

Serum Interleukin 6 Is a Major Predictor of Bone Loss in Women Specific to the First Decade Past Menopause*

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ABSTRACT

The role of serum interleukin 6 (IL-6) as a predictor of bone loss was examined in a population-based, longitudinal study of 137 postmenopausal German women, 52–80 yr old at baseline. Serum IL-6 and other biochemical parameters were measured in baseline blood or urine specimens. Repeat standardized measures of bone mineral density (BMD) at the femur (total hip) and the lumbar spine (L2–L4) were taken by dual x-ray absorptiometry an average of 3.3 yr apart. Medical history and anthropometric measures were obtained from standardized interview and examination. Crude and age-adjusted mean serum IL-6 levels were significantly lower in postmenopausal women with than without hormone replacement therapy at baseline. Among nonusers of hormone replacement therapy, serum IL-6 concentrations were highly predictive of femoral bone loss, independently of potential confounders and plasma sex hormones. Statistical interaction between serum IL-6 and menopausal age or menopausal age group (>10 vs. ≤10 yr) indicated that the effect of IL-6 on bone loss

weakened with increasing distance from menopause and was no longer significant in women more than 10 yr after menopause. Among women up to 10 yr past menopause (n = 39), serum IL-6 was the single most important predictor of femoral bone loss, accounting for up to 34% of the total variability of change in BMD. The unadjusted linear model predicted an annual 1.34% (95% confidence interval, 0.67–2.01) decrease in total hip BMD per log unit increase in serum IL-6. A similar, although nonsignificant, effect of serum IL-6 on vertebral bone loss was restricted to women within the first 6 yr after menopause (n = 18). These epidemiological data show that serum IL-6 is a predictor of postmenopausal bone loss, and that the effect appears to be most relevant through the first postmenopausal decade. Whether these findings reflect pathogenetic differences between early and postmenopausal bone loss, and whether serum IL-6 also predicts fracture risk need further elucidation. (*J Clin Endocrinol Metab* 86: 2032–2042, 2001)

ACCCELERATED BONE loss due to menopause-induced estrogen deficiency is believed to be the most important risk factor for postmenopausal or involutional osteoporosis (1). There is recent evidence that the protective effect of estrogen on the skeleton may also be important in older postmenopausal women (2–6). However, the underlying mechanisms remain to be elucidated. According to current concepts of estrogen action in bone, a direct effect of estrogen on bone cell function may be involved as well as an indirect effect on extraskeletal calcium homeostasis (4, 7). Besides, in women, the mechanism may vary with time after menopause (4).

During the past decade, considerable evidence has accumulated that one of the pathways by which estrogen may exert a protective effect on the skeleton may be by governing the effect of cytokines on bone remodeling. Estrogen defi-

ciency dramatically alters the dependency of bone cells on several cytokines, including interleukin 6 (IL-6), IL-1, and tumor necrosis factor (TNF) (8–10). For example, ovariectomy-induced stimulation of osteoclastogenesis in mice can be prevented by neutralizing antibodies against IL-6 (11) or abolishment of IL-6 function by gene knockout (12).

Although these data provide strong evidence for the involvement of cytokines in bone loss due to sex hormone deficiency, most of the results have been derived from *in vitro* and animal studies. It is therefore still unclear whether this pathophysiological model of bone loss can be fully applied to humans, and whether serum IL-6 concentrations would be useful in predicting future bone loss. To our knowledge, the relationship between serum IL-6 and bone loss has not previously been studied in postmenopausal women. Two studies have examined the cross-sectional relationship of circulating IL-6 levels to bone mineral density (BMD) (13) or markers of bone turnover (14) in women, and no significant association was observed. It is possible that an effect of IL-6 on bone is missed in cross-sectional analysis. Besides, the effect may be modified by menopausal age. Cell culture studies by Pacifici and colleagues (15, 16) and by our group (17) suggest that increased cytokine production in osteoblasts and their precursors may be restricted to the early

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postmenopausal phase. Against this background, the aim of the present study was to determine the predictive effect of serum IL-6 concentrations on femoral and vertebral bone loss in a population-based, longitudinal study of postmenopausal women and to examine whether the effect persists across the entire range of menopausal age.

Subjects and Methods

Study population and setting

The University of Heidelberg Medical Center is one of eight German centers participating in the European Prospective Osteoporosis Study (EPOS) (18). The main objectives of this multicenter study, details of the sampling process, and results of the baseline survey, known as the European Vertebral Osteoporosis Study (EVOS), have been reported previously (19, 20).

In the framework of EPOS, an age- and sex-stratified random sample of birth cohorts between 1910–1940 was drawn from the population registry of the town of Eppelheim in the vicinity of Heidelberg, Germany. Between January 1992 and March 1993, 297 men and 283 women or 58% of eligible and contactable persons who were then 51–82 yr of age participated in the EPOS baseline survey (20–23). The present study is based on female cohort members who also completed a follow-up examination in 1995–1996 after an average of 3.3 yr (range, 2.6–4.3 yr); follow-up rates from baseline among women were 62% ($n = 176$). The study was conducted with approval of the ethics committee on clinical investigations at the University of Heidelberg Medical Center and after the participants gave written consent.

IL-6 measures were complete in 166 women. We excluded 7 women considered pre- or perimenopausal because they reported regular menses during the preceding 12 months. Another 22 women were excluded for preexisting conditions or medication known to affect bone loss. Of these, 2 women had chronic bone disease (primary hyperparathyroidism, multiple myeloma), 1 woman had renal failure and was treated with genuine vitamin D, 5 women were receiving long-term oral corticosteroid therapy, and 14 women received treatment with fluoride or bisphosphonates between baseline and follow-up. This left 137 postmenopausal women for the present investigation.

Women receiving hormone replacement therapy (HRT) at baseline ($n = 21$) and women who had started HRT during follow-up ($n = 9$) were included for cross-sectional analysis of the association between HRT and serum IL-6. They were excluded, however, from longitudinal analyses of the predictive effect of cytokines on bone loss. As these analyses also required knowledge of the exact menopausal age, the main study population was reduced to 89 postmenopausal women, who had not undergone hysterectomy before menopause and had never used HRT ($n = 82$) or stopped HRT at least 1 yr before the baseline survey ($n = 7$).

Interview and postal mailers

A standardized interview based on the EVOS questionnaire (24) was conducted at baseline by trained personnel to assess medical history including risk factors of osteoporosis and, in women, reproductive history. In women, menopausal state was determined from the answer to the question: "are you still having menstrual periods?" and "if not, when was your last menstrual period?" Women who had stopped menstruating for at least 1 yr were considered postmenopausal. Among women without hysterectomy before menopause, the age at menopause was calculated from the reported year of the last menses as the age at the cessation of menses plus 1 yr (25).

Information on HRT and osteotropic drug therapy between baseline and follow-up was obtained by postal mailers. A standardized questionnaire was sent to surviving members of the German EVOS population twice a year between September 1, 1993, and March 1, 1996. Postal follow-up was complete in 84% of the Heidelberg cohort and in 100% of those who came back for a second clinic visit. Participants were asked if they had been taking any prescription drug for osteoporosis during

the preceding 6 months and, if so, to write down the trade name of the drug. In addition, women were asked if they had newly started HRT, and a space was provided to fill in the trade name of the preparation as well as the month and the year it was first taken.

At the time of the second bone density measurement, participants were asked to answer a short standardized questionnaire regarding major changes in health status since baseline. They were specifically asked if they had been immobilized for more than 2 weeks, been newly diagnosed by a physician with arthritis or chronic gastrointestinal disease, or received long-term corticosteroid treatment.

Evaluation of spinal x-rays

Standardized lateral x-rays of the thoracic and lumbar spine were taken at baseline and at follow-up according to the EVOS protocol (19). Spinal x-rays were evaluated by a combination of vertebral morphometry and radiological expert reading for differential diagnosis of vertebral deformity as previously described in detail (26). Severe osteophytosis was defined in the presence of at least one grade 3 or grade 4 osteophyte according to the classification by Nathan (27).

Anthropometric measurements

Standardized measurements of height in centimeters and weight in kilograms were taken at baseline and at follow-up in light clothing with shoes removed. A Seca stadiometer calibrated to 1 cm and Seca scales calibrated to 0.1 kg were used for this purpose. The body mass index (BMI) in kilograms per m^2 was calculated from concurrent measurements of height and weight, and the change in BMI was calculated as the difference between BMI at follow-up and baseline.

BMD measurements

BMD was measured by dual x-ray absorptiometry using the same equipment (QDR 1000, Hologic, Inc., Waltham, MA) and measurement protocol at baseline and at follow-up. Posterior-anterior scans of the left proximal femur and the lumbar spine were performed according to the guidelines of the European Concerted Medical Action for the Quantitation of Osteoporosis (23, 28, 29). The right proximal femur was scanned in only a few cases with a history of hip replacement on the left side. The average of vertebral bone density measurements at L2–L4 and total femoral BMD were chosen for the present analysis. All BMD measurements were calibrated to the semianthropomorphic European Spine Phantom (ESP) (30) as previously described (23, 29). Absolute bone loss in milligrams per cm^2 was calculated as the difference between the follow-up and baseline values; relative bone loss was expressed as the percent change relative to the baseline value.

Quality control procedures included daily measurements of the phantom provided by the manufacturer to assure machine stability. Repeat measurements of the ESP were used to calculate machine precision by dividing the SD at a particular specified density by that density. Based on five independent measurements of the ESP repeated three times at yearly intervals, the mean machine precision was 0.41% (range, 0.24–0.70) at 0.5 g/ cm^2 , 0.63% (range, 0.29–0.97) at 1.0 g/ cm^2 , and 2.03% (range, 1.01–3.04) at 1.5 g/ cm^2 . *In vivo* precision was calculated from two measurements of femoral and vertebral BMD in nine young healthy volunteers with measurements 4–12 weeks apart. The mean short-term precision was 1.1% (range, 0.2–2.5) for total hip BMD and 2.8% (range, 0.6–6.0) for BMD at the lumbar spine.

Baseline and follow-up BMD scans were submitted to external review and centralized evaluation at the Institut für Funktionsanalyse im Gesundheitswesen GmbH (IFH, Hamburg, Germany) according to a standardized protocol based on the manufacturer's recommendations (Institut für Funktionsanalyse im Gesundheitswesen GmbH Quality Management Center, Hologic, Scan Acquisition, and Analysis Guidelines). Of 176 women who participated in both surveys, a total of 168 had complete BMD measurements at both skeletal sites. In four cases missing values were due to the subject's refusal or bilateral hip replacement. The remainder of four BMD scans (two at the lumbar spine and two at the

proximal femur) had to be excluded from the analysis because of false positioning or artifacts.

Biochemical assays

Nonfasting morning venous blood samples were drawn at baseline, separated within 3 h after phlebotomy, and aliquoted. A serum sample was immediately processed in a routine laboratory for a chemistry panel including liver enzymes, creatinine, and total alkaline phosphatase. Serum alkaline phosphatase was measured by an automated colorimetric assay as previously described in detail (31). Laboratory upper normal ranges were 170 U/L for serum alkaline phosphatase, 114.9 $\mu\text{mol/L}$ (1.3 mg/dL) for serum creatinine, 15 U/L for aspartate aminotransferase (AST, SGOT), and 18 U/L for alanine aminotransferase (ALT, SGPT). Additional serum and heparinized plasma aliquots were kept frozen at -80°C until assayed for more specific biochemical parameters. Spot urine specimens were protected from light exposure, and aliquots were stored at -30°C .

IL-6 was measured in previously unfrozen serum samples using a highly sensitive amplified commercial ELISA with an alkaline phosphatase signal amplification system (Quantikine HS, human IL-6 immunoassay, R&D Systems, Inc., Minneapolis, MN). The assay has a sensitivity of 0.012 IU/mL (0.094 pg/mL). The intraassay coefficient of variation was 9%, and the interassay coefficient of variation was 16%. Results were expressed in picograms per mL and converted to international units per mL using the National Institute of Biological Standards and Control/WHO IL-6 International Reference Standard 89/548 (conversion factor = 0.131). In the subgroup of women less than 10 yr after menopause, human IL-1 β (high sensitivity), soluble IL-1 receptor type I, soluble IL-1 receptor type II, IL-1 receptor antagonist, TNF α (high sensitivity), soluble TNF receptor type I, and soluble TNF receptor type II were later measured in remaining serum or plasma aliquots by ELISA (R&D Systems, Inc.).

Circulating levels of sex hormones, adrenal androgens, and sex hormone-binding globulin (SHBG) were assayed in previously unfrozen plasma aliquots as described previously (22). The detection limit for plasma 17 β -estradiol was 20 pmol/L (5.4 pg/mL).

Biochemical markers of bone metabolism and calciotropic hormones were measured in an endocrinological research laboratory from previously unfrozen serum aliquots. The methods of analysis have been previously described in detail (21, 31, 32). Human intact PTH was measured with a two-site luminometric immunoassay (Magic Lite, Bayer Corp., Fernwald, Germany) at an upper normal range of 65 ng/L. The intra- and interassay coefficients of variation were less than 7% and less than 9% for serum bone-specific alkaline phosphatase (S-BAP), less than 10% and less than 15% for serum osteocalcin, less than 5% and less than 12% for 25-hydroxyvitamin D $_3$, less than 6% and less than 11% for human intact PTH, and 10% and 15% for urinary pyridinoline and deoxypyridinoline.

Serum concentrations of TSH were determined with a high-sensitivity TSH-coated tube assay (Johnson & Johnson, Rochester, NY) with a lower normal cut-off of 0.3 mIU/L as previously described (33).

Statistical analysis

SAS software (version 6.12, SAS Institute, Inc., Cary, NC) was used for data analysis. Mean differences in serum IL-6 and other continuous variables between postmenopausal women with and without HRT at baseline or between women within or beyond the first menopausal decade were tested by the general linear model procedure for ANOVA and covariance in unbalanced designs. The strength of cross-sectional associations was assessed by simple and partial Pearson correlation analyses. The distribution of circulating cytokines, SHBG, endogenous sex hormones, and most biochemical markers of bone turnover (serum osteocalcin and urinary cross-links) showed considerable deviation from the normal distribution; hence, these data were transformed to a natural logarithmic scale. For these variables, reported group means from analyses of variance or covariance represent the antilog of mean logarithmic data, which corresponds to the geometric mean in the more

intuitive original measurement scale. Estimates from linear regression models can be related to the original measurement scale by taking the antilogarithm of the log units change.

Linear regression techniques were applied to assess the determinants of serum IL-6 concentrations at baseline and the predictive effect of serum IL-6 on bone loss. Baseline variables examined for their cross-sectional relationship to serum IL-6 included anthropometric variables (age, menopausal age, and BMI), serum creatinine, behavioral factors (physical activity, cigarette smoking, and alcohol consumption), comorbidity (elevated liver enzymes, suppressed TSH, and history of diabetes mellitus), and plasma SHBG and endogenous sex hormone levels. The estrone/androstenedione ratio was computed as an index of aromatase activity. These variables as well as baseline measures of calcium intake, serum levels of calciotropic hormones, and change in health status between baseline and follow-up (change in BMI, immobilization >2 weeks, and history of arthritis or chronic gastrointestinal disease) were examined as confounders of the effect of serum IL-6 on bone loss. The effect of individual factors was first examined in univariate linear models. Main factors added to the multivariable prediction models were serum IL-6, menopausal age, BMI, serum creatinine, serum intact PTH, and, in separate models, plasma SHBG and sex hormone levels. Lifestyle variables and comorbidity were added to multivariable models if they were univariately related to the outcome variable (serum IL-6 or bone loss) at the $P < 0.150$ significance level. The homogeneity of the effect of serum IL-6 on bone loss across menopausal age was tested by including a product term with menopausal age as a continuous or dichotomous (>10 vs. ≤ 10 yr) variable. A cut-off of 10 yr was chosen as the accelerated phase of postmenopausal bone loss is considered to extend through the first decade past menopause (4). $P < 0.05$ was considered statistically significant, based on two-sided tests.

Results

Characteristics of the study population

Women using HRT at baseline ($n = 21$) had significantly lower mean serum levels of IL-6 ($P < 0.001$) than 116 nonusers (Table 1). This group difference remained significant after age adjustment (0.15 vs. 0.21 IU/mL; $P = 0.013$), but was considerably reduced after adjustment for age, BMI, and serum creatinine (0.16 vs. 0.20 IU/mL; $P = 0.083$). No difference in mean serum IL-6 was observed between 103 never users and 13 previous users of HRT (data not shown). Analyses regarding the predictive effect of serum IL-6 on bone loss were restricted to 89 women of known menopausal age who did not receive HRT at baseline or during follow-up. These 89 women did not significantly differ from all 116 nonusers of HRT with respect to baseline characteristics (Table 1).

Descriptives of lifestyle and comorbidity with possible impact on bone loss among the main study population are summarized in Table 2. A total of 11 women had vertebral fractures or severe osteophytosis at the lumbar spine. As both conditions lead to inaccurate measurements of vertebral BMD, these women were excluded from analyses involving vertebral bone loss as an end point.

Women within 10 yr after menopause ($n = 39$; mean menopausal age, 6.2 yr; range, 1–10 yr) did not significantly differ from older postmenopausal women ($n = 50$; mean menopausal age, 18.8 yr; range, 11–39) with respect to baseline lifestyle habits or comorbidity. On the average, women in the first decade after menopause were significantly younger (58.9 vs. 68.2 yr; $P < 0.001$) and more obese (mean BMI, 29.4 vs. 27.2 kg/m 2 ; $P = 0.035$) than women of older menopausal

TABLE 1. Baseline characteristics of the study population

Variable	Postmenopausal women HRT at baseline	Postmenopausal women no HRT at baseline	Main study population ^a
Age (yr)	21, 56.7 (3.2)	116, 63.2 (6.7)	89, 64.1 (6.6)
Age at menopause (yr)	10, 51.2 (3.2)	97, 50.9 (4.6)	89, 50.9 (4.7)
Menopausal age (yr)	10, 6.7 (4.4)	97, 12.7 (8.0)	89, 13.3 (8.1)
BMI (kg/m ²)	21, 25.3 (2.6)	116, 27.9 (4.8)	89, 28.2 (5.0)
Serum creatinine (μmol/L)	21, 77.0 (10.5)	116, 77.6 (13.4)	89, 77.7 (14.1)
BMD total hip (mg/cm ²)	21, 944.8 (143.7)	113, 872.5 (157.1)	86, 864.3 (158.3)
BMD lumbar spine (L2–L4; mg/cm ²)	21, 977.0 (156.3)	115, 948.3 (175.4)	89, 950.5 (172.4)
Serum IL-6 (IU/mL) ^b	21, 0.14 (0.09)	116, 0.21 (0.15)	89, 0.22 (0.15)

Values are baseline biomedical measurements, expressed as number, mean (SD).

^a Nonusers of HRT at baseline or during follow-up with known menopausal age.

^b Geometric means presented; $P < 0.001$ for the difference between users and nonusers of HRT at baseline.

TABLE 2. Descriptives on lifestyle and medical history among 89 postmenopausal women not using HRT, 52–80 yr at baseline

Behavioral characteristics at baseline, n (%)	
Heavy or very heavy physical activity since age 50 yr	14.6 (13)
Current leisure time exercise ≥ 1 h/week	29.2 (26)
Current cigarette smoking	10.1 (9)
Alcohol intake during past year ^a	
Never	12.5 (11)
<1 day a week	54.5 (48)
1–4 days a week	15.9 (14)
≥ 5 days a week	17.0 (15)
Calcium intake from food during past week	
Very low (score <7)	12.4 (11)
Low (score 7–14)	21.3 (19)
Intermediate (score ≥ 14)	38.2 (34)
Highest (score ≥ 7 and milk daily)	28.1 (25)
Health status at baseline, n (%)	
Serum liver enzymes elevated beyond normal ^b	20.2 (18)
Serum TSH <0.3 mIU/L	11.2 (10)
History of type 2 diabetes mellitus ^a	9.1 (8)
Osteoporosis by WHO criteria ^c	32.6 (28)
Severe osteophytosis at lumbar spine ^b	2.2 (2)
Vertebral fractures at lumbar spine	10.1 (9)
Health status since baseline, n (%)	
Immobilization >2 weeks	3.4 (3)
History of arthritis	7.9 (7)
History of chronic gastrointestinal disease	5.6 (5)

^a Information on alcohol use and history of diabetes mellitus missing in one woman.

^b See *Subjects and Methods* for upper normal range of liver enzymes and grading of osteophytes.

^c BMD t score ≤ -2.5 at femoral neck or lumbar spine against reference values for young females (34). $n = 86$ with bone mineral density measurements at both sites.

age. They were also less likely to lose weight between baseline and follow-up (mean change in BMI, +0.46 *vs.* −0.41 kg/m²; $P = 0.029$) and to have osteoporosis at the hip or lumbar spine (18% *vs.* 44.7%; $P = 0.008$) according to WHO criteria (34).

Older postmenopausal women had significantly lower mean serum 25-hydroxyvitamin D concentrations than early postmenopausal women (Table 3), and the difference increased after adjustment for age (38.6 *vs.* 69.0 nmol/L; $P = 0.002$). No group differences were observed with respect to crude mean levels of plasma SHBG and endogenous sex hormones, biochemical markers of bone turnover, and serum intact PTH. This was also true after adjusting for age, BMI, and serum creatinine, except for higher mean adjusted bio-

available testosterone levels (0.20 *vs.* 0.14 nmol/L; $P = 0.040$) in early *vs.* older postmenopausal women. About 20% of women in each group had plasma estradiol levels below 36.71 pmol/L (10 pg/mL).

Distribution of bone loss by menopausal age

Average bone loss at the hip was more pronounced in older than in younger postmenopausal women, and the reverse was observed for vertebral bone loss (Table 3). However, none of these differences was statistically significant. A quadratic model best described the relationship between menopausal age and bone loss, suggesting an initial decline in rates of bone loss with increasing distance from menopause, and a new rise in bone loss rates in women of older menopausal ages (Fig. 1, A and B). For relative annual rates of femoral bone loss, the fitted curve took the shape of an inverse J (Fig. 1A). Starting from an average annual bone loss of approximately 0.5%/yr, rates of bone loss slightly declined during the first 10 yr after menopause and continuously rose thereafter up to an average rate of about 2.5%/yr. An inversely U-shaped curve characterized the relationship of vertebral bone loss rates with menopausal age (Fig. 1B), reflecting that the highest rates of bone loss at an average of 1%/yr were observed in women within the first years after menopause and again in late menopause. No significant linear or curvilinear association was observed between biological age and femoral or vertebral bone loss.

Determinants of serum IL-6 concentrations

Crude mean serum IL-6 concentrations did not significantly differ between early and late postmenopausal women (Table 3). As illustrated in Fig. 2, an effect of menopausal age group on serum IL-6 was at least in part masked by the fact that biological age was linearly and positively related to serum IL-6 in older, but not in younger, postmenopausal women. Among women beyond 10 yr after menopause, age explained 7% of the variability in serum IL-6, although the effect did not reach statistical significance ($P = 0.061$) due to a limited sample size.

In univariate linear models, serum IL-6 was significantly and positively correlated with BMI ($r = 0.30$; $P = 0.004$) and serum creatinine ($r = 0.23$; $P = 0.031$), and a nonsignificant, inverse association existed with serum intact PTH ($r = -0.18$; $P = 0.094$). Behavioral factors, such as smoking, alcohol consumption, and exercise habits or comorbidity at baseline

TABLE 3. Descriptives on change in BMD and baseline biochemical parameters by menopausal age group among 89 postmenopausal women not using HRT, 52–80 yr at baseline

Characteristic	Women ≤ 10 yr past menopause [n, mean (SD)]	Women > 10 yr past menopause [n, mean (SD)]
Annual change in BMD		
Total hip mg/cm ²	39, -4.71 (11.12)	47, -5.73 (15.07)
%	39, -0.47 (1.25)	47, -0.80 (2.04)
Lumbar spine ^a mg/cm ²	35, -3.87 (15.09)	43, 0.91 (17.90)
%	35, -0.40 (1.46)	43, 0.07 (1.98)
Serum IL-6 (IU/mL) ^b	39, 0.23 (0.14)	50, 0.21 (0.16)
Plasma sex hormone levels ^b		
Total estradiol (pmol/L)	39, 46.8 (29.0)	49, 43.7 (13.5)
Estrone (pmol/L)	39, 115.3 (34.6)	49, 103.6 (44.7)
SHBG (nmol/L)	39, 55.5 (34.2)	49, 62.2 (52.0)
Bioavailable testosterone (nmol/L)	36, 0.17 (0.13)	48, 0.15 (0.30)
DHEAS (μ mol/L)	39, 2.35 (1.57)	49, 1.90 (1.85)
Androstendione (nmol/L)	39, 1.86 (1.34)	49, 1.97 (1.17)
Estrone/androstendione ratio	39, 0.06 (0.19)	48, 0.05 (0.05)
Calcitropic hormones		
Serum intact PTH (ng/L)	38, 46.5 (17.2)	46, 41.6 (17.8)
Serum 25-hydroxy-vitamin D (nmol/L) ^c	32, 60.1 (32.3)	38, 46.1 (26.9)
Markers of bone turnover		
Urinary DPD (nmol/mmol Cr) ^b	39, 7.8 (2.9)	48, 7.6 (4.4)
Urinary PYD (nmol/mmol Cr) ^b	39, 29.7 (10.9)	48, 30.0 (15.7)
Serum osteocalcin (μ g/L) ^b	39, 9.7 (3.2)	50, 9.8 (4.9)
Serum TAP (U/L)	39, 132.2 (38.8)	50, 123.6 (27.3)
Serum BAP (μ g/L)	39, 13.1 (5.5)	47, 12.7 (5.4)

DHEAS, Dehydroepiandrosterone sulfate; DPD, deoxypyridinoline; PYD, pyridinoline; TAP, total alkaline phosphatase; BAP, bone-specific alkaline phosphatase.

^a Women with fractures at lumbar vertebrae (n = 9) or severe osteophytosis (n = 2) excluded.

^b Geometric means presented.

^c $P = 0.052$.

showed no association ($P > 0.150$) with IL-6 levels in univariate analyses (data not shown). Univariate associations of serum IL-6 to endogenous sex hormones, SHBG, and adrenal androgen precursors were not statistically significant, but the positive direction of the correlation between total plasma estradiol and serum IL-6 levels needs mentioning ($r = 0.18$; $P = 0.096$).

In multivariable analysis a higher biological age, early postmenopausal status (within 10 yr after menopause), higher BMI, higher serum creatinine, and lower serum intact PTH were all independently related to higher serum IL-6. In addition, the estrone/androstenedione ratio significantly contributed to the model (Table 4). Associations with other sex hormones or SHBG, as evaluated in separate models, were not significant. We examined whether the relationship between sex hormones or SHBG with serum IL-6 was modified by menopausal age group. As BMI was positively related to serum IL-6 as well as to plasma estradiol levels ($r = 0.38$; $P < 0.001$), we also considered a modifying effect of BMI. Adding the respective product terms to multivariable regression models revealed no interaction with menopausal age group. However, the effect of circulating estradiol was modified by BMI, suggesting that a significant and inverse relationship between plasma estradiol and serum IL-6 was diminished with increasing BMI ($\beta = -1.6930$; $P = 0.013$ per log unit increase in estradiol and $\beta = 0.0702$; $P = 0.006$ for the interaction with BMI). Similar interactions with BMI were observed for plasma estradiol when used as a group variable

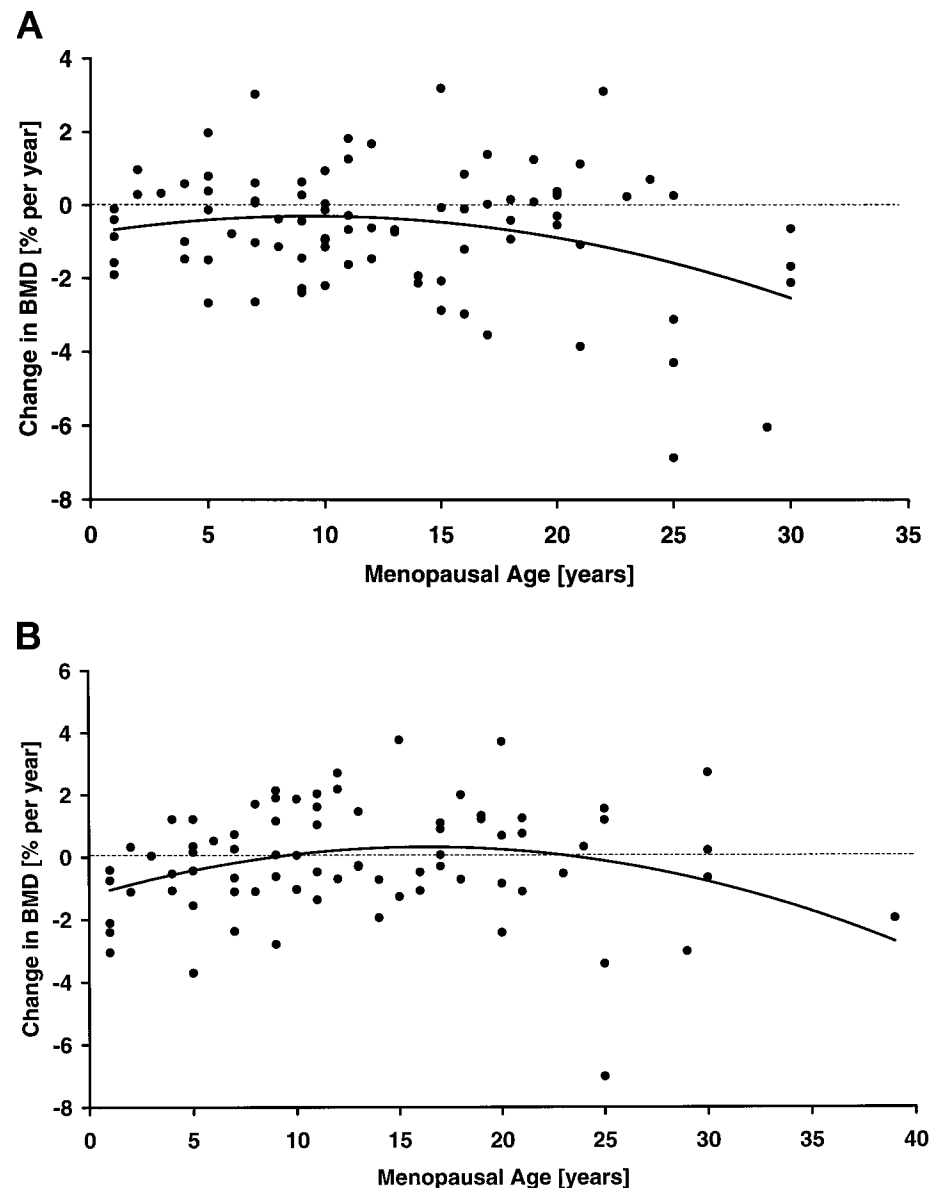
(>36.71 vs. ≤ 36.71 pmol/L) as well as for circulating plasma estrone and dehydroepiandrosterone sulfate (data not shown). Plasma bioavailable testosterone showed no linear relationship to serum IL-6 in multivariable models with or without interaction terms included.

Predictive effect of serum IL-6 on bone loss

Higher serum IL-6 concentrations were strongly related to higher femoral bone loss in univariate linear regression models (Table 5). On the other hand, endogenous sex hormones, including plasma estradiol as a continuous or dichotomous variable (cut-off, 36.71 pmol/L), were not predictive of bone loss in univariate or menopausal age- and BMI-adjusted models. However, a significant protective effect of plasma estradiol, estrone, and bioavailable testosterone on femoral bone loss was apparent in models including serum intact PTH and an interaction between PTH and sex hormones. In models also adjusting for menopausal age and BMI, the protective effect was most pronounced for bioavailable testosterone ($\beta = 21.8471$; $P = 0.004$ per log unit increase in bioavailable testosterone and $\beta = -0.5097$; $P = 0.002$ for the interaction with PTH) and estradiol as a dichotomous variable ($\beta = 23.3418$; $P = 0.013$ for total plasma estradiol levels ≥ 36.71 vs. < 36.71 pmol/L and $\beta = -0.4787$; $P = 0.030$ for the interaction with PTH).

The effect of serum IL-6 on bone loss was not explained by sex hormones or SHBG. This was true in bivariate models and in multivariable models also adjusting for menopausal

FIG. 1. Annual relative change (percent per year) in total hip BMD (A) and vertebral BMD (B) by baseline menopausal age among postmenopausal women without HRT, 52–80 yr at baseline. Regression equations from the quadratic regression models are given by: % change in femoral BMD = $0.0977(\text{menopausal age}) - 0.0052(\text{menopausal age})^2 - 0.7508$ ($P = 0.064$ for the quadratic term) and % change in vertebral BMD = $0.1882(\text{menopausal age}) - 0.0058(\text{menopausal age})^2 - 1.2100$ ($P = 0.012$ for the quadratic term).



age, BMI, serum creatinine, serum intact PTH, and the interaction with PTH (Table 5). Adding interactions between estrogens and BMI did not change the results. Bioavailable testosterone in interaction with PTH was the only sex hormone that remained predictive of femoral bone loss independently of IL-6.

The IL-6 effect also persisted in multivariable regression analysis adjusting for menopausal age, BMI, serum intact PTH, and other factors related to bone loss at the hip in univariate models at the $P < 0.150$ significance level. These included an elevation of liver enzymes at baseline as well as a decrease in BMI and bone-related morbidity (history of chronic gastrointestinal disease, arthritis, or immobilization) between baseline and follow-up. Testing for the homogeneity of the IL-6 effect across menopausal age, a product term between serum IL-6 and menopausal age was added and significantly contributed to the model (Table 5). The direction of the interaction suggests that a significant effect of IL-6 on

bone loss fades with increasing distance from menopause. Apart from serum IL-6, a higher menopausal age, initial elevation of liver enzymes, and morbidity during follow-up remained independently predictive of increased bone loss at the hip (data not shown). A significant interaction between serum IL-6 and menopausal age was also evident from a separate multivariable model fitting the regression of femoral bone loss on menopausal age as a dichotomous variable (>10 vs. ≤ 10 yr), biological age, and additional covariates as described above ($\beta = -13.2762$; $P = 0.002$ per log unit increase in IL-6 and $\beta = 13.6372$; $P = 0.024$ for the product term). From this model, estimates of the independent effect of serum IL-6 on femoral bone loss can be calculated as $\beta = -13.2762$ among early and $\beta = 0.3610$ among late postmenopausal women.

Figure 3, A and B, graphically depicts the univariate relationship between serum IL-6 and absolute femoral bone loss in early and late postmenopausal women, as derived

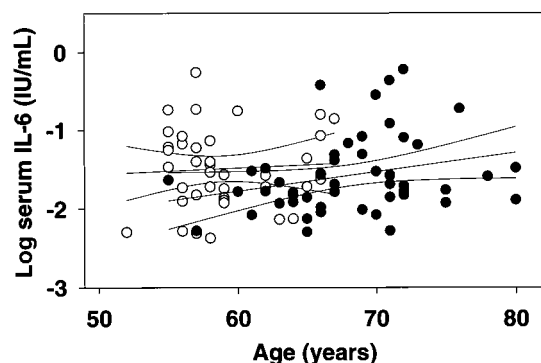


FIG. 2. Linear regression of serum IL-6 concentrations on biological age at baseline among 89 postmenopausal women without HRT stratified by menopausal phase. $\circ$, Early postmenopausal women (≤ 10 yr after menopause; $n = 39$); $\bullet$, women of older menopausal age (> 10 yr after menopause; $n = 50$). The effect of age on serum IL-6 (natural log scale) is described by the following linear regression equations: change in log serum IL-6 = $0.0080(\text{age}) - 1.9591$ ($r^2 = 0.004$; $P = 0.718$) in early postmenopausal women; change in log serum IL-6 = $0.0245(\text{age}) - 3.2422$ ($r^2 = 0.071$; $P = 0.061$) in older postmenopausal women.

from analyses stratified for menopausal age group. In women up to 10 yr after menopause (Fig. 3A), IL-6 explained 34% of the variability in absolute bone loss [$\beta = -12.7650$; $\text{SE}(\beta) = 2.9389$; $P < 0.001$]. In contrast, serum IL-6 was not predictive of femoral bone loss [$\beta = -5.4754$; $\text{SE}(\beta) = 4.5507$; $P = 0.235$] among women of older menopausal age (Fig. 3B). A test for difference in slopes of regression lines from univariate models was not statistically significant ($P = 0.197$). As shown above, the difference increased to statistical significance after accounting for the effect of covariates ($\beta = -13.2762$ vs. $\beta = 0.3610$; $P = 0.024$). Results were similar for relative bone loss at the hip. For example, the unadjusted linear model predicted an annual 1.34% decrease in total femoral BMD per log unit increase in serum IL-6 among early postmenopausal women [$\beta = -1.3397$; $\text{SE}(\beta) = 0.3398$; $r^2 = 0.30$; $P < 0.001$].

Further analyses stratified for menopausal age group confirmed that serum IL-6 was by far the strongest single determinant of femoral bone loss among women within the first decade after menopause ($n = 39$). Other factors univariately related to higher femoral bone loss in this group of early postmenopausal women included a higher BMI, higher total plasma estradiol, higher serum creatinine, higher initial femoral BMD, and a history of immobilization between baseline and follow-up. Among sex hormones, only bioavailable testosterone showed a significant protective effect, again in interaction with serum intact PTH. Adjusting for total plasma estradiol alone or in combination with BMI and the interaction between estradiol and BMI did not explain the IL-6 effect on femoral bone loss. The effect was also independent of BMI and serum creatinine or bioavailable testosterone in interaction with intact PTH or initial femoral BMD and immobilization. Because of the small sample size, the effects of these various sets of possible confounders had to be examined in separate linear regression models. A predictive

effect independent of IL-6 was observed only for higher initial total hip BMD ($r^2 = 0.13$; $P = 0.027$) and immobilization during follow-up ($r^2 = 0.11$; $P = 0.042$), and for bioavailable testosterone in interaction with PTH ($r^2 = 0.10$; $P = 0.085$). Serum IL-6 was consistently found to be the most important predictor ($r^2 = 0.17$ – 0.33) in all models.

Among women more than 10 yr after menopause, factors univariately related to higher bone loss included a higher menopausal age, lower initial femoral BMD, elevation of liver enzymes at baseline, decrease in BMI since baseline, and bone-related morbidity during follow-up. Consistent with our observations in the entire study population, plasma levels of endogenous sex hormones, in particular estrone and bioavailable testosterone, were predictive of bone loss in interaction with serum intact PTH. The small sample size precluded more complex multivariable analyses to assess the independent effect of individual predictors of bone loss in older postmenopausal women.

A linear effect of higher serum IL-6 levels to higher bone loss at the lumbar spine was also restricted to the early postmenopausal phase. Up to 6 yr after menopause, we observed a similar, albeit nonsignificant, effect of serum IL-6 on vertebral bone loss ($\beta = -13.4085$; $r^2 = 0.16$; $P = 0.101$), but the number of observations in this subgroup was reduced to only 18 women.

Predictive effect of biochemical markers of bone turnover on bone loss

For comparison, we also assessed the predictive effect of markers of bone turnover on bone loss among postmenopausal women. Higher serum S-BAP was the only biochemical marker to predict increased bone loss. In univariate linear regression models, this marker explained 5% and 8% of the total variability of bone loss at the hip or at the lumbar spine respectively. Similar results were obtained from multivariable models controlling for menopausal age, BMI, serum creatinine, change in BMI, comorbidity factors, and seasonal variation. Interaction terms between markers and menopausal age or menopausal age group did not significantly contribute to these models. The significant effect of serum IL-6 on femoral bone loss as well as the interaction with menopausal age group persisted in multivariable models, also adjusting for S-BAP (data not shown).

Discussion

In the present study serum IL-6 was predictive of femoral bone loss in postmenopausal women. The effect lessened with increasing distance from menopause, and was no longer significant in women beyond the first postmenopausal decade. In women up to a menopausal age of 10 yr, no other hormonal, biochemical, or anthropometric variable approached IL-6 in its ability to predict femoral bone loss or explained the IL-6 effect.

A similar pattern for the effect of serum IL-6 on vertebral bone loss was observed, although the association was considerably weaker and less consistent. This observation seems puzzling, given the fact that the spine is the predominant skeletal site of accelerated bone loss. However, many other

TABLE 4. Determinants of serum IL-6 concentrations among 89 postmenopausal women not using HRT, 52–80 yr at baseline

Independent variable	Unit change	β^a	95% CI	P value	r ²
Menopausal age group	>10 vs. ≤10 yr	−0.30	−0.56; −0.03	0.032	0.06
Age	5 yr	0.12	0.02; 0.22	0.022	0.07
BMI	1 SD	0.19	0.09; 0.29	<0.001	0.16
Serum creatinine	1 SD	0.13	0.03; 0.23	0.010	0.09
Serum PTH	1 SD	−0.15	−0.25; −0.05	0.004	0.11
Estrone/androstendione ratio ^b	1 SD	−0.11	−0.21; −0.01	0.031	0.06

CI, Confidence interval.

^a Predicted change in log units of serum IL-6 (international units per mL) per unit increase in independent variable, adjusted for all other variables in the model, based on 82 observations with complete data.

^b Transformed to the natural log scale.

TABLE 5. Prediction of annual femoral bone loss (milligrams per cm²) by serum IL-6 among 89 postmenopausal women not using HRT, 52–80 yr at baseline

Model	Unit change	β^a	95% CI	P value	r ²
Univariate					
Serum IL-6	1 log unit	−8.70	−14.16; −3.24	0.002	0.10
Multivariate, model 1 ^b					
Serum IL-6	1 log unit	−9.23	−15.22; −3.24	0.004	0.11
Multivariate, model 2 ^c					
Serum IL-6	1 log unit	−18.58	−30.36; −6.79	0.003	0.12
Serum IL-6 × menopausal age	1 log unit × yr	0.93	0.06; 1.79	0.040	0.06

^a Predicted annual change in total hip BMD (milligrams per cm²) per log unit increase in serum IL-6.

^b Adjusted for menopausal age, BMI, serum creatinine, serum intact PTH, plasma estradiol, and the interaction between estradiol and PTH, based on 80 observations with complete data.

^c Adjusted for menopausal age, BMI, serum intact PTH, elevation of liver enzymes, change in BMI and bone-related morbidity (immobilization, arthritis, or chronic gastrointestinal disease) during follow-up, based on 81 observations with complete data.

associations were also much weaker for the spine than for the femur, pointing, rather, at a measurement problem. This is consistent with findings in other population-based studies, which have suggested that measurements of vertebral BMD changes may be misleading with respect to the overall changes in skeletal BMD (35–38). Alternatively, an effect of serum IL-6 on cancellous bone may be best seen within very close distance to menopause; it would then be masked in our study due to the small number of women within the first few postmenopausal years.

The observation that the predictive effect of IL-6 on bone loss was restricted to the first 10 yr after menopause confirms results from cell culture studies previously reported by our group and others. *In vitro* studies of cytokine secretion in circulating human mononuclear cells (15, 16) and human bone marrow cells (17) have shown increased secretion rates of IL-6 and related cytokines in cells from early, but not late, postmenopausal women compared with baseline levels in cells from premenopausal women. The fact that the IL-6 effect on bone loss is modified by menopausal age may explain why an association between serum IL-6 and BMD was not detected in a previous cross-sectional study by other researchers (13). We found a cross-sectional association of initial femoral BMD to serum IL-6 only after stratification for menopausal age group, and the relationship was evident in women of younger, but not older, menopausal age (data not shown).

The precise mechanism for the restriction of the association between IL-6 and bone loss to early menopause remains to be elucidated. There are two possible explanations for the

observed differential effect of circulating IL-6 levels on bone loss among early and older postmenopausal women. First, there may be a selective increase in the sensitivity of bone cells toward IL-6 in early menopause. Indeed, recent *in vitro* studies have demonstrated that ovariectomy up-regulates the expression of the two subunits of the IL-6 receptor in stromal cells in ex vivo murine bone marrow cultures (39). Secondly, elevated serum IL-6 concentrations in older postmenopausal women may be less likely to reflect increased IL-6 secretion in bone due to estrogen deficiency. Serum IL-6 concentrations are known to rise with age, as has been shown in the present analysis and in a previous cross-sectional study of 80 healthy women by others (14). We were able to demonstrate that a higher biological age and early postmenopausal status (within 10 yr after menopause) were significantly and independently related to higher serum IL-6 levels. Although the metabolic pathways underlying the age-related increase in serum IL-6 are still subject to research, recent data point to a possible link with atherosclerosis (40, 41).

This leaves us with the question of what factors may determine individual differences in serum IL-6 concentrations during the early postmenopausal phase. Although current HRT users were found to have lower serum IL-6 than non-users in the present study and in one previous population-based study (42), a relationship between serum IL-6 and endogenous estrogens was not obviously present among younger or older postmenopausal women. A link between endogenous estrogen activity and serum IL-6 was indicated by the observed inverse association of serum IL-6 to the

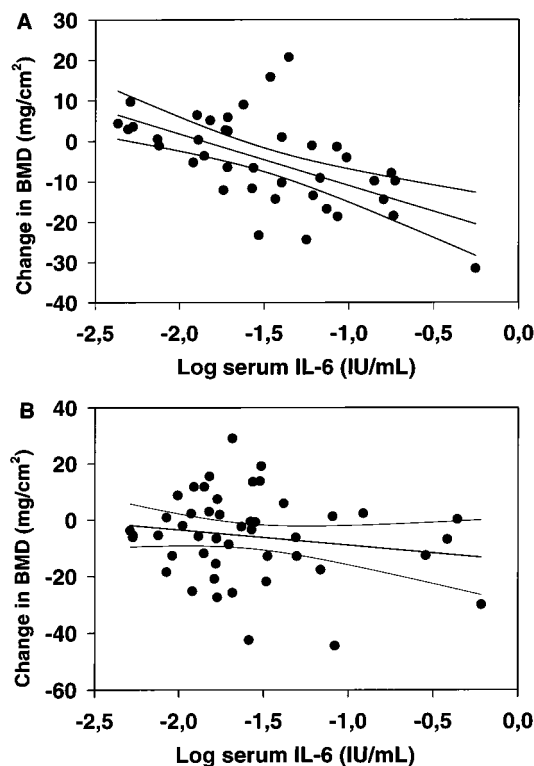


FIG. 3. Annual change (milligrams per cm²) in femoral BMD (total hip) by serum IL-6 levels (IU per mL) in 89 postmenopausal women without HRT. Women are stratified according to baseline menopausal age into those 10 yr or less (A) and those more than 10 yr (B) after menopause. The slope of the regression line significantly differs from zero among women within the first decade after menopause [$n = 39$; change in femoral BMD (mg/cm²) = $-12.7650 (\log \text{ serum IL-6}) - 23.7331$ ($r^2 = 0.338$; $P < 0.001$), but not in women more than 10 yr postmenopause [$n = 47$; change in femoral BMD (mg/cm²) = $-5.4754 (\log \text{ serum IL-6}) - 14.4868$ ($r^2 = 0.031$; $P = 0.235$)].

estrone/androstenedione ratio and to serum intact PTH. However, as previously reported by McKane and colleagues (14), IL-6 and total plasma estradiol in our population were rather positively related in univariate linear models. We found that the association between serum IL-6 and residual estrogen concentrations was modified by BMI, as an inverse relationship between IL-6 and endogenous estrogens was increasingly concealed at higher levels of BMI. Results from a recent population-based study (42) support our observation that obesity, as estimated by BMI, is strongly correlated with higher serum IL-6 concentrations, and that BMI and estrogen status may interact in the determination of circulating IL-6. It remains to be shown how environmental, hormonal, and genetic factors act together in the determination of IL-6 expression and serum IL-6 levels after menopause. The first evidence of an association between bone mass and polymorphisms in the IL-6 gene or related genes is emerging (43, 44).

Differences in residual estradiol, SHBG, or other sex hormone concentrations did not explain the IL-6 effect on bone loss, nor were they related to bone loss in univariate or menopausal age- and BMI-adjusted linear models. Based on the results from a large population-based cohort study of postmenopausal women 65 yr and over, estradiol levels be-

low 18.36 pmol/L (5 pg/mL) may be critical in the prediction of bone loss (2, 3) and osteoporotic fractures (2, 5). We cannot exclude that the immunological assay system we used may have not been sensitive enough to demonstrate a relationship between plasma estradiol and bone loss. On the other hand, plasma estradiol, as both a continuous and a dichotomous variable (cut-off, 36.71 pmol/L), as well as estrone and bioavailable testosterone did show a protective effect on femoral bone loss in interaction with serum intact PTH in our dataset, whereas SHBG demonstrated an inverse association. This supports the concept that there is a later, cytokine-unrelated effect of endogenous estrogens on bone metabolism, perhaps mediated by an effect on extraskeletal calcium homeostasis (4).

As IL-6 is known to exert its osteoclastogenic effects as part of a complex cytokine network (10), we subsequently determined serum concentrations of related cytokines (IL-1 β , soluble IL-1 receptor type I and type II, IL-1 receptor antagonist, TNF α , and soluble TNF receptor type I and type II) from remaining serum or plasma aliquots in the subset of early postmenopausal women (data not shown). None of these cytokines was significantly related to bone loss. This could result from a greater analytical variability or a more pronounced difference between systemic cytokine levels and those in the local bone environment.

In the present study biochemical markers of bone turnover were not predictive of bone loss in postmenopausal women, with the exception of serum S-BAP. This is in apparent contrast to previous findings demonstrating a predictive effect of markers of bone resorption, in particular pyridinium cross-links, on fracture risk in elderly women (45, 46). A potential bias toward a younger and healthier population sample, as described below, may have been responsible for underestimation of the ability of these markers to predict bone loss in women of the present study. On the other hand, there is increasing evidence that biochemical markers of bone turnover may be poor predictors of bone loss during the early postmenopausal years (47, 48). As low bone mass is one major risk factor for fragility fractures in the elderly (49), and accelerated bone loss in early postmenopause is believed to be the most important contributing factor to low bone mass in women (1), the ability of IL-6 to specifically predict bone loss during the early postmenopausal years may be of great clinical significance.

The strengths of the present study lie in the population-based setting and the longitudinal design. Furthermore, we were able to analyze the relationship between IL-6 and bone loss in the light of other endogenous and environmental factors known or suspected to be related to age-related bone loss (3, 35, 50–52).

Our study also has several major limitations. First, the number of women was small, limiting multivariable analyses and the precision of estimates derived from the prediction models. Secondly, as response rates to the initial survey at baseline were only 58%, we cannot rule out that our results were affected by selection bias. Comparisons of age, health and functional status between participants and nonparticipants in the Heidelberg EVOS cohort as well as for all Ger-

man study centers combined indicated that a selective participation of younger and healthier individuals already occurred at baseline (53). Loss of individuals during follow-up, which is of concern to all longitudinal studies, almost certainly added to this effect. As older, less frail, and chronically ill subjects are most likely to be underrepresented in the study population, it will not be truly representative of all postmenopausal women, and the findings have to be interpreted with care. Bone loss as well as the predictive ability of IL-6, sex hormones, and biochemical markers of bone turnover may have been underestimated, particularly among older postmenopausal women.

In summary, this is the first study to show that serum IL-6 is a predictor of postmenopausal bone loss, and that the effect appears to be most relevant through the first postmenopausal decade. In line with previous results from *in vitro* and animal studies, these findings support the hypothesis that IL-6 is an important mediator of bone loss during the first accelerated phase of bone loss, whereas other mechanisms may be more relevant to bone loss in later postmenopausal life. However, our correlative data cannot prove a cause-effect relationship. Serum IL-6 could be merely a marker of some related causal pathomechanism. Notably, the IL-6 effect on femoral bone loss was not explained by residual estradiol levels, although there was evidence for an association between lower endogenous estrogen activity and higher IL-6 levels. Further studies are warranted to elucidate the mechanisms underlying our observations and to determine whether serum IL-6 also predicts the risk of osteoporotic fracture.

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