able for multiplexing up to 12-plex (11). These features would allow simple, fast, and high-throughput screening procedures. In the future, it would be valuable to perform larger-scale studies to confirm our preliminary data and to further resolve the analytical issues such as reference intervals, assay specificity and sensitivity, and experimental and biological variations.

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References

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Urinary Osteocalcin Is a Useful Marker for Monitoring the Effect of Alendronate Therapy, Kaisa K. Ivoaška,1 Kim Pettersson,2 Arja Nenonen,3,4 Kirsti Uusi-Rasi,3 Ari Heinonen,3 Pekka Kannus,3 and H. Kalervo Viääänäen1* (Institute of Biomedicine, Departments of 1 Anatomy and 2 Biotechnology, University of Turku, Turku, Finland; 3 The UKK Institute for Health Promotion Research, Tampere, Finland; 4 Rheumatism Foundation Hospital, Heinola, Finland; * address correspondence to this author at: Institute of Biomedicine, Department of Anatomy, University of Turku, Kiinamyllynkatu 10, FI-20520 Turku, Finland; fax 358-2-3337352, e-mail kalervo.vaananen@utu.fi)

A drawback in the use of bone mineral density (BMD) measurements to monitor the effect of antiresorptive treatment is that 1 or 2 years of successful treatment may be necessary before significant increases in BMD can be detected (1). Biochemical markers can enable dynamic and rapid measurement of total body skeletal metabolism and may be clinically useful, particularly for detecting the effects of osteoporosis therapy (2). Circulating osteocalcin (OC), a bone-specific protein produced by osteoblasts, is widely used as an index of bone formation (3, 4). Fragments of OC are also found in urine (5–7), and the measurement of urinary OC (U-OC) is another method for monitoring bone metabolism (8–11). We recently described 3 immunoassays for measuring U-OC (11). Here we report the use of U-OC assays to monitor the effect of alendronate, a bisphosphonate and bone resorption inhibitor widely used to treat osteoporosis (12).

The study included 164 healthy women, postmenopausal for 1–5 years [mean (SD) age, 53.1 (2.3) years], described in detail previously (13). The study was a 1-year, double-blind, randomized, placebo-controlled intervention trial with 2 experimental groups: one receiving alendronate (5 mg/day Fosamax; Merck & Co) and the other receiving placebo (donated by Merck & Co). All participants in both study groups received a daily supplement of calcium carbonate (630 mg) and vitamin D3 (200 IU = 5 μg; Citracal + D; Mission Pharmacal). We collected 24-h urine samples and serum samples after a 12-h fast at baseline and after 3, 6, and 12 months. The samples were stored at −70 °C. U-OC was measured with U-MidOC, U-LongOC, and U-TotalOC assays, which all have unique specificities toward different naturally occurring U-OC fragments (11). Results were normalized for urinary creatinine determined in accordance with the alkaline picrate reaction. Intact OC and N-terminal midfragment of OC were measured in serum samples with an in-house immunoassay (S-TotalOC) (14). All measurements were performed blinded and in duplicate. BMD of the lumbar spine was measured at baseline and after 12 months with dual-energy x-ray absorptiometry (Norland XR-26; Norland Inc.), according to standard procedures. Seventy-six women in both the alendronate and placebo groups had both serum and urine analyzed for OC at baseline. After 3 months, OC results were obtained for 59 women in the alendronate group and for 69 women in the placebo group; after 6 months, for 60 in the alendronate group and 65 in the placebo group; after 12 months, for 51 in the alendronate group and 66 in the placebo group. Statistics were calculated after logarithmic transformation, and comparisons were made with one-way ANOVA, with Tukey or Dunnett post hoc adjustment. We used the SAS Enterprise Guide 2 program (SAS 8.2, SAS Institute) and considered P < 0.05 as statistically significant. All results are shown as the median (interquartile range).

In the alendronate group, both U-OC and serum OC (S-OC) concentrations decreased significantly from baseline (P < 0.001). After 3 months, U-MidOC, U-LongOC, and U-TotalOC values decreased to 41.5 (32.4–59.3)%, 33.7 (25.8–53.0)%, and 59.2 (41.7–81.5)% of baseline, respectively (Fig. 1, A–C). U-OC concentrations were unchanged during the remaining follow-up period of 3–12 months. The magnitude of decrease in S-OC was smaller
than the decrease in U-OC. After 3 months, S-OC concentrations had decreased to 69.4 (58.9–81.2)% of baseline (Fig. 1D). S-OC values tended to decrease further over 3–6 months ($P = 0.016$), but the decrease was not statistically significant after a post hoc test ($P = 0.067$). A decrease over 3–6 months was not detected for any of the U-OC assays ($P = 0.73–0.92$).

All study participants received calcium and vitamin D$_3$ supplementation; therefore, a reduction in bone turnover was expected in the placebo group, although to a lesser extent than in the alendronate group. In the placebo group, the concentrations relative to baseline after 3 months were as follows: U-MidOC, 75.4 (61.0–89.2)%; U-LongOC, 70.3 (51.3–91.7)%; U-TotalOC, 98.5 (79.3–133.0)%; and S-TotalOC, 88.4 (79.2–105.6)% (Fig. 1, A–D). All OC assay results differed significantly ($P < 0.001$) between the placebo and alendronate groups during the entire follow-up period. After 3 months, when the effects of calcium and vitamin D supplementation were taken into account, the specific net decrease in U-OC attributable to alendronate was 34%–40% depending on the assay, being largest for U-TotalOC and statistically significant for all assays ($P < 0.001$).

BMD values in the alendronate and placebo groups at baseline were 0.960 and 0.945 g/cm$^2$, respectively. During the 1-year treatment, lumbar spine BMD increased in the alendronate group by 3.7% (0.8%–6.3%; $P < 0.001$) and in the placebo group by 0.8% (−1.3% to 2.4%; $P = 0.22$). For the entire study population, changes in OC assay results after 3 months were statistically significantly negatively correlated to changes in BMD after 12 months [Spearman correlation coefficient ($r$), −0.18 to −0.40, depending on OC assay; $P < 0.05$ for all correlations]. For the treatment group only, $r$ values were smaller and statistically significant only for the U-LongOC assay ($r = −0.27; P < 0.05$).

Bone turnover markers are not clinically useful unless the change in an individual patient is greater than would be expected from normal variability of the marker. Least significant change (LSC) is the minimum change between 2 successive results in an individual that would imply a real biological response. It defines a threshold as the change greater than the precision error of the method in a...
single individual (15, 16). One-sided LSC values (15, 16) at \( P < 0.05 \) were calculated as described (16, 17) (Table 1), and participants with a decrease greater than the LSC at 3 months (increase at 12 months for BMD) were considered responders to the treatment. LSC values for U-MidOC, U-LongOC, and U-TotalOC assays were 54\%, 63\%, and 41\%, respectively, indicating that a substantial decrease in U-OC must be detected before the result can be considered a response to an antiresorptive agent (Table 1). Most of the women classified as responders by OC assays were also considered responders on the basis of change in BMD above LSC. Of the nonresponders by OC assays (\( n = 24, 25, 29, \) and 13 for U-MidOC, U-LongOC, U-TotalOC, and S-TotalOC, respectively), approximately one third were responders on the basis of BMD change (\( n = 8, 8, 13, \) and 4, respectively; Table 1). Correspondingly, some of the responders by change in BMD (\( n = 24 \)) were classified as nonresponders by OC assays (\( n = 8, 8, 13, \) and 4, respectively).

The specificity of the methods was determined as the percentage of nonresponders in the placebo group, and sensitivity as the percentage of responders in the alendronate group. The specificities of all OC assays were high (92.8\%–100\%), but the sensitivities were lower (50.8\%–59.3\%; Table 1). We also determined the areas under the ROC curves (AUC). There were no significant differences in the performance of OC assays in the ROC analysis (AUC, 0.802–0.839; SE, 0.036–0.040; Fig. 1E and Table 1). All 4 OC assays performed at 3 months distinguished alendronate and placebo groups more effectively than did BMD measured at 12 months (AUC, 0.718; SE, 0.046; Fig. 1E).

Bone turnover markers may be used when BMD changes are too small to be used clinically, particularly within the first 6 months after therapy initiation (18). In women receiving antiresorptive therapy, short-term changes in bone turnover markers are related to long-term changes in BMD and may also predict long-term increases in BMD (19–21). Furthermore, greater reductions in bone turnover shortly after initiation of treatment are associated with fewer fractures in the future (22, 23). Reduction of bone turnover by alendronate decreases circulating OC concentrations (24, 25), and changes in S-OC correlate with changes in BMD (19). Our results demonstrate that various molecular forms of U-OC also decrease in response to alendronate treatment in postmenopausal women and that there are no major differences between assays detecting different fragments.

Because antiresorptive agents affect primarily bone resorption, a reduction is usually seen first in markers of bone resorption, and markers of formation decrease later (2). The kinetics of U-OC and S-OC in response to alendronate were distinct from each other. U-OC concentrations reached a plateau after 3 months, but there was a trend for S-OC concentrations to decrease further until 6 months. Differences in the kinetics of U-OC and S-OC in response to alendronate have also been observed previously in smaller cohorts (8, 10). U-OC and S-OC may therefore reflect different aspects of bone turnover, and the molecular forms of OC in urine may be associated more with resorption than the molecular forms of OC in circulation, as has been suggested (8, 10, 11, 26). Measurements at earlier time points would have been valuable for clarification of the short-term kinetics.

The AUC for U-OC and S-OC measured at 3 months were larger than the AUC for BMD, performed at 12 months, demonstrating that S-OC and U-OC are both suitable for monitoring short-term effects of alendronate on bone metabolism in postmenopausal women. The response in bone turnover markers may be even more profound in osteoporotic women, who have higher baseline bone marker values. Measurement of OC in urine rather than in serum may be advantageous because of the improved stability of OC in urine (11, 27), lack of pro-

### Table 1. Performance of OC immunoassays in monitoring alendronate treatment after 3 months of therapy.

<table>
<thead>
<tr>
<th></th>
<th>U-MidOC</th>
<th>U-LongOC</th>
<th>U-TotalOC</th>
<th>S-TotalOC</th>
<th>BMD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV(\text{a,b} ) %</td>
<td>7.4</td>
<td>8.0</td>
<td>16</td>
<td>5.0</td>
<td>0.7</td>
</tr>
<tr>
<td>CV(\text{c,d} ) %</td>
<td>22</td>
<td>26</td>
<td>-7.0</td>
<td>5.9</td>
<td>1.7</td>
</tr>
<tr>
<td>LSC(\text{e,f} ) %</td>
<td>53.5</td>
<td>63.0</td>
<td>40.5</td>
<td>17.9</td>
<td>4.3</td>
</tr>
<tr>
<td>Responders in alendronate group, n</td>
<td>35</td>
<td>34</td>
<td>30</td>
<td>46</td>
<td>24</td>
</tr>
<tr>
<td>Nonresponders in alendronate group, n</td>
<td>24</td>
<td>25</td>
<td>29</td>
<td>13</td>
<td>35</td>
</tr>
<tr>
<td>Nonresponders by assays but classified as responders by BMD, n</td>
<td>8</td>
<td>8</td>
<td>13</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Sensitivity,(g) %</td>
<td>59.3</td>
<td>57.6</td>
<td>50.8</td>
<td>78.0</td>
<td>40.7</td>
</tr>
<tr>
<td>Specificity,(h) %</td>
<td>100.0</td>
<td>95.7</td>
<td>92.8</td>
<td>68.1</td>
<td>91.3</td>
</tr>
<tr>
<td>AUC (SE)(i)</td>
<td>0.839 (0.036)</td>
<td>0.838 (0.037)</td>
<td>0.802 (0.040)</td>
<td>0.804 (0.040)</td>
<td>0.718 (0.046)</td>
</tr>
</tbody>
</table>

\(\text{a} \) Parameters for BMD are calculated from measurements performed after 12 months.

\(\text{b} \) CV\(\text{a,b} \), analytical variability. The CV\(\text{a,b} \) was determined as the mean CV of all duplicated measurements.

\(\text{c} \) CV\(\text{c,d} \), biological variability. The CV\(\text{c,d} \) was determined as the mean of the changes observed at 3 months in the placebo group, receiving only vitamin D\(_3\) and calcium supplementation.

\(\text{d} \) One-sided LSC at \( P < 0.05 \) was defined as LSC = \( 2.33 \times \sqrt{(CV_{\text{a,b}}^2 + CV_{\text{c,d}}^2)} \) (16, 17). Participants who showed a decrease greater than the LSC at 3 months (increase at 12 months for BMD) were considered responders to the treatment.

\(\text{e} \) Sensitivity was determined as the percentage of responders in the alendronate group (\( n = 59 \)).

\(\text{f} \) Specificity was determined as the percentage of nonresponders in the placebo group (\( n = 69 \)).

\(\text{g} \) The values for AUC (SE) were calculated by ROC curve analysis.
found seasonal variation (27), and simple and noninvasive sample collection. In addition to 24-h samples, as used in this study, OC can be measured from spot urine samples collected as the first or second morning void (9, 11). Moreover, U-OC responds to therapy more rapidly than S-OC.

In summary, the results suggest that U-OC is a useful marker for monitoring short-term changes in bone metabolism in response to antiresorptive therapy in postmenopausal women.

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Effect of Specimen Anticoagulation on the Measurement of Circulating Platelet-Derived Growth Factors, Robert Zimmermann, Julia Koenig, Juergen Zingsem, Volker Weisbach, Erwin Strasser, Juergen Ringwald, and Reinhold Eckstein (Department of Transfusion Medicine and Hemo-

Platelets (PLTs) contain an assortment of growth factors (GFs), in particular PLT-derived GFs (PDGFs), transforming GFs (TGFs), and vascular endothelial GF (VEGF) (1). By topical release and action on these GFs simultaneously with thrombus formation and bleeding cessation, PLTs initiate the processes of wound repair, angiogenesis, and defense against infectious agents (1). Released GFs may also have distant effects if they reach the fluid compartment of the circulating blood. Increased serum PDGF concentrations during and after hemodialysis, attributable to PLT-membrane contact at artificial surfaces, may be directly involved in the increased frequency of atherosclerosis in hemodialysis patients (2). The role of circulating GFs in many other nonmalignant clinical conditions has also been investigated (3–8).

Increased concentrations of VEGF have been found in a wide spectrum of malignancies (9–16). Many researchers assume a prognostic relevance of this finding because