Urinary Osteocalcin as a Marker of Bone Metabolism

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Background: Osteocalcin (OC) is produced by osteoblasts during bone formation, and circulating OC has been used in clinical investigations as a marker of bone metabolism. OC is excreted into urine by glomerular filtration and can be found in urine as midmolecule fragments.

Methods: We developed and evaluated three immunoassays (U-MidOC, U-LongOC, and U-TotalOC) for the detection of various molecular forms of urinary OC (U-OC). We evaluated the association of U-OC with other markers of bone turnover and with bone mass in 1044 elderly women. In addition, we studied seasonal and circadian variation of U-OC.

Results: U-OC correlated with other bone turnover markers [Spearman correlation (r), 0.30–0.57; P < 0.0001], demonstrating the association between U-OC and skeletal metabolism. There was a significant association between bone metabolism assessed by U-OC quartiles and bone mass assessed by total body bone mineral content (P < 0.0001). The seasonal effects appeared to be rather small, but we observed a significant circadian rhythm similar to the one reported for serum OC with high values in the morning and low values in the afternoon.

Conclusions: The three immunoassays had unique specificities toward different naturally occurring U-OC fragments. U-OC concentrations measured with any of these assays correlated with bone turnover rates assessed by conventional serum markers of bone metabolism. The measurement of OC in urine samples could be used as an index of bone turnover in monitoring bone metabolism.

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Bone turnover markers provide dynamic and rapid measures of total body skeletal metabolism and may be clinically useful tools, e.g., in monitoring osteoporosis therapy. At least some markers appear promising for determinations of fracture risk, rate of bone loss, or disease severity [for reviews, see Refs. (1–3)]. Osteocalcin (OC) is a small, bone-specific protein (Mₚ ~ 5800) produced by osteoblastic cells during bone formation (4–6). A fraction of OC enters the blood, where it can be detected, and circulating OC has been widely measured to assess bone turnover (7, 8). In addition to the newly synthesized OC derived from osteoblasts, the circulating OC pool probably also includes molecules derived from the resorption process when OC embedded in the bone matrix is released (9, 10).

The main route of circulating OC catabolism is renal filtration and degradation (11), and immunoreactive OC is present in urine (12). Urinary OC (U-OC) is a heterogeneous pool of various OC fragments (13–15) reflecting potentially diverse degradation cascades and consist mainly of the middle portion of the molecule truncated at both the amino and carboxy termini. Intact, unfragmented OC has not been found in urine.

The introduction of serum OC (S-OC) assays into routine clinical practice has been limited by the presence of multiple forms of OC in the circulation and differences among assays in the ability to detect these forms (16). It is unclear whether OC fragments are generated by proteolysis of intact OC in blood, are formed during biosynthesis, or whether some fragments are derived directly from bone resorption. OC molecules of resorptive origin would be of clinical interest, but the circulating resorptive forms may be masked in serum samples by de novo-synthesized OC.
intact OC, which is inevitably recognized by most immunoassays in addition to the shorter fragments. Circulating intact OC and large fragments are degraded in circulation or peripheral organs (11), whereas smaller OC fragments might be more resistant to degradation and accumulate in urine. Urine could therefore potentially offer a better source for OC fragments of resorptive origin. In addition, urine samples can be collected easily, and the problems related to the instability of S-OC after sampling (17) are likely to be less severe for U-OC, which is presumably an end-product of fragmentation.

U-OC is attractive as a new candidate marker of bone turnover despite such limitations as the gradual age-related renal impairment and the tendency to show higher preanalytical variability than serum assays (18). The latter could be related to the use of creatinine for normalizing urinary marker values, which contributes a second source of variability (19). Urine as a sample material is, however, intriguing because it traditionally has been used for measurement of resorption markers and not markers of osteoblastic origin.

At the moment, there are no commercial methods available for the measurement of U-OC. Most assays are probably unable to detect the small fragments present in urine, and the concentrations in urine are low. RIAs for U-OC have used a polyclonal guinea pig antibody against OC purified from human bone (20) or a polyclonal chicken antibody against a synthetic midmolecule peptide sequence (residues 12–36) (14), and two-site immunofluorometric assays (13) use murine monoclonal antibodies (Mabs) for various epitopes of human OC (21) in two different two-site combinations. Limited data are available on the analytical performance of U-OC assays and the possible confounding factors that might have an influence on the measured U-OC, such as biological variation and stability of immunoreactivity after sampling.

In this report we describe the development and characterization of three immunoassays for the measurement of OC fragments in human urine and provide data on preanalytical variability.

Materials and Methods

Mabs and calibration

Mabs 6F9, 2H9, and 3H8 have been described in detail previously (21). Mab 6F9 binds to residues 7–19 of OC, and Mabs 2H9 and 3H8 recognize OC fragment 20–43. Mab 3H8 prefers γ-carboxyglutamic acid (Gla)-containing forms of OC. The antibodies were biotinylated with a 50-fold molar excess of biotin–isothiocyanate (bio-Mabs) and labeled with a 200-fold molar excess of europium(III) chelate (Eu-Mabs) as described previously (21). A synthetic peptide of hOC amino acids 1–43 (Gla at positions 17, 21, and 24; Advanced Chemtech) was used to generate two sandwich assays, bio-6F9/Eu-3H8 (U-MidOC) and bio-2H9/Eu-6F9 (U-LongOC), and one competitive assay, bio-hOC/Eu-3H8 (U-TotalOC). A synthetic peptide of hOC amino acids 1–43 (Gla at positions 17, 21, and 24; Advanced Chemtech) was used as a calibrator. The calibrator was diluted in 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 g/L NaN3 (pH 7.75) buffer containing 10 g/L bovine serum albumin and stored in aliquots at −70°C.

Immunoassay procedures for urine OC

For the U-MidOC and U-LongOC assays, 400 ng of bio-6F9 (U-MidOC) or bio-2H9 (U-LongOC) in 50 μL of Delfia Assay Buffer was added to the wells of streptavidin-coated microtiter plates, incubated with continuous shaking at room temperature (22°C) for 30 min, and washed twice with Delfia Wash Solution. The samples or calibrators (10 μL) were added, followed by 100 ng of Eu3+-labeled 3H8 (U-MidOC) or Eu3+-labeled 2H9 (U-LongOC) in 50 μL of Assay Buffer containing 5 mmol/L EDTA. After 2 h of shaking at room temperature (22°C), the plates were washed six times, and 200 μL of Delfia Enhancement Solution was added. Before time-resolved fluorescence measurement in a Victor2 Multilabel Counter, the plates were shaken for 30 min. Streptavidin-coated plates were purchased from Innovac Diagnostics, and Delfia Assay Buffer, Delfia Wash Solution, Delfia Enhancement Solution, and the Victor2 Counter were from Perkin-Elmer Life Sciences/Wallac.

For the U-TotalOC assay, a competitive assay design was used. Streptavidin-coated microtiter plates were pre-washed once and coated with bio-hOC (1.7 ng/well) in 50 μL of Assay Buffer for 30 min with continuous shaking. The plates were washed four times, and samples or calibrators (30 μL) were added together with 2 ng of Eu3+-labeled 3H8 in 100 μL of Assay Buffer. The plates were incubated for 1 h, after which the wells were washed four times and 200 μL of Delfia Enhancement Solution was added. After incubation for 30 min with shaking, the fluorescence was measured by time-resolved detection.

Calibrators and samples were analyzed in duplicate in all assays.

Assay evaluation

We determined assay imprecision with control samples prepared by diluting a urine sample (obtained as the first morning void from an 8-year-old boy) in zero calibrator in two concentrations, low and high (respective OC concentrations, 9.1 and 44.7 μg/L in the U-MidOC assay, 0.08 and 0.55 μg/L in the U-LongOC assay, and 125 and 380 μg/L in the U-TotalOC assay). Within-run imprecision was defined as the mean CV of all the measurements (duplicates) performed for the cohort of elderly women (n = 1019; details below). We determined total imprecision by calculating the variation for control samples from 15 separate assays (25 for the
We evaluated dilution linearity by diluting three urine samples into zero calibrator in two-, four-, and eightfold dilutions and calculated the linearity for each dilution as a percentage of the value obtained from the diluted sample compared with the value obtained from undiluted sample. We evaluated the recovery of added analyte by adding a known amount of calibrator peptide to three individual urine samples containing a known amount of OC and calculating the result as the percentage of the added analyte recovered. The exact amounts of added analyte were 79 and 19 μg/L for U-MidOC assay, 10.5 and 2.4 μg/L for the U-LongOC assay, and 219 and 75 μg/L for the U-TotalOC assay.

We determined the ability of each assay to recognize different molecular forms of OC present in human urine by use of OC fragments isolated from a urine sample. The sample was collected as the first morning void from a 11-year-old boy and was stored at −70 °C. The total urine OC pool was isolated and fractionated as described previously (15). Briefly, the urine sample was extracted with a C_{18} solid-phase column followed by immunoaffinity chromatography involving Mabs 6F9, 2H9, and 3H8 (all in one column). Different molecular forms were separated by fractionation on a C_{4} HPLC column, and collected fractions were analyzed for OC with all three assays. To determine the molecular masses and sequences of isolated fragments, we analyzed fractions containing OC by matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-MS) and N-terminal sequencing as described previously (15).

**IN VITRO STABILITY AND CIRCADIAN AND SEASONAL VARIATIONS**

Fresh first morning void urine was collected from three healthy females and stored at 4 °C until delivered to the laboratory within 2 h after collection. Samples were stored in aliquots either at 4 °C or room temperature (22 °C) for 0 h, 2 h, 4 h, 8 h, 1 day, 3 days, or 5 days. After incubation, samples were frozen and stored at −70 °C until assayed simultaneously.

To study circadian variation, we had nine healthy young females (median age, 24.5 years; interquartile range, 24–26 years) following their typical daily routine collect all urine voids during 1 day at 0600 (first morning void), 0900, 1200, 1500, 1800, 2100, and 2400, and the following morning at 0600. First morning voids were first stored at 4 °C and frozen at −70 °C within 2 h. The samples collected during the daytime were immediately stored at −70 °C, and samples collected in the evening were temporarily stored at −20 °C and then at −70 °C the following day.

We evaluated seasonal variations using samples from the cohort of elderly women described below. Urine samples were collected throughout all seasons and stored at −80 °C. The number of samples collected each month from January to December was 96, 118, 111, 127, 83, 53, 32, 50, 88, 104, 112, and 69, respectively.

**COHORT OF ELDERLY WOMEN**

We randomly selected 1044 women, all 75-years-old, from the population files of the city of Malmö, Sweden, during 1995–1999. An invitation to participate was sent to 1604 women the week after their 75th birthday. One hundred and fifty-two women did not participate because of illness, 376 women declined to enter the study, and 32 women were not reached despite several attempts; no exclusion criteria were used. Informed consent was obtained for all women, and all parts of the study were approved by the local ethics committee (22). Urine samples were obtained as the first morning void and stored at −80 °C. Nonfasting serum samples were obtained between 0800 and 1300 and stored at −80 °C. Urine was available for 1019 women and serum for 1024 women. Urine samples were analyzed for three U-OC fragments and urine creatinine and serum samples for two markers of bone formation and two markers of bone resorption (details of the measurements are given in the following sections). Bone mineral content and areal bone mineral densities (aBMDs) of the hip, lumbar spine, and total body were assessed together with serum and urine sampling; results from the hip (femoral neck) were obtained for 951 women, from the lumbar spine for 974 women, and from the total body for 931 women.

**OTHER MEASUREMENTS**

Serum bone-specific alkaline phosphatase (S-BoneALP) was measured by the Metra BAP immunoassay (Quidel Corporation). Serum intact and N-mid-OC (S-TotalOC) was measured by an in-house immunoassay (23). Briefly, S-TotalOC is a one-step sandwich assay based on Mabs bio-2H9 and Eu-6F9 and synthetic OC peptide 1–49 as a calibrator with within- and between-run CVs <5% and <8%, respectively. Serum C-terminal cross-linked telopeptides of type I collagen (S-CTX) was measured by the Elecsys β-CrossLaps immunoassay (Roche Diagnostics). Serum tartrate-resistant acid phosphatase 5b (S-TRACP5b) was measured by a solid-phase, immunofixed, enzyme activity assay (within-run CV, 1.8%; between-run CV, 2.2%) as described previously (24). Urine creatinine was measured in accordance with the Jaffe reaction. All measurements of each individual assay were made within the same laboratory. Bone mineral content (BMC) and aBMD of the femoral neck, lumbar spine (L2–L4), and total body were assessed by a dual-energy x-ray absorptiometry technique (Lunar® DPX-L).
**Statistical Analysis**

All results from U-OC measurements were corrected for urine creatinine before analyses and are presented as the median (interquartile range) unless otherwise stated. The Shapiro–Wilk test for gaussian distribution and the Levene test for homogeneity of variance were applied for all markers, and because of nongaussian distributions, statistical calculations were done only after logarithmic transformation or with a nonparametric test if logarithmic transformation did not give a gaussian distribution. Comparisons between the groups were made with one-way ANOVA with Tukey or Dunnett post hoc adjustment in the case of gaussian distribution and with the nonparametric Wilcoxon test followed by Bonferroni adjustment when appropriate if distributions were not gaussian.

Nonparametric Spearman correlation was used for correlation analysis. The Statistical Analysis System Enterprise Guide 2 program (SAS 8.2; SAS Institute) was used in all analyses, and \( P < 0.05 \) was considered statistically significant.

**Results**

**Assay Characteristics**

Dose–response curves for the U-MidOC and U-LongOC assays (Fig. 1A) and the U-TotalOC assay (Fig. 1B) are shown as the means of eight individual calibration curves, and the concentrations are shown as equivalents of the calibrator fragment 1–43 (\( \mu \text{g/L} \)). For the U-MidOC assay, the calibration series covered the range from 0.3 to 90 \( \mu \text{g/L} \). Because immunoreactivities observed in urine with the U-LongOC assay were lower, the calibration curve for that assay covered the range from 0.04 to 30 \( \mu \text{g/L} \). The competitive U-TotalOC assay was less sensitive, with a measurement range of 9–840 \( \mu \text{g/L} \). This was, however, sufficient to measure OC concentrations in the majority of clinical urine samples. In the cohort of elderly women, only 2.3% of samples (25 of 1019) had U-TotalOC results below the lowest calibrator, and the majority of samples (857 of 1019) had U-TotalOC results <300 \( \mu \text{g/L} \) (without creatinine correction). The within-run variations calculated for the low and high controls, respectively, were 2.7% and 1.7% for the U-MidOC assay, 9.1% and 6.2% for the U-LongOC assay, and 9.7% and 18% for the U-TotalOC assay. The result was similar when the variation was calculated from the cohort of elderly women, in which the mean CVs for all measurements were 1.7% for the U-MidOC assay, 4.3% for the U-LongOC assay, and 14% for the U-TotalOC assay. The total variation for low and high controls, respectively, was 12% and 6.1% in the U-MidOC assay, 14% and 11% in the U-LongOC assay, and 27% and 23% in the U-TotalOC assay.

The detection limits were 0.17 \( \mu \text{g/L} \) (U-MidOC), 0.03 \( \mu \text{g/L} \) (U-LongOC), and 28 \( \mu \text{g/L} \) (U-TotalOC). Assay linearity and analytical recovery are summarized in Table 1.

The assays varied in their ability to recognize different naturally occurring molecular forms of U-OC. Prepubertal urine contained two major pools of immunoreactive OC fragments eluting at distinct times during HPLC fractionation (peak I and a twin peak II/III; Fig. 2A). According to MALDI analysis, the first pool (peak I) consisted of OC fragments starting predominantly from residue Asp\(^{14}\). The monoisotopic mass of the most predominant ion produced by peak I was 2126.93 (\([M+H]^+\); Fig. 2B), which corresponds to the calculated monoisotopic molecular mass of the human OC fragment containing residues 14–31 (theoretical mass, 2126.89 for \([M+H]^+\)). In contrast, the double peak II/III contained fragments with more extended amino termini starting from residue Gly\(^7\) or Pro\(^9\). The predominant ion produced by peak II was 2808.37 (\([M+H]^+\); Fig. 2B), which corresponds to the calculated molecular mass of the human OC fragment containing residues 7–31 (theoretical mass, 2808.24 \([M+H]^+\)). Theoretical molecular masses were calculated without Gla modification because \(\gamma\)-carboxylation is destroyed in MALDI-MS and is not included in the molecular masses of observed ions (25). The identities of

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**Fig. 1.** Dose–response curve for the two-site assays, U-MidOC (squares) and U-LongOC (triangles; A) and the competitive assay, U-TotalOC (B). Open symbols represent CVs calculated from eight consecutive assays, each one performed with duplicate measurements. In A, both scales are logarithmic. In B, the response is shown as relative fluorescence compared with the zero calibrator (value of 1) on a linear scale and the calibrator amount on a logarithmic scale (the curve on a linear scale is shown in the inset).
the fragments were confirmed by N-terminal sequencing. The U-TotalOC assay detected U-OC fragments present in all three peaks, reflecting the total concentration of immunoreactive OC in urine. The two-site assay U-MidOC recognized only OC fragments present in peaks II and III and failed to detect the more truncated fragments present in peak I. The two-site assay U-LongOC detected only the longest fragments present in peak III, but not as effectively as U-MidOC.

**Biological and Preanalytical Variability**

We evaluated seasonal variation was by comparing results obtained from different individuals sampled in different months throughout the year. The number of individuals in each month was different, being smallest for July (n = 32) and largest for April (n = 127). There was no statistically significant trend for U-OC values with regard to sampling month when U-OC was measured with either the U-LongOC (P = 0.25, nonparametric Wilcoxon test) or U-TotalOC (P = 0.93; Fig. 3B) assay. For the U-MidOC values, there was a minor trend (P = 0.02) for greater values in autumn compared with the rest of the year (Fig. 3A). We observed a circadian rhythm for U-OC with all three assays (Fig. 3, C and D). U-OC values were highest during the night and early morning and decreased in the afternoon. This was followed by an increase later in the evening and night.

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**Table 1. Linearity of dilution and recovery of added analyte.**

<table>
<thead>
<tr>
<th>Dilution linearity, % of undiluted value</th>
<th>Recovery, %</th>
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<tbody>
<tr>
<td></td>
<td>U-MidOC</td>
</tr>
<tr>
<td></td>
<td>U-MidOC</td>
</tr>
<tr>
<td>Undiluted</td>
<td>100</td>
</tr>
<tr>
<td>1:2</td>
<td>110.0 (2.2)</td>
</tr>
<tr>
<td>1:4</td>
<td>103.9 (2.9)</td>
</tr>
<tr>
<td>1:8</td>
<td>93.2 (10.1)</td>
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</tbody>
</table>

* Results are calculated for each dilution as the percentage of the value obtained from the diluted sample to the value obtained from the undiluted sample [mean (SD); n = 3].

* Recovery results are shown as a percentage of the measured concentration compared with the calculated concentration for a known amount of added analyte [mean (SD); n = 3].

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**Fig. 2. U-OC assays have distinct specificities for different U-OC fragments.**

(A), three pools of fragments (I, II, and III) obtained by HPLC fractionation of U-OC were differentially recognized by the U-TotalOC (I + II + III; □), U-MidOC (II + III; ■), and U-LongOC (III; ▲) assays. The fragments identified in particular pools are listed beside the peaks and the numbers refer to the N- and C-terminal residues of fragments (i.e., 1–49 refers to intact OC). (B), representative reflector mode MALDI-MS spectra for HPLC peaks I and II. The mass values refer to the monoisotopic forms, and the insets display isotopic patterns of selected ions. An ion of 2127 Da corresponding to OC fragment 14–31 was detected in peak I, and the most prominent ion produced by peak II was at 2808 Da, corresponding to OC fragment 7–31.
The results of stability studies are shown in Table 2. The immunoreactivity detected by the two-site assays U-LongOC and U-MidOC did not differ from the initial value when urine samples were stored at either 4 °C or 22 °C for up to 5 days ($P = 0.19–0.99$, one-way ANOVA with Dunnett test). Concentrations measured by the U-TotalOC assay appeared to be smaller after incubation at either 4 or 22 °C compared with the initial values. The decrease in immunoreactivity was more pronounced at 4 °C and for the longest timepoints, i.e., 3 and 5 days.

Fig. 3. Seasonal (A and B) and circadian (C and D) variation of U-MidOC (A and C) and U-TotalOC (B and D).

There was a minor trend in U-MidOC, with values being higher at the end of the year (A), whereas there were no changes in U-TotalOC (B) and U-LongOC (data not shown) values. Individual samples are shown with crosses, and median values in each month are shown with circles connected by a line. The number of individuals in each month from January to December was 96, 118, 111, 127, 83, 53, 32, 50, 88, 104, 112, and 69. Circadian rhythms were observed in U-MidOC (C) and U-TotalOC (D; nine individuals; lines). ■ indicates the mean, and error bars indicate the SE. The profile for U-LongOC was similar to that for U-MidOC (data not shown). The highest value for U-MidOC was observed in the first morning void at 0600 (set as 100%) and the lowest at 1500 and 1800 (62% and 63% of the 0600 value). The highest value for U-Total was observed at 0900 (113% of the first morning void at 0600) and the lowest at 1800 (62% of 0600 value). crea, creatinine.

| Table 2. Stability of U-OC in samples stored at 4 and 22 °C.\textsuperscript{a} |
|---|---|---|
| 4 °C | U-MidOC | U-LongOC | U-TotalOC |
| 2 h | 102.2 (1.6) | 102.9 (4.8) | 95.9 (2.4) |
| 4 h | 102.3 (5.3) | 97.0 (2.9) | 89.7 (3.5)\textsuperscript{a} |
| 8 h | 102.7 (7.2) | 102.8 (10.3) | 98.5 (1.3) |
| 24 h | 102.0 (1.8) | 102.7 (2.9) | 90.6 (3.2)\textsuperscript{a} |
| 3 days | 97.7 (4.9) | 101.2 (10.0) | 81.7 (3.5)\textsuperscript{a} |
| 5 days | 97.4 (0.3) | 93.9 (10.5) | 78.1 (6.0)\textsuperscript{a} |

<table>
<thead>
<tr>
<th>22 °C</th>
<th>U-MidOC</th>
<th>U-LongOC</th>
<th>U-TotalOC</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 h</td>
<td>104.5 (2.8)</td>
<td>118.9 (38.7)</td>
<td>101.7 (2.8)</td>
</tr>
<tr>
<td>4 h</td>
<td>97.9 (4.2)</td>
<td>102.4 (17.9)</td>
<td>96.2 (5.2)</td>
</tr>
<tr>
<td>8 h</td>
<td>106.6 (3.7)</td>
<td>107.5 (22.8)</td>
<td>91.8 (7.5)</td>
</tr>
<tr>
<td>24 h</td>
<td>101.5 (2.5)</td>
<td>105.7 (8.8)</td>
<td>88.4 (9.1)</td>
</tr>
<tr>
<td>3 days</td>
<td>102.5 (5.4)</td>
<td>100.0 (11.2)</td>
<td>83.6 (9.5)</td>
</tr>
<tr>
<td>5 days</td>
<td>93.6 (5.4)</td>
<td>84.9 (11.0)</td>
<td>79.5 (7.7)\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Statistically different from the initial value ($P < 0.05$, one-way ANOVA with Dunnett test).
(P = 0.0001 and <0.0001, respectively, one-way ANOVA with Dunnett test). In addition, results obtained already after 4 h (P = 0.02) and 1 day (P = 0.04) were different from the initial value. We also observed a similar trend in samples incubated at 22 °C, but it was less pronounced, presumably because of higher variation, and we did not observe a statistically significant difference compared with the initial value until after 5 days (P = 0.01).

ASSOCIATION WITH BONE MASS

U-OC concentrations measured with the U-MidOC, U-LongOC, or U-TotalOC assays had a statistically significant negative correlation with BMC measured at the total body, spine, or hip (P <0.0001 for all associations). Women were further classified into four groups according to U-OC values, 1 being the lowest quartile of U-OC and 4 the highest, and the total body BMC in each quartile group is shown in Fig. 4A. There was a significant negative association between BMC and U-OC quartiles (P <0.0001 for all assays, Wilcoxon nonparametric test). BMC values were lower in the high turnover quartiles, and women in the highest quartile for U-OC (high bone turnover) had significantly lower BMC values than women within the lowest U-OC quartile (P <0.001 for all assays, Wilcoxon nonparametric test with Bonferroni adjustment). There was also a significant negative association between t-scores for femoral neck BMD and U-OC (Fig. 4B). In addition, the percentage of individuals with a t-score below −2.5 was greater (>39–42%) in quartile 4 than in quartile 1 (>22–26%; P <0.001). The results were similar whether U-OC was measured with the U-MidOC, U-LongOC, or U-TotalOC assay.

CORRELATION WITH BONE TurnOVER RATE

U-OC correlated significantly (P <0.0001) with bone turnover rate assessed by the serum markers of bone turnover used in this study (Table 3). The outcome of correlation analysis with the U-MidOC and U-LongOC assays was nearly identical. U-MidOC and U-LongOC values were strongly correlated with the bone resorption marker S-CTX, and the r value for U-OC and S-CTX was 0.51 (95% confidence interval, 0.46–0.56) for both U-OC assays. U-MidOC and U-LongOC results also were strongly correlated with S-TRACP5b [r = 0.44 (0.39–0.49) and 0.41 (0.41–0.46), respectively]. Interestingly, the correlation of U-MidOC and U-LongOC with the bone formation marker S-BoneALP appeared to be lower [r = 0.38 (0.32–0.43) and 0.36 (0.30–0.41), respectively], than the correlation with resorption markers. Correlation coefficients for U-TotalOC and bone markers were slightly smaller than for the two other U-OC assays. The r value for U-TotalOC and S-CTX was 0.38 (0.33–0.43), and that for U-TotalOC and S-TRACP5b was also 0.38 (0.33–0.43). The correlation coefficient for U-TotalOC and S-BoneALP was only marginally smaller, 0.30 (0.24–0.36).

We also evaluated the correlation between serum and U-OC. U-MidOC and U-LongOC values were strongly correlated with S-OC [r = 0.57 (0.52–0.61) and 0.55 (0.51–0.59), respectively]. The correlation between U-TotalOC and S-OC [r = 0.30 (0.23–0.35)] was lower and equal to the correlation for U-TotalOC and S-BoneALP [r = 0.30 (0.24–0.36)].

In addition to the large cohort of elderly women, U-OC was also evaluated in a smaller cohort of pre- and postmenopausal women (n = 91), and U-OC detected with any of these assays was associated with bone turnover rate and was able to distinguish between postmeno-
pausal women receiving or not receiving hormone replacement therapy (See the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol51/issue3/).

Discussion

We have developed three novel immunoassays for the detection of different molecular forms of U-OC and demonstrated that U-OC is a valid turnover marker for the assessment of bone metabolism. All U-OC assays were carefully characterized, the exact molecular structure of measured OC was verified, and some potential confounders were investigated to clarify the biology and clinical significance of U-OC.

Immunoreactive U-OC can be classified into at least two main categories according to the size of fragments, the longer fragments starting from residues Gly7 or Pro9, and the shorter ones having residue Asp14 or Leu16 at the NH2 terminus (15). We developed assays that detected longer and shorter fragments (U-TotalOC) or longer fragments exclusively (U-MidOC and U-LongOC), and U-OC measured with all three assays was able to provide insight into bone metabolism in the cohort of elderly women. In this cohort, U-OC correlated significantly with other markers of bone turnover, namely, S-CTX, S-TRACP5b, S-BoneALP, and S-TotalOC, emphasizing the connection between U-OC and bone turnover rate. However, although we observed a significant correlation between U-OC and conventional serum markers of bone turnover, the correlation between different markers generally appeared to be rather modest [Spearman correlation (r), 0.30–0.57 in this cohort], which should also be taken into account when indexes to assess the balance between resorption and formation are considered (1, 26). The distinct significant association between total body BMC and bone metabolism assessed by U-OC quartiles further demonstrates the connection between U-OC and skeletal metabolism. In addition to the association with BMC, we also evaluated the association of U-OC with aBMD assessed at the femoral neck. Femoral neck BMD as assessed by dual-energy x-ray absorptiometry is currently the most widely used marker to assess bone and the key diagnostic tool in osteoporosis (27, 28). It should be recognized, however, that the assessment of regional BMD in a particular skeletal site reflects only events taking place at that site and thus only in a fraction of the skeleton; it also provides information only in two dimensions. Bone turnover markers reflect the total body skeletal metabolism; thus, it may not be physiologically correct to try to relate biochemical markers such as U-OC to BMD. A topic for further studies would be the combination of short-term monitoring of U-OC, or turnover in general, with long-term follow-up of changes in BMC or BMD to understand the correlation between bone markers and bone mass.

The performance of all three U-OC assays was highly similar in the tested groups, and we found no clear evidence for qualitative differences with regard to U-OC fragments in different clinical samples. In particular, the discriminatory power in the quartile analysis and the correlation with bone turnover markers were nearly identical for U-MidOC and U-LongOC (which actually detects a small subset of fragments detected by U-MidOC). The U-OC assays were simple to perform, and the detection limits and dynamic ranges were adequate for measurement of urine samples, with the exception of a few too dilute urine samples, which were excluded from the evaluations. The variations in the competitive U-TotalOC assay were greater than for the two-site assays, and the total variation for U-TotalOC was particularly high (>20%). This was observed primarily for the control samples, and the calibration curve for the U-TotalOC assay constructed with synthetic OC was highly reproducible with overlapping calibration curves and within-assay CVs <10%. The high total variation may partly be attributable to the control samples, which were prepared by diluting urine containing very high concentration of U-OC and collected from a prepubertal boy. The molecular fragments generated in this setting and their relative

| Table 3. Correlation coefficients for U-OC* and other bone markers. |
|---------------------------------|-----------------|-----------------|-----------------|
|                                | U-MidOC         | U-LongOC        | U-TotalOC       |
| S-CTX, ng/L                    |                 |                 |                 |
| r (95% CI)                     | 0.51 (0.46–0.56)| 0.51 (0.46–0.56)| 0.38 (0.33–0.43)|
| P                              | <0.0001         | <0.0001         | <0.0001         |
| S-TRACP5b, U/L                 |                 |                 |                 |
| r (95% CI)                     | 0.44 (0.39–0.49)| 0.41 (0.35–0.46)| 0.38 (0.33–0.43)|
| P                              | <0.0001         | <0.0001         | <0.0001         |
| S-BoneALP, U/L                 |                 |                 |                 |
| r (95% CI)                     | 0.38 (0.32–0.43)| 0.36 (0.30–0.41)| 0.30 (0.24–0.36)|
| P                              | <0.0001         | <0.0001         | <0.0001         |
| S-TotalOC, µg/L                |                 |                 |                 |
| r (95% CI)                     | 0.57 (0.53–0.61)| 0.55 (0.51–0.60)| 0.30 (0.24–0.35)|
| P                              | <0.0001         | <0.0001         | <0.0001         |

* U-OC results in µg OC/mmol creatinine.

** CI, confidence interval.
proportions may be different from those of postmenopausal women and older individuals, and a premenopausal undiluted adult urine sample or urine obtained from, e.g., a postmenopausal woman shortly after ovariectomy would probably serve as a more appropriate model for control samples in the future. By reducing the analytical variability by careful validation, the value of U-OC in clinical settings could become even more pronounced.

Measurements were performed with urine samples collected as the first morning void, but we used a second morning void and 24-h urine collections as well and found them to be appropriate sample material (data not shown). The concentrations were highest and most easily measurable in the first morning voids, which can be recommended as a sample material for further studies. Furthermore, the immunoreactivity in urine samples seemed to tolerate short-term storage at 4 °C or even room temperature before freezing at −20 or −70 °C. This in vitro stability of OC in urine facilitates the collection and storage of urine samples, and in this aspect U-OC appears to be superior to S-OC. However, U-OC may exhibit some prestorage variability resulting from sample handling immediately after the collection. Despite the carefully controlled collection and storage of urine samples in the stability study, we were unable to address this aspect in the current study in detail, and this remains to be evaluated.

Circulating OC concentrations demonstrate a circadian rhythm characterized by a decrease during the morning to a low around noon. This is followed by a gradual increase that peaks after midnight. The difference between the highest and lowest values ranges from 10% to 20% depending on the assay (29,30). Pronounced diurnal variation has also been observed for resorption markers such as urinary collagen type I cross-linked N-telopeptides (31) and S-CTX (32). Resorption markers reach their maximum and minimum at around 0500 and 1400, respectively, and the magnitude of variation appear to be higher for collagen degradation products than, for example, for S-OC (32). Taylor et al. (12) found a diurnal variation of U-OC after the one reported for S-OC, i.e., high concentrations in the morning and low concentrations in the afternoon. Our results with all three U-OC assays were consistent with this rhythm. In addition to circadian variability, minor seasonal fluctuations in S-OC, with concentrations decreasing from January to July and then increasing to a peak in winter, have been reported (30,33). More recent studies, however, have found no indication of an important wintertime increase and only a borderline summertime increase, if any (34). The effect of sampling season on urine OC values has not been investigated previously. We studied the seasonal variability in the cross-sectional study of elderly women and did not detect any major seasonal variation in U-OC concentrations. There was a small but statistically significant tendency for higher values in late autumn and winter when U-OC was measured with the U-MidOC assay, which might be related to the summertime increase in sunlight and vitamin D. The results obtained with the two other assays displayed no significant variability regarding the month of sampling, which may be related to the lower sensitivities of these assays to detect minor changes. Nevertheless, the seasonal effects appeared to be rather small in comparison with other sources of measurement noise and not of sufficient magnitude to confound the use of U-OC measurements.

The clinical properties of the U-OC assays appear to be distinct from those of S-OC assays. The correlations between the U-OC assays and the well-known resorption marker S-CTX were significantly higher, without overlapping confidence limits, than the correlation with the bone formation marker S-BoneALP. The correlations between the U-OC assays and another resorption marker, S-TRACP5b, also tended to be higher than those between the U-OC assays and S-BoneALP, but the confidence limits were slightly overlapping. We also recently showed that U-MidOC and U-LongOC assay results were predictive for fractures in a large prospective study of elderly women (22). Briefly, when women in the highest quartile of bone turnover markers were compared with all other women, the odds ratios for sustaining a clinical vertebral fracture were 2.71 (95% confidence interval, 1.50–4.89) for the U-MidOC and 2.75 (1.52–4.96) for the U-LongOC assays. Together with the resorption markers S-CTX and S-TRACP5b, these two U-OC assays were the only bone turnover markers that were statistically significant, and the odds ratios for the U-OC assay results were comparable to the odds ratios for S-CTX [1.94 (1.05–3.58)] and S-TRACP5b [2.28 (1.26–4.15)]. Interestingly, the four S-OC assays evaluated were not predictive for fractures. Differences between U-OC and S-OC have also been published by others. Gundberg et al. (35) observed discordance between the measurements of S-OC and urinary excretion of its breakdown product (Gla) in patients with Paget disease, which is characterized by high bone turnover with an increased number and appositional rate of osteoblasts seen in the affected sites with no net bone loss (36). In such patients, urinary excretion of Gla was normal, but S-OC concentrations were increased threefold (35). More recently, Åkesson et al. (37) monitored patients after tibia osteotomy (n = 14) and found that U-OC values had kinetics similar to those for resorption markers such as urinary deoxypyridinoline and distinct from the kinetics of S-OC. Different kinetics were also observed for S-OC and U-OC (measured by a competitive assay for the U-OC 14–28 fragment) when alendronate was administered to osteoporotic postmenopausal women (n = 19), and the changes in U-OC, but not in S-OC, were correlated with changes in resorption markers (serum or urinary collagen type I cross-linked N-telopeptides and CTX) (14). We have observed similar differences in the kinetics of U-OC and S-OC in the follow-up of postmenopausal
women (n = 76) after alendronate treatment in a placebo-controlled alendronate intervention trial (38).

Osteoclastic bone resorption in vitro releases immunoreactive OC, both intact and fragmented molecules, from bone matrix (10, 39). Because bone hydroxyapatite is able to absorb only intact OC molecules but not OC fragments (40, 41), circulating intact OC, whether released from biosynthesis in osteoblasts or derived from resorption, may traffic back to the skeleton. Circulating intact OC, which is not adsorbed to hydroxyapatite, and the largest OC fragments are likely to be degraded by proteases in the circulation or peripheral organs (11, 42). Small OC fragments are more resistant to degradation and probably accumulate in the urine as midmolecule fragments because the middle portion of the molecule is more resistant to proteolysis because of structural properties of the molecule (15, 43, 44). Because of the rapid clearance from the circulation to bone or by proteolytic activity, S-OC probably reflects rather acute changes in bone metabolism, whereas OC fragments accumulating in the urine serve more as an index of basal bone turnover rate, most likely predominantly resorption.

In summary, we have developed three immunoassays for the detection of OC fragments in urine. The assays have unique specificities toward different naturally occurring U-OC fragments. U-OC concentrations followed a circadian rhythm similar to the one reported for S-OC, but we observed no major seasonal variations. High concentrations of OC in urine were associated with low bone mass, and U-OC detected with any of these assays correlated with bone turnover rate assessed by conventional serum markers of bone metabolism. Furthermore, there is accumulating evidence that U-OC assays have distinct properties compared with S-OC assays in clinical settings. The measurement of U-OC thus provides an additional means to monitor bone metabolism and may have applications in diagnostics related to disorders of bone metabolism.

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