

# Undercarboxylated Osteocalcin Measured with a Specific Immunoassay Predicts Hip Fracture in Elderly Women: The EPIDOS Study\*

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## ABSTRACT

Increased levels of circulating undercarboxylated osteocalcin (ucOC), measured indirectly with the hydroxyapatite (HAP) binding assay, have been shown to predict hip fracture risk in a small group of elderly institutionalized women.

The aim of this study was to confirm these findings in a prospective cohort study (EPIDOS prospective study) of 7598 healthy, independently living women over 75 yr of age. One hundred and four women who sustained a hip fracture during a 22-month follow-up period were age matched with 255 controls who did not fracture. Baseline samples were collected before hip fracture for measurement of total OC and ucOC, assessed either with the HAP binding assay or directly with a new enzyme-linked immunosorbent assay (ELISA). This direct ELISA uses human recombinant noncarboxylated OC as a standard and two monoclonal antibodies, one of which was raised against the 14–30 Glu synthetic peptide. We found that the intra- and interassay variations are less than 11%, and this assay exhibits a 5% cross-reactivity with purified human bone OC, used as a source of carboxylated OC. ucOC levels measured with this ELISA correlated well with the HAP binding assay in the population of 359 elderly women ( $r = 0.82$ ;  $P < 0.0001$ ).

We estimated the risk of hip fracture for women with levels of ucOC in the highest quartile of values for the 255 controls. We found that increased levels of ucOC measured by ELISA were associated with increased hip fracture risk with an odds ratio (OR) of 1.9 (95% confidence interval, 1.2–3.0), and the ELISA had a greater sensitivity than the HAP assay. In contrast, total OC was not associated with hip fracture risk. After adjustment for femoral neck bone mineral density (BMD) and mobility status assessed by gait speed, ucOC still predicted hip fracture with an OR of 1.8 (1.0–3.0). Women with both femoral neck BMD in the lowest quartile and ucOC in the highest quartile were at higher risk of hip fracture, with an OR of 5.5 (2.7–11.2), than those with only low BMD or high ucOC levels.

In conclusion, we have developed a new specific ELISA for serum ucOC, with low cross-reactivity with carboxylated OC and increased specificity and sensitivity over the HAP assay. Using this new ELISA, we found that ucOC, but not total OC, predicts hip fracture risk independently of femoral neck BMD in elderly women drawn from the general population. Thus, ucOC measurement could be combined with bone mass determination to improve the assessment of hip fracture risk in elderly women. (*J Clin Endocrinol Metab* 82: 719–724, 1997)

**O**STEOCALCIN (OC) is a bone-specific protein of 49 amino acids that is synthesized by osteoblasts and odontoblasts (1). A fraction of neosynthesized OC is released in blood and is widely used as a sensitive marker of bone formation (2). OC contains three residues of  $\gamma$ -carboxyglutamic acid (Gla) at positions 17, 21, and 24 of the amino acid sequence, which are responsible for its high affinity for hy-

droxyapatite (HAP) (3, 4). The synthesis of OC is vitamin D and vitamin K dependent. Indeed, the promoter of the OC gene contains a vitamin D-responsive element that directly stimulates the transcription of OC (5), and vitamin K is necessary for the posttranslational  $\gamma$ -carboxylation of glutamic acid residues in proosteocalcin (6).

In a cross-sectional study, serum concentrations of vitamin K<sub>1</sub> and menaquinones 7 and 8, two major components of circulating vitamin K<sub>2</sub>, were significantly lower in elderly women with hip fracture than in age-matched healthy controls (7). Patients with spinal crush fracture were also reported to have very low concentrations of circulating menaquinones (8). These findings suggest that vitamin K deficiency could result in alterations in bone strength and thus in increased risk of fracture. Warfarin, a potent vitamin K antagonist, impairs the  $\gamma$ -carboxylation of glutamic acid, which is reflected by an increase in the fraction of circulating undercarboxylated OC (ucOC) (4). Interestingly, we have previously shown that ucOC is significantly increased with age, especially in elderly women (9). Levels return to normal values for young adults after treatment with low doses of vitamin K<sub>1</sub> (10). We also showed that ucOC negatively correlated with hip bone mineral density (BMD) (11) and that the fraction of ucOC is a powerful predictor of hip fracture

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risk in a group of elderly women (12, 13). However, these results were obtained in a small group of elderly institutionalized women who are known to be under nutritional deficiency and, therefore, are not representative of the general population.

In addition, in all previous studies, ucOC was assessed with indirect assays, based on the different affinities of normal carboxylated OC and ucOC for HAP or barium sulfate (3, 14, 15). These methods do not give accurate levels of the undercarboxylated fraction of OC, as a significant fraction of fully decarboxylated OC binds nonspecifically to these chemical compounds used to precipitate carboxylated OC. In addition, these techniques are cumbersome, their long term precisions are somewhat variable, and there is a clear need for a more convenient, accurate, and precise method to be used in clinical studies.

The aims of this study were to develop a direct and specific enzyme-linked immunosorbent assay (ELISA) for measuring ucOC in serum and to determine the value of serum ucOC to prospectively predict the risk of hip fracture in a large cohort of healthy independently living women over 75 yr of age (EPIDOS study). Results obtained with direct ELISA were compared to the predictive value of ucOC, assessed indirectly with the conventional HAP binding assay.

## Subjects and Methods

### Subjects

This study is part of a large prospective study on the risk factors for hip fracture in elderly French women, named EPIDOS. During a period of 2 yr (January 1992 through December 1993), 7598 healthy female volunteers, 75 yr of age or older, were recruited from population-based lists in 5 French cities (Amiens, Lyon, Montpellier, Paris, and Toulouse). All women were ambulatory, and 90% of them were living independently. At the baseline visit, a fasting serum sample was collected, and they were given a lifestyle questionnaire and mobility tests, such as gait speed. Gait speed was assessed by the time required to perform a 6-m walk at the usual pace (average of 2 attempts). During an average 22-month follow-up, 124 women sustained a hip fracture. We then performed a nested case control study where each hip fracture patient was matched with 3 controls (C) from the same cohort according to age ( $\pm 1$  yr) and time of recruitment ( $\pm 1.5$  months). None of the controls sustained any fracture during the follow-up period. Of the 496 elderly women, 5 patients were excluded because of the presence of primary hyperparathyroidism (2 hip fracture and 1 C) or chronic hemodialysis (2 C). Patients receiving the following treatments known to influence calcium metabolism were also excluded: bisphosphonate ( $n = 13$ ), fluoride ( $n = 6$ ), and calcium and/or vitamin D<sub>3</sub> ( $n = 52$ ). For each hip fracture patient excluded, the 3 age-matched controls were also excluded to perform the statistical analysis. A total of 104 patients with hip fracture and 255 elderly controls were analyzed. After exclusion, 53% of hip-fractured patients were still matched with 3 controls, the remaining were associated with 2 age-matched controls.

This study was approved by the local ethical committee, and written informed consent was obtained from all participants.

### Methods

Serum calcium, creatinine, albumin, and total alkaline phosphatase activity were measured by standard laboratory methods. Serum 25-hydroxyvitamin D (25OHD) was measured by a RIA after extraction (Inctar, Stillwater, MN).

### Total OC

Levels were measured using an immunoradiometric assay (IRMA; ELISA-Osteo, CIS BioInternational, Gif-sur-Yvette, France) that recog-

nizes a large N-terminal midfragment in addition to the intact molecule. This assay recognizes fully carboxylated and noncarboxylated OC with the same affinity (16).

### Measurements of ucOC by the HAP binding assay

This method is based on the lower affinity of ucOC for HAP compared to fully carboxylated OC and has been previously described (14). Briefly, 250  $\mu$ L serum were incubated with 7.5 mg HAP (calcium phosphate tribasic type IV, Sigma Chemical Co., St. Louis, MO) in an Eppendorf tube, mixed end over end for 1 h at 4 C, and then centrifuged (3000 rpm, 15 min). This concentration of HAP has been shown to provide the best discrimination between fully carboxylated human OC and thermally decarboxylated human OC. OC measured in the supernatant with IRMA for total OC represents a valid estimate of the undercarboxylated fraction of OC. The ucOC level was expressed either as an absolute value (nanograms per mL) or as a percentage of the total OC.

### Measurements of ucOC by ELISA

*Standard.* Recombinant human Glu-OC (Biotechnology Research Laboratory, Takara Shuzo Co., Otsu, Shiga, Japan) was used as a standard. This recombinant protein was calibrated by amino acid analysis and by IRMA for total OC.

*Monoclonal antibodies.* Two monoclonal antibodies (OC4-5 and OCG3) were used in the ELISA. OC 4-5 has been obtained from immunization of BALB/c mice with 14-30 GluOC synthetic peptide linked to nitrocellulose as a carrier. OC G3 was obtained by the same method, using bovine OC linked to keyhole limpet hemocyanin as an immunogene. This antibody recognizes the 21-31 sequence of human OC, but does not distinguish Gla-OC from Glu-OC (17).

### Procedure

Two micrograms of OC 4-5 in 200  $\mu$ L 0.1 mol/L phosphate-buffered saline (PBS) were added in each well of a microtiter plate (MaxiSorp F16, Nunc, Roskilde, Denmark) and incubated overnight at 4 C. The wells were then washed three times with PBS and saturated with 200  $\mu$ L BlockAce, Snow Brand Milk Products Co., Ltd., Sapporo, Japan (mainly consisting of skim milk) at room temperature for 2 h. This block solution was then discarded, and 100  $\mu$ L of standard (human Glu-OC protein derived from recombinant DNA) or unknown samples (4-fold diluted in BlockAce; 25% in PBS) were added. After 1 h of incubation under slight agitation at room temperature, wells were washed three times with PBS, and 100  $\mu$ L monoclonal antibody OCG3 (3000-fold diluted) labeled with horseradish peroxidase were added to each well. After 1 h of incubation under slight agitation at room temperature, unbound labeled antibody was removed by washing three times with PBS and 100  $\mu$ L substrate solution (1 mg/mL OPD in 50 mmol/L citrate buffer, pH 5.0, and 0.01% H<sub>2</sub>O<sub>2</sub>) were added to each well. After 15 min of incubation in the dark, the reaction was stopped with 100  $\mu$ L HCl (1 mol/L), and absorbance was read at 492 nm with an automated plate reader.

All serum samples were stored at -80 C until assayed, and biochemical measurements were performed in a blinded manner, *i.e.* without knowledge of the presence or absence of fracture.

### Statistical analysis

The correlation coefficient for comparison between the indirect HAP assay for ucOC and the ELISA was calculated using linear regression analysis.

Clinical and biochemical characteristics between the two groups were assessed by ANOVA, in which the hip fracture patients were compared pairwise to the age-matched controls. The relative risks of hip fracture for increased ucOC level or total OC were estimated using the odds ratio (OR) before and after adjustments for confounding factors such as femoral neck BMD and gait speed, which were considered as continuous variables. The ORs were obtained from conditional logistic regression on matched sets to take into account the nested case-control design of the study. All analyses were performed using Statistical Analysis Software (SAS Institute, Cary, NC).

**Results**

*ELISA for ucOC*

*Specificity of the capture antibody for ucOC.* To determine the specificity of OC 4-5 for ucOC, competitive inhibition of <sup>125</sup>I-labeled recombinant human Glu-OC binding by recombinant human Glu-OC and by osteocalcin purified from human bone that is mainly carboxylated was tested. As shown in Fig. 1, the concentration that inhibits 50% of the binding of <sup>125</sup>I-labeled recombinant human Glu-OC was about 100-fold lower for recombinant human Glu-OC (IC<sub>50</sub> = 7 × 10<sup>-7</sup> mol/L) than for human bone osteocalcin (IC<sub>50</sub> = 5 × 10<sup>-5</sup> mol/L).

*Cross-reactivity with human bone OC.* Known amounts of purified human bone OC were measured with the ELISA for ucOC. As shown in Table 1, the estimated cross-reactivity of ELISA for human bone OC was about 5%.

*Analytical performances.* A typical standard curve of the ELISA is shown in Fig. 2. The detection limit of the method, defined as being the minimum detectable concentration equivalent to twice the SD of the zero standard, was 0.2 ng/mL. The interassay variation evaluated by repeated measurements (n = 10) of three sera (mean ucOC levels, 0.46, 1.7, and 2.7 ng/mL) was less than 11.4%. The intraassay variation assessed by 10 measurements of two sera (mean ucOC, 0.46 and 2.7 ng/mL) was less than 9%. The recovery test was performed by adding known amounts of recombinant human Glu-OC (0.5, 1, 2, and 4 ng/mL) to 2 serum samples. The recovery ranged from 77–103%. The dose dilution curves of various normal sera (samples A, B, and C) were parallel to the standard curve (Fig. 2).

*Correlation with HAP binding assay.* Serum ucOC levels were measured in elderly women (Cohort EPIDOS; n = 359) using both HAP binding assay and ELISA. There was a significant correlation between the two methods (Fig. 3); ucOC levels

measured by HAP binding assay explained about 67% of those determined by ELISA.

*ucOC as a predictor of hip fracture risk in elderly women*

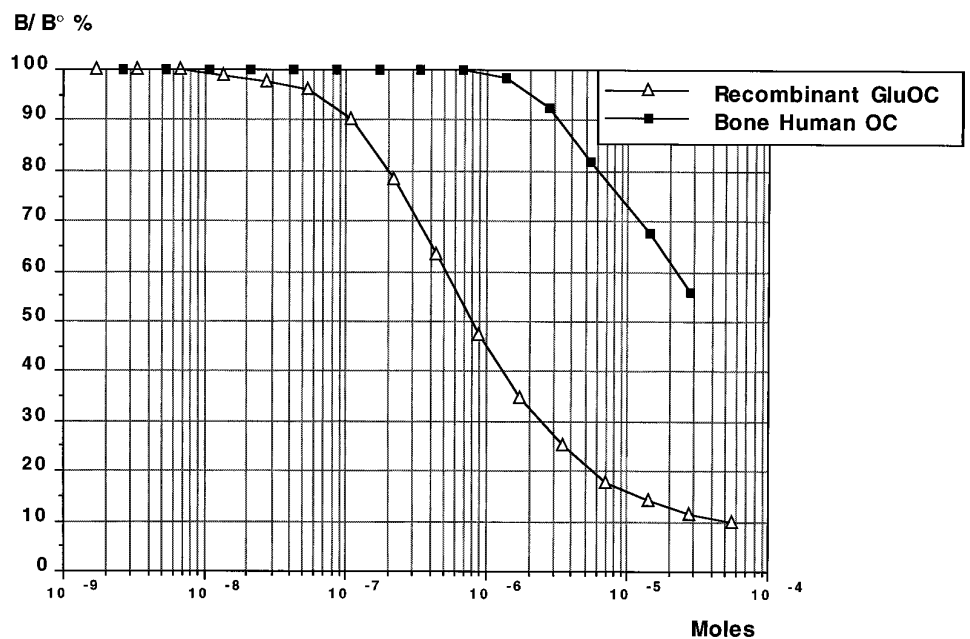
Baseline characteristics of elderly controls and patients with hip fracture are shown in Table 2. There was no significant difference in weight, serum calcium, creatinine, 25OHD, or total alkaline phosphatase activity between the two age-matched groups. Femoral neck BMD measured with dual energy x-ray absorptiometry using a Lunar DPX+ device (Lunar, Madison, WI) was significantly decreased in patients who subsequently sustained a hip fracture. Gait speed, a fall-related factor, was significantly lower in the group of hip fracture subjects. Albumin levels were also significantly decreased in hip fracture patients compared to those in age-matched controls.

Levels of total OC and ucOC measured by either HAP binding assay or ELISA were similar in the group of hip fracture patients and the controls (Table 3). When ucOC levels were measured by HAP and expressed as a percentage of the total OC (%ucOC), mean values were higher in patients who will sustain a hip fracture than in age-matched controls (Table 3).

The relative risk of sustaining a hip fracture was then calculated for levels of total OC, ucOC (ELISA and HAP methods), and %ucOC (HAP method) in the highest quartile

**TABLE 1.** Estimated cross-reactivity of ELISA for human bone OC

	Added purified human bone OC (ng/mL)			
	50	25	12.5	6.25
Conc. measured in the ELISA for UcOC (ng/mL)	2.13	1.26	0.8	0.28
Cross-reactivity (%)	4.3	5	6.4	4.5



**FIG. 1.** Specificity of the capture antibody for recombinant human Glu OC Δ and for human bone OC ■. Results are expressed as percentage of maximal binding (B°)

FIG. 2. Typical standard curve of the ELISA and dilution of various sera (samples A, B, and C). The upper scale gives in  $\mu\text{L}$  the volume of serum present in the well.

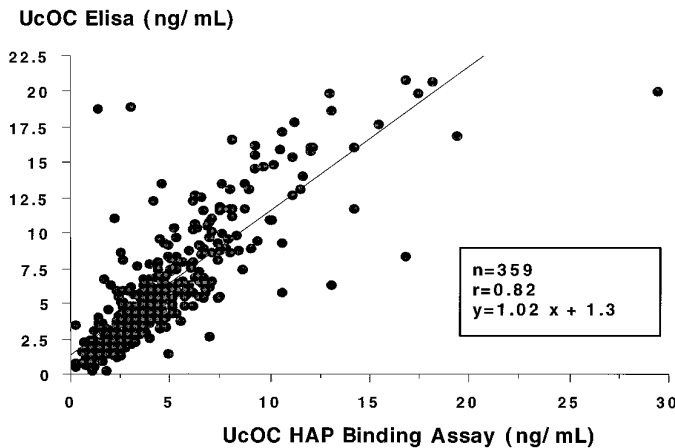
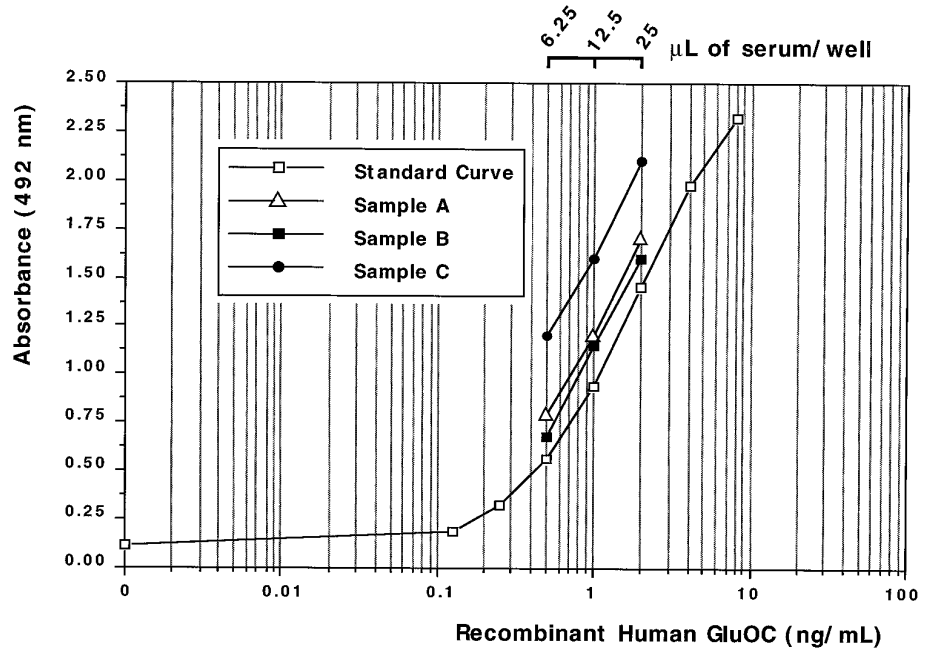


FIG. 3. Correlation study of the HAP binding assay and the ELISA for ucOC determination. Serum samples of 359 elderly women were analyzed simultaneously by both assays. Linear regression analysis:  $r = 0.82$ ;  $y = 1.02x + 1.3$ .

of the values for the 255 controls, using the OR. As shown in Table 4, elderly women with baseline ucOC measured by ELISA in the highest quartile, who represented 29% of the population, were at increased risk of hip fracture, with an OR of 1.9. Similarly, %ucOC levels in the highest quartile were associated with an increased hip fracture risk, with an OR of 2. In contrast, increased levels of ucOC measured with the conventional HAP method were not significantly associated with increased hip fracture risk.

ucOC measured with both methods was significantly and negatively correlated with femoral neck BMD ( $P < 0.0002$ ;  $r = -0.19$ ). To determine whether the association between ucOC and hip fracture risk was due to the correlation between ucOC and bone mass, adjustment for femoral neck BMD was performed. After adjustment, ucOC (ELISA method) and %ucOC (HAP method) levels in the highest quartile remained significantly associated with increased

TABLE 2. Baseline characteristics of hip fracture patients and age-matched controls

	Controls (n = 255)	Hip fracture (n = 104)	P
Age (yr)	82.3 $\pm$ 4.4	82.4 $\pm$ 4.5	0.80
Wt (kg)	59.8 $\pm$ 11	57.6 $\pm$ 10	0.07
Serum calcium (mg/L)	94 $\pm$ 3.7	93.6 $\pm$ 3.1	0.33
Serum creatinine (mg/L)	10.7 $\pm$ 2.8	10.2 $\pm$ 2.7	0.13
Serum albumin (g/L)	45.2 $\pm$ 3.2	44.5 $\pm$ 3.0	0.03
Serum 25OHD (ng/mL)	14.6 $\pm$ 8.6	15.9 $\pm$ 12.4	0.17
Serum total alkaline phosphatase (IU/L)	69.3 $\pm$ 24.3	69.3 $\pm$ 26.8	0.97
Gait speed (m/s)	0.81 $\pm$ 0.20	0.76 $\pm$ 0.21	0.02
Femoral neck BMD ( $\text{g}/\text{cm}^2$ )	0.705 $\pm$ 0.11	0.646 $\pm$ 0.10	<0.0001

Values are the mean  $\pm$  1 SD.

TABLE 3. Baseline levels of total OC and ucOC in elderly women

	Controls (n = 255)	Hip fracture (n = 104)	P
Total OC (ng/mL)	27.5 $\pm$ 11.2	28.0 $\pm$ 12.5	0.70
ucOC ELISA (ng/mL)	5.8 $\pm$ 4.1	6.7 $\pm$ 4.8	0.09
ucOC HAP (ng/mL)	4.4 $\pm$ 3.3	5.1 $\pm$ 3.7	0.13
% ucOC HAP	15.1 $\pm$ 6.8	17 $\pm$ 7.5	0.04

Values are the mean  $\pm$  1 SD.

risk of hip fracture, with ORs of 1.8 [confidence interval (CI), 1.1–3.0] and 1.8 (CI, 1.1–2.9), respectively.

Because of a slower gait speed in the EPIDOS population, which is a significant predictor of hip fracture risk with an OR of 3.2 (CI, 1.2–10), adjustment for this parameter was also performed. After adjustment for gait speed, ucOC (ELISA method) and %ucOC levels in the highest quartile remained significantly associated with an increased risk of hip fracture, with ORs of 1.9 (CI, 1.1–3.1) and 1.7 (CI, 1.0–2.8), respectively. As shown in Fig. 4, elderly women with both low femoral neck BMD, defined by the lowest quartile, and either ucOC (ELISA method) or %ucOC (HAP method) levels in the high-

**TABLE 4.** Increased ucOC levels as predictors of hip fracture in elderly women

	Odds ratio (95% confidence interval): highest quartile of elderly controls	<i>P</i>
Total OC (ng/mL)	1.3 (0.7–2.1)	0.39
ucOC ELISA (ng/mL)	2.0 (1.2–3.2)	<0.008
ucOC HAP (ng/mL)	1.6 (0.9–2.7)	0.07
% ucOC HAP	2.0 (1.3–3.3)	<0.004

est quartile were at higher risk of hip fracture, with ORs of 5.5 (CI, 2.7–11.2) and 4.4 (CI, 2.3–8.4), respectively, than those with only one of these two independent risk factors, *i.e.* with either low BMD or high ucOC levels.

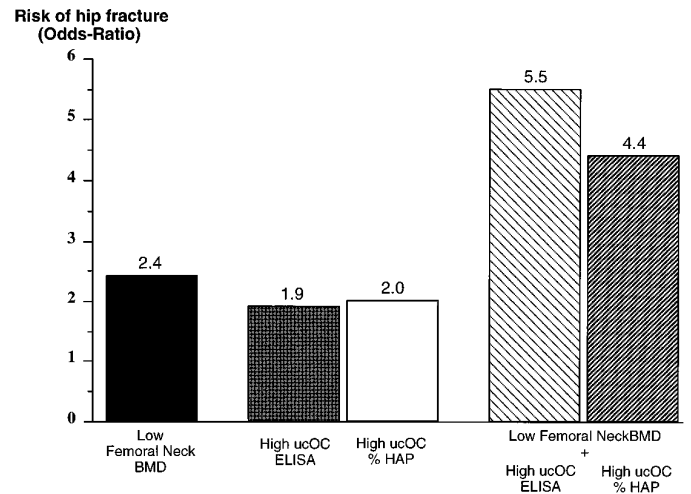
### Discussion

In this study, we found that increased levels of ucOC, but not total OC, measured by a new direct and specific immunoassay, predict hip fracture in elderly women, independently of bone mass.

The immunoassay for ucOC uses two monoclonal antibodies; one of these recognizes specifically the 14–30 sequence in which the three glutamic acid are not carboxylated and recombinant human Glu OC as a standard. A typical test of dilution and recovery gave adequate results, indicating that this assay can be used to measure undercarboxylated OC in all serum samples. In addition, adding various amount of purified human bone OC in the ELISA, we determined that this assay consistently cross-reacts by about 5% with carboxylated OC. This figure compares well with, for example, the liver alkaline phosphatase cross-reactivity (16%) of bone-specific immunoassay recently developed (18), bone and liver isoenzymes, and carboxylated and noncarboxylated OC, differing only in posttranslational modifications.

We observed a significant correlation between undercarboxylated OC measured by immunoassay and that obtained by the HAP assay in a large population of elderly women ( $r = 0.81$ ). However, HAP levels explained only 67% of the variance in the levels measured by the ELISA, suggesting that a significant proportion of molecules detected by the HAP method are different from those recognized in the immunoassay. Circulating OC is not a single 49-amino acid peptide. Actually, we recently showed that in addition to the intact molecule, several fragments of OC circulate, and that all together could represent about 50% of the total OC levels (16). In addition, it has been shown that in bone, the degree of carboxylation of the three glutamic acid residues may be different (19). Thus, partly carboxylated OC molecules with one, two, or three Gla residues could also be present in serum, adding to the heterogeneity of circulating OC. It would not be surprising if the HAP assay and the ELISA measured different OC moieties, although this has not yet been investigated. We also found that levels measured by ELISA were about 30% higher, on the average, than those determined by HAP assay. Indeed, it has been shown that the HAP binding assay underestimates ucOC levels because a significant proportion of decarboxylated OC does not bind specifically to HAP (14).

We previously found that increased ucOC levels measured with the conventional HAP method predict the subsequent



**FIG. 4.** Combination of the assessment of bone mineral density (BMD) and ucOC levels to predict hip fracture risk in elderly. Women with both low femoral neck BMD and high levels of ucOC (ELISA) or % ucOC (HAP method) were at higher risk of hip fracture than women with either low femoral neck BMD or high levels of ucOC or % ucOC.

risk of hip fracture in elderly institutionalized women followed prospectively for 3 yr (12, 13). However, one could argue that the results of that study may not be valid in the general population, as institutionalized women are known to be in poor nutritional and overall health status and, therefore, are more likely to be vitamin K deficient. Interestingly, in this study, we were able to confirm that although the differences observed between the two groups were small, increased levels of serum ucOC measured by a direct ELISA are still predictive of hip fracture risk in a large cohort of healthy elderly women, most of them living at home. In addition, the predictive value was independent of bone mass and mobility status, two major hip fracture risks (20–22), suggesting that ucOC measurement could be used in combination with these tests to identify a subgroup of women at very high risk for hip fracture. Indeed, we found that women with both low femoral neck BMD and high levels of ucOC were at higher risk to sustain hip fracture than women with only one of these two independent risk factors. Similar findings were obtained with levels of ucOC measured by the HAP method and expressed as a percentage of the total OC. In contrast, increased levels of ucOC measured by the HAP method and expressed as an absolute value were not consistently associated with increased hip fracture risk. This suggests that actually the direct ELISA, with increased precision and specificity, would be more sensitive to predict hip fracture risk than the indirect HAP method when results are expressed as an absolute value. As this new immunoassay is rapid (5 h) and requires a small volume of serum (25  $\mu$ L), it should certainly represent an improvement over the cumbersome and serum-consuming HAP or barium sulfate indirect assays.

The mechanisms underlying the association between increased serum ucOC and increased hip fracture risk are still unknown. We previously showed in the same prospective study that total OC and bone alkaline phosphatase were not predictive of hip fracture risk, indicating that an increase in

the overall bone turnover rate would not be responsible for that association (23). One of the most obvious reasons would be a decrease in the bone matrix content of OC as shown in warfarin-treated rats (4). The effect of this modification on bone metabolism is unknown, as the function of OC is unclear, although some studies have suggested a role for OC in the initiation of bone resorption (24–26), in the maintenance of the remodeling of trabecular bone (27), or, recently, in the inhibition of bone formation (28). However, although the mechanism by which ucOC is linked to bone fragility is not yet understood, increased serum ucOC levels probably reflect the low levels of both vitamin D and vitamin K that characterized the elderly population not only living in institutions (29) but also living at home (30). Thus, ucOC could represent an integrated and bone-specific index of a deficiency of those vitamins.

In conclusion, we have developed a new direct, specific, and convenient immunoassay for serum ucOC with low cross-reactivity with carboxylated OC. Using this immunoassay in a large cohort of healthy women, we have shown that ucOC, but not total OC, predicts hip fracture risk independently of bone mass in elderly women taken from the general population.

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