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## Homocysteine Levels and the Risk of Osteoporotic Fracture

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### ABSTRACT

#### BACKGROUND

Very high plasma homocysteine levels are characteristic of homocystinuria, a rare autosomal recessive disease accompanied by the early onset of generalized osteoporosis. We therefore hypothesized that mildly elevated homocysteine levels might be related to age-related osteoporotic fractures.

#### METHODS

We studied the association between circulating homocysteine levels and the risk of incident osteoporotic fracture in 2406 subjects, 55 years of age or older, who participated in two separate prospective, population-based studies. In the Rotterdam Study, there were two independent cohorts: 562 subjects in cohort 1, with a mean follow-up period of 8.1 years; and 553 subjects in cohort 2, with a mean follow-up period of 5.7 years. In the Longitudinal Aging Study Amsterdam, there was a single cohort of 1291 subjects, with a mean follow-up period of 2.7 years. Multivariate Cox proportional-hazards regression models were used for analysis of the risk of fracture, with adjustment for age, sex, body-mass index, and other characteristics that may be associated with the risk of fracture or with increased homocysteine levels.

#### RESULTS

During 11,253 person-years of follow-up, osteoporotic fractures occurred in 191 subjects. The overall multivariable-adjusted relative risk of fracture was 1.4 (95 percent confidence interval, 1.2 to 1.6) for each increase of 1 SD in the natural-log-transformed homocysteine level. The risk was similar in all three cohorts studied, and it was also similar in men and women. A homocysteine level in the highest age-specific quartile was associated with an increase by a factor of 1.9 in the risk of fracture (95 percent confidence interval, 1.4 to 2.6). The associations between homocysteine levels and the risk of fracture appeared to be independent of bone mineral density and other potential risk factors for fracture.

#### CONCLUSIONS

An increased homocysteine level appears to be a strong and independent risk factor for osteoporotic fractures in older men and women.

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**O**STEOPOROSIS IS A MAJOR HEALTH problem that is characterized by low bone mineral density, deterioration of bone microarchitecture, and an increased risk of fracture.<sup>1</sup> Osteoporotic fractures are associated with increased morbidity and mortality and with substantial economic costs.<sup>2-4</sup>

It has been hypothesized that the metabolism of homocysteine is involved in osteoporosis. Homocystinuria, a rare autosomal recessive disease characterized by markedly elevated levels of plasma homocysteine, has several clinical manifestations involving the eyes, the vasculature, and the central nervous system. The presence of homocystinuria is associated with the early onset of generalized osteoporosis.<sup>5,6</sup> The underlying pathophysiological mechanism for the occurrence of early osteoporosis in patients who have homocystinuria is not completely understood. However, *in vivo* and *in vitro* studies support the concept that a homocysteine-associated disturbance in collagen cross-linking in bone is involved.<sup>7-11</sup>

In the general population, a mildly elevated plasma level of homocysteine, termed hyperhomocysteinemia, is a common condition. Hyperhomocysteinemia is recognized as a major risk factor for atherosclerotic and thromboembolic disease<sup>12</sup> as well as for cognitive impairment, including that seen in Alzheimer's disease.<sup>13,14</sup> Although a previous study suggested the possible involvement of increased plasma homocysteine levels in age-dependent bone loss,<sup>15</sup> the role of moderately elevated plasma homocysteine levels in diseases of the skeletal system — in particular, osteoporotic fracture — is unknown. To examine the influence of homocysteine on osteoporosis, we studied the relationship between circulating levels of homocysteine and the incidence of fracture in two independent, prospective studies of three groups of men and women 55 years of age or older.

## METHODS

### STUDY SUBJECTS

We analyzed data from two independent samples, one sample from the Rotterdam Study consisting of two cohorts of subjects, and the other sample from the Longitudinal Aging Study Amsterdam (LASA). The Rotterdam Study is a prospective, ongoing population-based, cohort study of persons 55 years of age or older residing in the Ommoord district of the city of Rotterdam, in the Netherlands. The study

was designed to investigate chronic, disabling diseases. The rationale and design of the study have been described previously.<sup>16</sup> The baseline examination included 7983 subjects. The medical ethics committee of the Erasmus Medical Center approved the Rotterdam Study.

Two independent, nonoverlapping samples of subjects were included in the present study. At baseline (1991–1993), a random sample of 562 subjects was studied (cohort 1), and at a follow-up visit (1995–1996), a second sample of 553 subjects was studied (cohort 2). Cohort 2 was originally recruited for a study of age-related changes in the brains of elderly persons,<sup>17</sup> and the exclusion criteria were dementia, blindness, and the presence of standard contraindications to the use of magnetic resonance imaging. The subjects in cohort 2 ranged in age from 60 to 90 years and were randomly selected, with stratification according to age (in five-year groups) and sex.

LASA is an ongoing cohort study of the predictors and consequences of changes in autonomy and well-being in older persons in the Netherlands. The procedures used in sampling and data collection have been described in detail elsewhere.<sup>18</sup> Briefly, a sample of persons 55 to 85 years of age, stratified according to age, sex, and level of urbanization of residence (number of addresses per square kilometer), was drawn from the population registers of 11 municipalities. At the baseline examination (in 1992 or 1993), 3107 subjects participated. The present study was performed with a subsample of 1291 persons who were interviewed at the time of the second collection of data (in 1995 or 1996) and who were 65 years of age or older on January 1, 1996. The medical ethics committee of the Vrije Universiteit Medical Center approved the study.

All the subjects in the Rotterdam Study and LASA who participated in the present study gave written informed consent.

### ASSESSMENT OF FRACTURES

In the Rotterdam Study, general practitioners monitored the subjects for incident fractures, which were reported by means of a computerized system. Events were classified independently by two research physicians according to the *International Statistical Classification of Diseases and Related Health Problems, 10th Revision (ICD-10-CM)*.<sup>19</sup> An expert in osteoporosis reviewed all coded events for final classification. For this study, follow-up ended on January 1, 2002.

In LASA, fractures that occurred between the

second examination (in 1995 or 1996) and the third examination (in 1998 or 1999) were recorded prospectively on a calendar. Information about fractures was noted retrospectively for respondents who did not participate in the follow-up. All reported fractures were verified by a physician.

To ensure sufficient statistical power, a fracture in any skeletal location was documented as an outcome measure. All fractures that were considered to be nonosteoporotic (i.e., fractures due to cancer or to an accident, such as a motor vehicle accident, and all hand, foot, skull, and facial fractures) were excluded. The period of follow-up was calculated as the time from enrollment in the study to the first fracture, death, or the end of the planned follow-up period, whichever occurred first. For subjects lost to the study during follow-up, the follow-up period was calculated as the time from enrollment to the date of the last contact with the subject.

#### MEASUREMENT OF BONE MINERAL DENSITY

Bone mineral density was measured by dual-energy x-ray absorptiometry at the femoral neck and lumbar spine (vertebrae L2–L4 in the Rotterdam Study and L1–L4 in LASA).<sup>20,21</sup> In the Rotterdam Study, bone mineral density was measured with the use of a Lunar DPX-L densitometer (Lunar), and in LASA, measurements were made with the use of a Hologic QDR 2000 densitometer (Hologic).

#### MEASUREMENT OF HOMOCYSTEINE LEVELS

Blood samples were placed on ice immediately, processed within 60 minutes, and kept frozen until homocysteine levels were measured. For cohort 1 of the Rotterdam Study, serum samples were obtained from subjects who were not fasting, and total homocysteine levels were determined as a fluorescence derivative with the use of high-pressure liquid chromatography.<sup>22,23</sup> For cohort 2 of the Rotterdam Study, plasma samples treated with sodium citrate were obtained from subjects who were not fasting, whereas in the LASA group, EDTA-treated plasma samples were obtained in the morning, after subjects had eaten a light breakfast. Total homocysteine levels were measured with the use of a fluorescence polarization immunoassay on an IMx analyzer (Abbott Laboratories).

#### POTENTIAL CONFOUNDERS

Height and weight were measured while the study subjects were wearing lightweight clothing and no shoes. Data on the body-mass index before the pres-

ent study were available for cohort 2 of the Rotterdam Study (over a period of approximately 2.4 years) and for subjects in LASA (over a period of approximately 3.0 years), and we calculated the change in the body-mass index between the most recent previous visit and enrollment in the present study. Current smoking status and the number of falls in the preceding year were assessed with the use of a questionnaire.

Levels of serum creatinine were measured with the use of standard laboratory procedures. In the Rotterdam Study, the presence of type 2 diabetes mellitus was defined by the current use of antidiabetic medication or by a nonfasting or post-load plasma glucose level above 11 mmol per liter. Peripheral arterial disease was evaluated as described previously.<sup>24</sup>

In cohort 1 of the Rotterdam Study, dementia was diagnosed with the use of the Mini-Mental State Examination and the Geriatric Mental State Schedule,<sup>25</sup> and dietary intake of calories, protein, calcium, 25-hydroxyvitamin D, folate, and vitamins B<sub>6</sub> and B<sub>12</sub> during the preceding year was assessed with the use of a food-frequency questionnaire.<sup>26</sup> In LASA, the presence of diabetes and peripheral arterial disease was assessed with the use of a detailed questionnaire concerning self-reported chronic disease.<sup>27</sup> Cognitive impairment was diagnosed with the use of the Mini-Mental State Examination; a score below 24 was considered to be positive for cognitive impairment. Levels of serum 25-hydroxyvitamin D were measured with the use of a competitive protein-binding assay (Nichols Institute Diagnostics).

#### STATISTICAL ANALYSIS

The distribution of the plasma homocysteine levels was skewed toward higher values. Therefore, we used natural-log-transformed values, which provided the best-fitting model for analyses in which the plasma homocysteine levels were treated as a continuous variable. In order to compare the homocysteine levels in the three cohorts with one another, sex-specific standard-deviation scores were calculated separately for each subject in each cohort. The standard-deviation score was calculated with the formula  $(\text{hcys}_i - \text{hcys}_m) \div \text{SD}$ , where  $\text{hcys}_i$  is the natural-log-transformed homocysteine level in the individual subject,  $\text{hcys}_m$  the mean natural-log-transformed homocysteine level in the cohort, and SD the standard deviation of the natural-log-transformed homocysteine level in the cohort. This cal-

ulation allowed us to determine the increase in the risk of fracture for each increment of 1 SD in the natural-log-transformed homocysteine level.

The relation between the risk of fracture and various homocysteine levels was evaluated with a quartile-based analysis. The quartiles were defined in a sex-specific and age-specific manner for each of the five-year categories.

Cox proportional-hazards regression analysis was used to estimate the risk of fracture. Data were either pooled or were analyzed for each study sample. When all subjects were included, the analysis was adjusted for the study cohort. All estimated risks of fracture were adjusted for age and sex. Additional analyses were adjusted for body-mass index, smok-

ing status, presence or absence of a history of recent falls, and serum creatinine levels. In further analyses, we also adjusted for recent changes in the body-mass index and for dietary intake of calories, protein, calcium, 25-hydroxyvitamin D, folate, and vitamins B<sub>6</sub> and B<sub>12</sub> (or for serum 25-hydroxyvitamin D), and the presence or absence of diabetes mellitus, dementia (or cognitive impairment), and peripheral arterial disease.

Population attributable risks were calculated with the use of the formula  $\{P(RR-1) \div [P(RR-1) + 1]\} \times 100$ , where P is the percentage of the population exposed and RR is the relative risk. We calculated the 95 percent confidence interval by determining the 95 percent confidence interval for log (P[RR-1]) on the basis of the standard errors for P and RR, with the use of the delta method, and transforming back to the 95 percent confidence interval for the population attributable risk.

**Table 1. Baseline Characteristics of Study Subjects.\***

Characteristic	Rotterdam Study		LASA (N=1291)
	Cohort 1 (N=562)	Cohort 2 (N=553)	
Women — no. (%)	351 (62)	278 (50)	663 (51)
Age — yr	70.3±8.8	73.6±7.9	75.6±6.6
Body-mass index†	26.5±3.9	26.3±3.6	26.8±4.2
Current smoker — %	24	21	18
Fall in previous year — %	21	NA	32
Homocysteine level — μmol/liter‡	15.9±5.7	11.9±4.3	14.7±6.0
Serum creatinine level — μmol/liter§	82.6±20.4	89.1±20.2	93.6±22.0
Bone mineral density — g/cm <sup>2</sup> ¶			
Lumbar spine	1.09±0.20	1.11±0.21	0.97±0.19
Femoral neck	0.85±0.13	0.83±0.15	0.70±0.13
Follow-up — yr	8.1±3.7	5.7±1.9	2.7±0.7
Loss to follow-up — %	2.3	3.8	4.3
Incidence of fracture — no./1000 person-yr	18.2	15.8	16.4

\* Plus-minus values are means ±SD. LASA denotes the Longitudinal Aging Study Amsterdam, and NA not available.

† The body-mass index is the weight in kilograms divided by the square of the height in meters.

‡ In the Rotterdam Study, homocysteine levels were determined in serum with the use of high-performance liquid chromatography for cohort 1 and in sodium citrate-treated plasma with the use of an immunoassay for cohort 2. In LASA, homocysteine levels were determined in EDTA-treated plasma with the use of an immunoassay.

§ To convert the values for creatinine to milligrams per deciliter, divide by 88.4.

¶ Measurements of bone mineral density were available for 474 subjects in cohort 1 of the Rotterdam Study, 475 subjects in cohort 2, and 515 subjects in LASA. The measurements used for subjects in cohort 2 of the Rotterdam Study had been obtained approximately 2.4 years before the study began. In the Rotterdam Study bone mineral density was measured with the use of a Lunar densitometer, and in LASA with the use of a Hologic densitometer. Bone mineral density was measured at the lumbar spine in vertebrae L1–L4 in the Rotterdam Study and in vertebrae L2–L4 in LASA.

## RESULTS

### BASELINE CHARACTERISTICS

Selected baseline characteristics of the study subjects in the three cohorts are shown in Table 1. The three cohorts differed significantly with regard to mean age and sex ratio. Mean homocysteine levels were different in all three cohorts; the levels increased with age and were higher in men than in women in all three cohorts (see the Supplementary Appendix, available with the full text of this article at [www.nejm.org](http://www.nejm.org)).

### HOMOCYSTEINE LEVELS AND FRACTURE RISK

During 11,253 person-years of follow-up, 191 subjects (135 women and 56 men) sustained an osteoporotic fracture; a majority were hip and wrist fractures (Table 2). High homocysteine levels were associated with an increased risk of fracture (Table 3). After adjustment for age and sex, the overall relative risk of fracture for each increment of 1 SD in the homocysteine level was 1.3 when all subjects were pooled. The risk of fracture with increasing homocysteine levels was similar in all three groups of subjects (data not shown). The risk was similar in men and women: 1.4 (95 percent confidence interval, 1.1 to 2.8) in men, and 1.3 (95 percent confidence interval, 1.1 to 1.5) in women.

Because the three cohorts differed with regard to age and sex distribution (Table 1), the subjects were grouped in sex- and age-specific quartiles according

to the homocysteine level. In all three cohorts, subjects in the highest quartile had an increase in the risk of fracture so that the risk was twice as high as the risk in each of the lower three quartiles. We subsequently analyzed the homocysteine levels divided into the highest quartile (risk group) and the lower three quartiles combined (reference group) (Table 3). The absolute cutoff values used to define the risk groups are described in the Supplementary Appendix. Subjects in whom homocysteine levels were

above the cutoff value had a risk of fracture that was two times as high as that for subjects with lower values. The risk estimates were similar in all three cohorts. The frequency of nontraumatic vertebral fracture was doubled in the highest quartile (risk group) (Table 2), although this trend did not reach statistical significance ( $P=0.26$ ). Figure 1 shows the cumulative incidence of fracture in the three cohorts according to the age-specific quartile of homocysteine levels.

**Table 2. Distribution of Types of Incident Fracture According to Study Cohort and the Quartile of Homocysteine Level.**

Type of Fracture	Rotterdam Study		LASA	Total	Homocysteine Level	
	Cohort 1	Cohort 2			Quartiles 1–3	Quartile 4
	<i>number (percent)</i>					
Hip	22 (27)	11 (22)	18 (31)	51 (27)	36 (29)	15 (23)
Wrist	21 (25)	14 (28)	18 (31)	53 (28)	37 (29)	16 (25)
Upper humerus	10 (12)	3 (6)	8 (14)	21 (11)	15 (12)	6 (9)
Vertebrae	7 (8)	7 (14)	2 (3)	16 (8)	8 (6)	8 (12)
Extremities*	19 (23)	10 (20)	8 (14)	37 (19)	25 (20)	12 (18)
Rib and sternum	3 (4)	3 (6)	3 (5)	9 (5)	3 (2)	6 (9)
Pelvis	1 (1)	2 (4)	1 (2)	4 (2)	2 (2)	2 (3)
Total	83 (100)	50 (100)	58 (100)	191 (100)	126 (100)	65 (100)

\* Extremities include the arm, leg, and ankle.

**Table 3. Results of Multivariate Analyses of the Relationship between Homocysteine Levels and the Risk of Fracture in the Three Study Cohorts.**

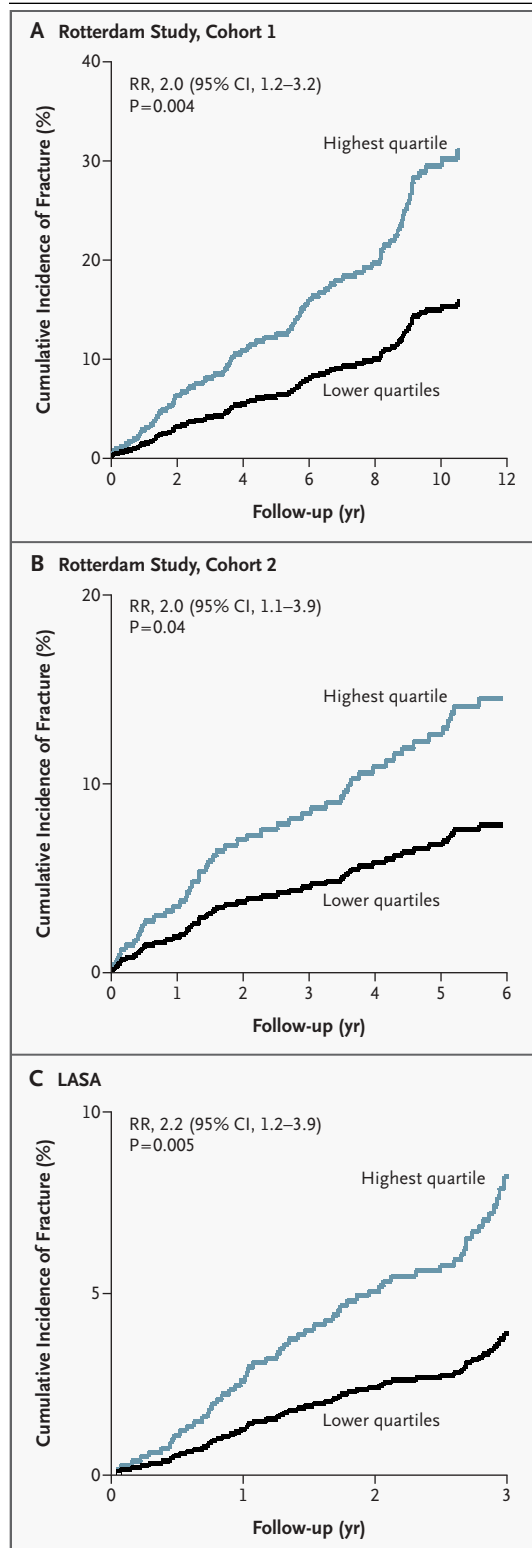
Analysis	Continuous Analysis*		Quartile-Specific Analysis†	
	No. of Fractures/ No. of Subjects	RR (95% CI)	No. of Fractures/ No. of Subjects	RR (95% CI)
Adjusted for age and sex	191/2406	1.3 (1.1–1.5)	191/2406	1.9 (1.4–2.6)
Multivariate 1‡	180/2332	1.4 (1.2–1.6)	180/2332	2.0 (1.4–2.8)
Multivariate 2§	180/2332	1.4 (1.2–1.6)	180/2332	2.0 (1.4–2.7)

\* Homocysteine levels were analyzed as a continuous measure. The relative risk (RR) is for each increment of 1 SD in the natural-log–transformed homocysteine value. CI denotes confidence interval.

† The homocysteine level was analyzed as a dichotomous variable. The relative risk is for subjects in the highest quartile of homocysteine levels, as compared with subjects in the three lower quartiles.

‡ This analysis included adjustments for age, sex, body-mass index, changes in body-mass index before entry into the study, smoking status, presence or absence of a history of recent falls, and serum creatinine level. Data for changes in previous body-mass index were not available for cohort 1 of the Rotterdam Study, and data for recent falls were not available for cohort 2.

§ This analysis included adjustments for age, sex, body-mass index, smoking status, and presence or absence of a history of recent falls, diabetes mellitus, dementia (in the Rotterdam Study) or cognitive impairment (in LASA), peripheral arterial disease, and serum creatinine level.



**Figure 1.** Cumulative Incidence of Fracture among Study Subjects with Homocysteine Levels in the Highest Age- and Sex-Specific Quartile as Compared with All Other Subjects.

RR denotes relative risk, and CI confidence interval.

**HOMOCYSTEINE LEVELS AND BONE MINERAL DENSITY**

As shown in Figure 2, after adjustment for age and sex, homocysteine levels were not associated with bone mineral density at either the femoral neck or the lumbar spine. When we included bone mineral density in the multivariate regression model, the risk estimates were not substantially changed.

**POSSIBLE CONFOUNDING VARIABLES**

The association between homocysteine levels and the risk of fracture was not reduced after adjustment for the body-mass index, changes in the body-mass index, smoking status, recent falls, serum creatinine levels, and the presence or absence of diabetes mellitus, peripheral arterial disease, and dementia or cognitive impairment (Table 3). In addition, the observed association between homocysteine levels and the risk of fracture in cohort 1 of the Rotterdam Study was not substantially reduced after adjustment for dietary intake of calories, protein, calcium, and vitamins (data not shown). The same analysis could not be performed for cohort 2 of the Rotterdam Study or for LASA, because data on dietary intake were not available for those cohorts. Instead, serum levels of 25-hydroxyvitamin D were used as a measure of nutritional status for subjects in LASA, and adjustment for this covariable did not alter the risk estimates.

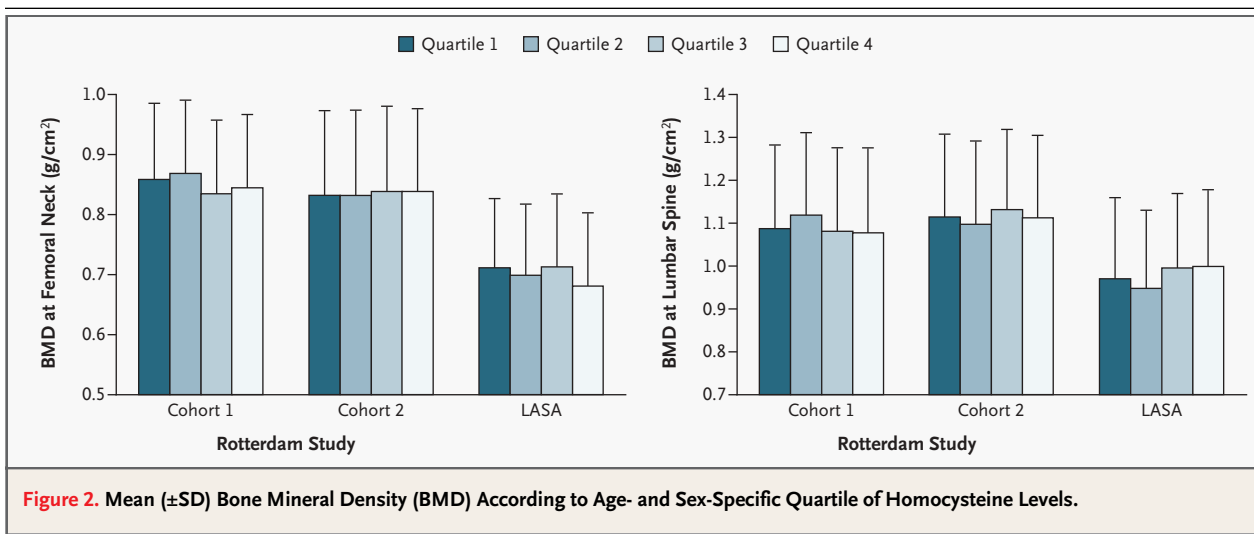
**POPULATION ATTRIBUTABLE RISK**

Table 4 shows the population attributable risks for the independent risk factors for fracture in the total study population. The risk of fracture that was attributable to a homocysteine level in the highest age-specific quartile was estimated at 19 percent. The association of a high homocysteine level with the occurrence of incident fractures was similar to the association of the risk of fracture with low bone mineral density, cognitive impairment, and recent falls.

**DISCUSSION**

Our analyses of data from three cohorts of subjects in two independent studies show a strong associa-





tion between increased homocysteine levels and the risk of osteoporotic fracture. The age- and sex-adjusted risk of fracture increased by 30 percent for each increase of 1 SD in the homocysteine level. A serum homocysteine level in the highest quartile doubled the risk of fracture. The magnitude of this effect is similar to that previously observed for the increase in the risk of cardiovascular disease and dementia according to homocysteine level.<sup>12-14,28</sup>

A novel aspect of this study is an examination of the relationship between the risk of fracture and homocysteine levels in a general, older population. The association appears to be consistent within this population, since we found similar risk estimates in the two cohorts of the Rotterdam Study and in LASA. The association appears to be independent of age, sex, and other risk factors for fracture, such as smoking, recent falls, dementia, diabetes mellitus, peripheral arterial disease, and nutritional deficiency. In view of the inherent limitation of measuring dietary intake by means of a questionnaire, nutritional deficiency cannot be completely ruled out as a confounder.

The calculated population attributable risk of the effects of increased homocysteine levels is considerable. A homocysteine level in the highest age-specific quartile conferred a 19 percent attributable risk in our population. The population attributable risks were similar for well-known risk factors for fracture, such as low bone mineral density, cognitive impairment, and recent falls, in the study population. A recent report showed that in the Rotterdam Study, the population attributable risks of

myocardial infarction associated with hypercholesterolemia and hypertension — two well-known risk factors — were 18 percent and 14 percent, respectively.<sup>29</sup> Thus, a high homocysteine level appears to have an effect whose size is similar to that of established risk factors for fractures and for cardiovascular disease.

There were considerable differences in the techniques used to assess homocysteine levels in the three study cohorts. For cohort 1 of the Rotterdam Study, serum samples were obtained, and for cohort 2, sodium citrate-treated plasma samples were obtained. In LASA, EDTA plasma samples were used. Furthermore, different methods were used to

Factor	Relative Risk (95% CI)	Population Attributable Risk (95% CI) %
Age >75 yr	2.3 (1.7–3.1)	31 (25–48)
BMD, lowest quartile	1.6 (1.1–2.3)	13 (2–25)
Current smoker	1.6 (1.1–2.3)	10 (4–23)
Fall in previous year†	1.9 (1.2–2.7)	20 (10–35)
Dementia and cognitive impairment†	2.5 (1.5–4.1)	15 (7–30)
Homocysteine level, highest quartile	1.9 (1.4–2.6)	19 (10–29)

\* All relative risks were adjusted for age and sex, except for an age of more than 75 years. CI denotes confidence interval, and BMD bone mineral density.

† Only data from cohort 1 of the Rotterdam Study and LASA were used to calculate the population attributable risk.

determine the homocysteine levels. These differences in method are known to influence the measurement of homocysteine levels.<sup>30-32</sup> Together with differences in the age and sex distribution among the three cohorts, the differences in method may explain the considerable variation in the mean homocysteine levels among the three cohorts. Despite these differences, an association between the homocysteine level and the risk of fracture was consistent in each cohort. Thus, this association appears to be independent of the method of measuring homocysteine levels. However, because of the large differences among the three cohorts, we refrained from using a single cutoff value for the presence of hyperhomocysteinemia.

According to a long-standing hypothesis, the mechanism underlying the association between the homocysteine level and the risk of fracture may involve interference by homocysteine in collagen cross-linking.<sup>7</sup> Homocysteine has been shown to interfere specifically with the formation of collagen cross-links and fibrils in solution.<sup>10</sup> In addition, lower amounts of collagen cross-links have been found in serum from patients who have homocystinuria — that is, persons with very high levels of circulating homocysteine — than in normal controls.<sup>11</sup> Because collagen cross-links are important for the stability and strength of the collagen network, interference in the formation of cross-links results in an altered bone matrix, which then results in fragile bone. Thus, increased homocysteine levels could lead to an increase in the risk of fracture through interference in collagen cross-linking. We therefore speculate that homocysteine interferes

with the development of the microarchitecture of bone independently of the amount of mineral in the bone. This notion was corroborated by the fact that we did not find evidence of a relationship between homocysteine levels and bone mineral density.

The association between elevated homocysteine levels and the risk of fracture should be confirmed in other large population studies. Proof of a causal relationship between increased homocysteine levels and bone disease could be established by intervention studies aimed at lowering the serum homocysteine level. Whereas randomized, controlled trials have shown that folic acid–based vitamin supplements can effectively reduce homocysteine levels<sup>33</sup> and reduce the rate of coronary restenosis,<sup>34</sup> additional studies are needed to assess whether the use of such therapy will reduce the risk of fracture.

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