S^2_I \) is the total variation derived from all values from all patients.

The index of individuality (II) was calculated according to the formula:

\[ II = \frac{S^2_{I + A}}{S^2_G} \]

where

\[ S^2_G = S^2_I - S^2_{I + A} \]

For the dynamic assessment of DPD values, to define the difference between 2 consecutive results that may indicate a change in patient status, we calculated the least significant change (LSC) according to the formula (10):

\[ \text{LSC} = 1.96 \times \sqrt{2 \times \frac{\sqrt{CV^2_I + CV^2_A}}{N}} \]

where \( CV_I \) and \( CV_A \) represent the calculated within-subject and analytical variabilities, respectively.

Before we performed these calculations, we applied 3 levels of outlier tests to remove outlier points (9), and because an outlier point was identified, 1 patient was excluded from the subsequent analysis.

We calculated correlation coefficients with Spearman rank analysis. \( P <0.05 \) was considered statistically significant.
Results of the DPD variability analysis are summarized in Table 1. Serum total calcium, ionized calcium, and total alkaline phosphatase were within the reference intervals in all patients (data not reported); interestingly, 25-hydroxyvitamin D concentrations were <12 µg/L in 12 individuals (18.7%) and <15 µg/L in 23 individuals (35.9%). CVs from the 3 daily determinations of DPD cross-links were 1.6%–84%, with a median of 18%. We observed a statistically significant correlation between the means of the 3 determinations and CVs for each individual (rS = 0.44; P < 0.01). 25-Hydroxyvitamin D concentrations inversely correlated (P < 0.05) with the mean DPD cross-links concentrations (rS = −0.34). After we divided the study population into 2 subgroups on the basis of 25-hydroxyvitamin D status, the correlation remained statistically significant (r = −0.36; P < 0.05) only in the group with concentrations >15 µg/L (n = 41). On the other hand, vitamin D data did not correlate with the CVs for DPD cross-links.

The LSC, calculated from the within-person variability and the analytical imprecision, for DPD results was 70.3%. It is well known that vitamin D deficiency is common in older individuals (11). Our data suggest that in the early postmenopausal years it is not unusual to observe 25-hydroxyvitamin D concentrations below the threshold of 15 µg/L in osteopenic patients. Although inversely correlated to DPD, 25-hydroxyvitamin D concentrations did not appear to affect DPD excretion variability in our study population.

Standardization of urine sampling allowed us to assess variability in DPD excretion in a cohort of women. We found a high biological variability in early postmenopausal women. It is known that the first years of the postmenopausal period are characterized by rapid bone turnover in most cases, and the biological variability of DPD excretion increases with the rate of bone turnover. Increased biological DPD cross-link variability seems to be correlated with bone turnover and represents an important clinical measurement to include in the evaluation of laboratory results.

The index of individuality has been used as a measure of the likely utility of population-based reference intervals (12), with the reference intervals being more useful if the ratio is >1.4. Our current results show that population-based reference values may be of more limited use for the correct interpretation of DPD concentrations because women in the first 5 years of the postmenopausal period may have values that are unusual for them but still lie within the population-based reference limits.

For biochemical markers of bone turnover, there are no uniform criteria establishing how large a difference between 2 consecutive measurements indicates progression of the disease. Individual responses can be interpreted only in relation to the within-person variability of the marker. In our study, the estimate of within-person variability over a short time did not reflect current clinical practice. In a routine clinical setting in which conditions are not strictly controlled, as in regular patient monitoring, it is likely that the variability will be greater because samples are not collected at the same time of day at all visits. Therefore, the LSC values described here probably underestimate the values that would be found in clinical practice.

Monitoring of bone turnover through DPD assessment in the first 5 years of menopause should take into account variation in the marker concentrations above calculated thresholds of indexes such as the LSC. Our data suggest that points of reference for detection or exclusion of disease progression be established specifically for other bone markers. Relatively large changes between values from sequential samplings should be expected for those analytes such as DPD that display high intraindividual variation during the early menopausal period.

### Table 1. Variability for DPD in 63 women in the first 5 years after menopause (after exclusion of 1 woman because of outlier results).

<table>
<thead>
<tr>
<th>DPD, nmol/mmol creatinine</th>
<th>Baseline Mean (SD)</th>
<th>95% confidence interval</th>
<th>1 week Mean (SD)</th>
<th>95% confidence interval</th>
<th>2 weeks Mean (SD)</th>
<th>95% confidence interval</th>
<th>CVa,%</th>
<th>CVb,%</th>
<th>CVc,%</th>
<th>Index of individuality</th>
<th>LSC, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8.2 (2.7)</td>
<td>7.6–8.9</td>
<td>9.3 (3.6)</td>
<td>8.4–10.2</td>
<td>8.9 (3.6)</td>
<td>8.0–9.8</td>
<td>11</td>
<td>20</td>
<td>28</td>
<td>1.09</td>
<td>70.3</td>
</tr>
</tbody>
</table>

a Includes imprecision for both DPD and creatinine.

References
Despite the increasing number of clinical applications of circulating Epstein–Barr virus (EBV) DNA analysis for the detection (1, 2), monitoring (3, 4), and prognostication (5, 6) of nasopharyngeal carcinoma (NPC), several fundamental questions concerning plasma EBV DNA, including its tissue origin and relationship with tumor mass, remain unanswered.

In the first part of this study, we investigated the relative contributions of tumor cells and other latently EBV-infected lymphoid tissues to the pool of circulating EBV DNA. Previous studies have shown that different EBV genotypes are harbored by latently EBV-infected lymphoid tissues and blood cells (7–10) in healthy persons and patients with malignancies. On the other hand, it is well established that the EBV is monoclonal in NPC tumor tissues and other EBV-associated cancers (11–14). Therefore, investigation of the genotype of plasma EBV DNA should reveal the relative contributions of tumor cells and other latently EBV-infected lymphoid tissues to the pool of circulating EBV DNA in NPC patients.

For this study, 25 patients with newly diagnosed NPC, 15 patients with systemic lupus erythematosus, and 13 renal transplant recipients were recruited and gave informed consent. This study was approved by the ethics committee of the Prince of Wales Hospital, Hong Kong. Plasma samples were collected from all patients, and tumor specimens for microdissection were obtained from 14 of the 25 NPC patients. DNA extracted from the plasma samples and the microdissected tumor tissues was used for the amplification of EBV DNA, targeting a region encoding the carboxy terminus of latent membrane protein (LMP-1), using the primers 5'-ATGGT-AATGCCTAGAAGTAAGAAGG-3' (LMP1f) and 5'-CATAGCCCTAGCGACTGCTG-5' (R2h). The PCR products were ligated to the pGEM®-T Easy Vectors (Promega), and the ligation products were transformed into competent Escherichia coli cells. Twelve bacterial clones were picked and sequenced with a BigDye® Terminator (Ver. 1.1) Cycle Sequencing Kit (Applied Biosystems). The sequences were aligned by the SeqScape® (Ver. 2.0) software (Applied Biosystems).

EBV DNA encoding the LMP-1 was successfully amplified from the plasma of all 25 NPC patients and 8 patients receiving immunosuppressive therapies, including 3 patients with systemic lupus erythematosus and 5 renal transplant recipients. The EBV genotypes detected in the plasma of the NPC patients and the immunosuppressed patients are shown in Table 1 (also see Table 1 in the Data Supplement accompanying the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue11). The sequences are referenced to the sequence of EBV strain B95-8 (accession no. V01555). Only the differences between the patients’ sequences and the B95-8 sequence are listed in Table 1. A locally predominant sequence was detected in the plasma of 15 NPC patients (patients N1 to N15) and 6 immunosuppressed patients (patients S1, S2, and R1 to R4). This locally predominant sequence is characterized by a 30-bp deletion (15) compared with the B95-8 sequence. Only 1 EBV genotype was detected in the plasma of 22 (88%) of the 25 NPC patients. In the remaining 3 NPC patients (12%; patients N13 to N15), 2 EBV genotypes were detected in the plasma. The nucleotide sequences of the minor and major genotypes differ by a single nucleotide substitution in each of the 3 patients. The minor genotype represents 11%–22% of the total number of clones in these 3 patients. With regard to the representation of the number of clones to the actual composition of plasma EBV DNA, Walling et al. (8) have shown that the ratios of the resulting clone sequences in an EBV genotyping assay would closely resemble the original sequence ratios before PCR. With regard to the tissue origin of plasma EBV DNA, we have shown that in each of the 14 patients with available tumor tissues, the LMP-1 nucleotide sequence of the predominant genotype was identical to the sequence of the tumor. In 8 of these 14 patients (N18 to N25), the EBV genotypes showed characteristic nucleotide changes that distinguished them from the locally predominant genotype. Our findings suggest that most of the circulating EBV DNA molecules in NPC patients are tumor derived.

In contrast, only samples from 3 (37.5%) of the 8 patients receiving immunosuppressive therapies (patients S1, R1, and R2) showed a single EBV genotype. Samples from 4 of the 8 patients (patients S2, S3, R3, and R5; 50%) showed 2 EBV genotypes, and the samples from 1 patient (R4; 12.5%) showed 3 EBV genotypes. In these 5 patients, the minor and the major genotypes differed by substitutions of 1 to 2 nucleotides in the nucleotide sequence. For the 4 patients showing 2 EBV genotypes, the minor genotype comprised 29%–40% of the total number of clones. For the patient showing 3 EBV genotypes (R14), there were 2 codominant EBV genotypes, and each of

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