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CIRCULATING LEVELS OF CARBOXY-METHYL-LYSINE (CML) ARE ASSOCIATED WITH HIP FRACTURE RISK:

THE CARDIOVASCULAR HEALTH STUDY

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Abstract

Advanced glycation end products (AGE) in bone tissue are associated with impaired biomechanical properties and increased fracture risk. Here we examine whether serum levels of the AGE carboxy-methyl-lysine (CML) are associated with risk of hip fracture.

We followed 3373 participants from the Cardiovascular Health Study (age 78 years; 39.8% male) for a median of 9.22 years. Rates of incident hip fracture were calculated by quartiles of baseline CML levels, and hazard ratios were adjusted for covariates associated with hip fracture risk. A sub-cohort of 1315 participants had bone mineral density (BMD) measurement.

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DISCLOSURES

All authors state that they have no conflicts of interest with regard to the contents of this article.

There were 348 hip fractures during follow-up, with incidence rates of hip fracture by CML quartiles of 0.94, 1.34, 1.18, and 1.69 / 100 participant-years. The unadjusted hazard ratio of hip fracture increased with each 1 SD increase (189 ng/ml) of CML level (1.27 [1.16, 1.40]; p<.001). Sequential adjustment for age, gender, race/ethnicity, BMI, smoking, alcohol consumption, prevalent CHD, energy expenditure, eGFR (based on cystatin C), and diabetes moderately attenuated the hazard ratio for fracture (1.17 [1.05, 1.31]; p=.006). In the cohort with BMD testing, total hip BMD was not significantly related to CML levels.

We conclude that increasing levels of CML are associated with hip fracture risk in older adults, independent of hip BMD. These results implicate AGE in the pathogenesis of hip fractures.

Keywords

Carboxy-methyl-lysine; hip fracture risk; bone quality; Cardiovascular Health Study; bone mineral density

INTRODUCTION

A characteristic of biological aging is the non-enzymatic glycosylation of long-lasting proteins, such as collagen (1). Approximately 90% of bone matrix protein consists of collagen. In contrast to enzymatic cross-linking of collagen fibrils, which orients collagen fibrils and contributes to tensile strength, non-enzymatic glycosylation of collagen weakens collagen's biomechanical properties (2). This occurs on account of three factors: (**1**) Glycosylation is a marker of ambient oxidative and carbonyl stress that increases with "biological" aging. While more extreme in diabetes (DM), glycosylation increases with age in all people. (**2**) Glycosylation products – termed advanced glycation end products (AGEs) - are inducers of oxidative stress and inflammation acting through the receptor for AGE (RAGE) to activate NF-kB and its downstream inflammatory proteins. (**3**) Accumulation of AGEs in bone leads to increased bone matrix stiffening and fragility. *In vitro* cell culture studies show that non-enzymatic glycosylation of collagen makes it resistant to osteoclastic bone resorption and decreases osteoblast differentiation and proliferation (3). Excess amounts of "old" bone result from these processes.

Carboxy-methyl-lysine (CML) is the major AGE epitope recognized by antibodies prepared against AGE proteins (4). It forms when oxoaldehyde glyoxal reacts with lysine. It reflects the combination of oxidation and glycation of proteins. CML accumulation has been shown to parallel AGE formation (4). In addition, CML appears to be the dominant component of AGEs; on average, 30% of lysine residues present on a protein are converted to CML after glycation.

Few clinical investigations have studied AGEs and bone fracture risk (5), relying upon cadaveric bone specimens and tissue AGE levels. These studies are time-consuming, expensive, and too invasive for clinical use. The measurement of circulating AGEs as a biomarker of fracture risk in population-based studies, remains rare (6) and is limited to cross-sectional studies. In the present study, we examine serum CML levels in a cohort of older adults to determine whether they are prospectively associated with hip fracture risk.

The cohort is drawn from the Cardiovascular Health Study (CHS), an ongoing population based study of individuals with a mean age of 78 years at the time of these measurements.

METHODS

The CHS is a prospective, observational population-based cohort study of 5,888 Medicare-eligible adults >65 years in 4 US communities (7). Two cohorts were recruited. In the original cohort, 5,201 eligible men and women were enrolled during 1989–1990. In the second recruitment, during 1992–93, an additional 687 predominantly black men and women were enrolled. Clinic examinations were performed at study baseline and at annual visits through 1998–1999, and again in 2005–2006. Participants were contacted by telephone annually between exams, and twice per year during 2000–2004 and 2007, when no clinic examinations occurred. All participants signed informed consent upon study entry.

The cohort for this analysis was taken from the 1996–1997 examination. At that point, 1180 of the original 5888 participants had died, and 296 were lost to follow-up or refused further visits. Of the remaining 4412 participants, 3373 had an adequate blood sample available.

Laboratory tests were done as previously reported (8). As a measure of renal function, cystatin C levels (mg/L) were measured from stored samples using a BNII nephlometer (Dade Behring Inc., Deerfield, IL) that utilized a particle enhanced immunonephlometric assay (N Latex Cystatin-C). Estimated cystatin glomerular filtration rates (ecGFR) were derived as: $-4.32 + 80.35 \times 1/\text{cyst C}$ (9). Information regarding smoking history, medication use, history of falling in the preceding year, amount of energy (kcal) expended per week based on the Minnesota Leisure Time Activity Questionnaire, and alcohol use were obtained at the time of the visit. Technicians directly measured weight, blood pressure, waist circumference, height, grip strength, and the time needed to walk 15 feet (in seconds). Frailty was defined using a phenotype that requires >3 of the following criteria to be present: unintended weight loss >10 lbs in the prior year; self-reported exhaustion most of the time; physical activity in the lowest 20% of CHS cohort (<383 Kcal/week in men; <270 Kcal/week in women); weakness as measured by grip strength (lowest 20% of CHS cohort: <23 kg/m^2 in men, <17 kg/m^2 in women); and slowness of walking (lowest 20% in each sex, adjusted for height) (10). Those with 1 or 2 criteria were considered “pre-frail”, an intermediate syndrome with increased risk for the development of frailty. Random urine specimens were used to measure urinary albumin to creatinine ratio; albuminuria was available for 2972 participants.

Measurement of Hip Fracture

Data on hip fracture was obtained through patient report and confirmed from hospital discharge codes. Incident hip fracture was indentified using *International Classification of Diseases, Ninth Revision (ICD-9)* codes from hospitalization records. CHS prospectively gathers all hospitalization data, including discharge summaries, from participants every six months. To ensure completeness of hospitalization records, data were checked against Medicare claims data to identify any hospitalizations that were not reported by the participant. Hip fracture was defined as *ICD-9* code of 820.xx. Admissions for pathologic

fractures (ICD-9 code 773.1x) and motor vehicle accidents (E810.xx- E825.xx) were excluded.

Measurement of CML: Measurement of CML

Fasting samples were stored at the University of Vermont, Burlington, VT at −80 degrees Celsius until assays for CML were measured using a competitive enzyme-linked immunosorbent assay (ELISA) (AGE-CML ELISA, Microcoat, Penzberg, Germany) (11). This assay has been validated (11, 12), is specific, and shows no cross-reactivity with other compounds. The minimum level of detectability of the assay is 5 ng/mL, below the concentration found in human studies. Both the intra- and inter-assay coefficients of variation were <5%.

Bone Mineral Density Measurement

A subset of the cohort from two field centers (n=1315) underwent BMD scanning 1–2 years before CML levels were obtained. BMD was measured by DXA (QDR 2000 or 2000±; Hologic, Inc, Bedford, MA). All scans were completed using the array beam mode. Scans were read blindly at the University of California, San Francisco reading center with Hologic software version 7.10. The coefficient of variation for total hip BMD was <0.75%.

Statistical Analysis

We graphically examined the distribution of CML levels. Because the distribution of CML is highly skewed at large values, we winsorized the top 1% (n=33, values above 1435 mg/ml). For descriptive purposes, participants were categorized by quartiles of the distribution of winsorized CML levels. We show mean ± standard deviations for continuous variables and counts (%) for categorical variables. Linear trend tests across quartiles of CML were used for continuous variables, and χ^2 test for categorical variables.

Kaplan-Meier curves were generated to describe the association between CML concentrations and time to incident hip fracture. Time to event was calculated as the interval in years from the baseline visit in 1996/1997 to the earliest date of first incident hip fracture, death, loss to follow-up, or end of follow-up on June 30, 2008. Cox proportional hazards models were used to estimate the hazard ratio (HR) of incident fracture associated with a 1 standard deviation increase in CML level (189 mg/ml). Adjustments were made in nested fashion as follows: unadjusted; Model 1 (demographic adjusted) adjusted for age, gender, race/ethnicity, and clinic site; Model 2 (osteoporosis adjustment) was adjusted for factors in model 1 and for factors known to be associated with risk of osteoporosis: prevalent coronary heart disease (13), smoking (14), BMI (14), alcohol use (14), level of physical activity (15), and baseline eGFR (16, 17). Model 3 additionally included a history of falls, which could be a consequence of CML levels. We also tested additional adjustment for log2-transformed albuminuria among individuals with available measurements. Age categories (<75, 75–84, 85+) were used as strata to improve the fit of the model. We evaluated the validity of the proportional hazards assumption graphically and numerically using Schoenfeld residuals and found no meaningful departures.

We examined the association of CML and fracture separately in men and women, and in those with and without diabetes, and tested for interaction with cross-product terms.

In the sub-cohort with BMD measurements, we calculated the correlation between CML levels winsorized at the upper 1st percentile with total hip BMD. We also examined the association of CML levels on total hip BMD adjusted for factors related to BMD using similar models as above (with frailty status added to Model 3).

Analyses were conducted using R (R Development Core Team (2009) (18).

RESULTS

The median age of the cohort was 78.0 years (IQ range, 75.0, 81.0 years). The median value of CML was 584 ng/ml (IQ range, 498, 703 ng / ml). Baseline characteristics of participants by quartiles of CML levels are shown in Table 1.

Over a median follow-up period of 9.22 years (IQ range, 5.12, 11.42 years), there were 348 hip fractures during 27,409 person-years of follow-up (Table 2). Survival free of hip fracture was lowest among individuals with the lowest CML levels, similar among those in intermediate quartiles, and highest among those with highest levels (log-rank $p<0.001$; Figure 1).

The unadjusted HR for hip fracture risk per 1-SD (189 ng/ml) increment in CML levels, winsorized at the upper 1% of the distribution, was 1.27 (1.15, 1.40) (Table 3). Adjustment for demographic factors had little impact on the HR. Further adjustment for factors associated with osteoporosis moderately attenuated the HR of hip fracture with CML levels. Further adjustment for falling - in the causal pathway for fractures - did not attenuate the association of CML level change with hip fracture risk, nor did additional adjustment for frailty status or albuminuria (Model 3 HR 1.17 [1.05, 1.31], p= .006).

When the cohort was stratified by gender, men and women had similar HR for hip fracture in association with CML in the fully adjusted model (Appendix, Table 1). There were also no differences in HR between participants with or without diabetes.

CML Levels and Total Hip Bone Density

The mean bone mineral density of the total hip was 0.83 mg ± 0.18 calcium/cm^2 (median 0.82 [IQ range, 0.70, 0.95]). The correlation between hip bone density and CML levels was small but statistically significant (r= −0.073; p=.01). In linear regression models, the association of CML with hip BMD was not statistically significant after adjustment for covariates in Models 2 or 3 (Appendix, Table 2). When we examined risk of hip fracture in the sub-cohort of participants with measured BMD, a 1 SD increase in winsorized CML levels remained significantly associated with fracture risk (HR 1.25 [1.02, 1.52]; p=.03) adjusted for Model 3 covariates and total hip BMD.

DISCUSSION

To the best of our knowledge, this is the first study to examine circulating levels of CML as a biomarker of hip fracture risk. The hazard ratio of hip fracture risk increased with increasing CML levels, even after adjustment for osteoporosis risk factors. Moreover, the association of increasing CML levels with hip fracture risk was independent of eGFR, albuminuria, and hip BMD.

There have been few clinical investigations of the contribution of bone, serum, or urine AGEs to fracture risk. Most reports have examined pentosidine, an AGE whose levels can be measured in bone through its fluorescent properties. Cadaveric models show a strong correlation between increased bone AGEs and bone fracture (19). In hip fracture specimens (20), pentosidine levels were elevated in cortical and cancellous bone compared to age-matched controls. Urine or serum pentosidine levels predicted vertebral fractures in postmenopausal women and older adults with DM (21). While these reports are consistent with the findings of this study, it should be noted that pentosidine leads to collagen cross-linking in bone, whereas CML is a chemical adduct on collagen with no cross-linking properties. The biological effects of these two AGEs may differ.

We did not find a significant relationship between hip BMD and circulating CML levels in regression models. This is consistent with the hypothesis that fracture risk in association with CML is through impaired bone quality, not through bone quantity (3). Whether pharmacological treatment of osteoporosis can modify this apparent effect is uncertain, but *in vitro* studies suggest opposing effects of bisphosphonates and AGEs on osteoblasts (22). Similarly, AGE crosslink breakers exist (23), although their effects on bone quality remain to be tested.

Advantages of this study include a well-characterized cohort with long follow up, information on multiple covariates, the examination of men and women, and rigorous ascertainment of study outcomes. The mean eGFR (based on cystatin levels) was >60 ml/minute in all quartiles of CML, mitigating the potential confounding effects of chronic kidney disease. A limitation of our study is that some covariates which impact bone, such as vitamin D, testosterone, and PTH levels, were not available. We had only a single measure of CML and hence cannot ascertain the degree to which biological variability may have led us to underestimate the magnitude of associations nor whether the trajectory of CML over time conveys additional information.

In conclusion, we observed a statistically significant prospective association of CML levels with incident hip fracture risk. The mechanisms of this association are uncertain, but the association was independent of BMD. Our findings suggest that AGEs may represent a promising marker for categorizing and perhaps even ameliorating future hip fracture risk (24).

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APPENDICES

Appendix, Table 1

Hazard ratios of hip fracture in men and women, and in those with and without diabetes, in association with a 1 standard deviation increase in CML levels (189 ng/ml) sequentially adjusted for the factors found in Table 3.

	MEN	WOMEN
Unadjusted	1.23 (1.12, 1.35) <.001	1.32 (1.18, 1.48) <.001
Model 1	1.28 (1.16, 1.42) <.001	1.33 (1.86, 1.50) <.001
Model 2	1.23 (1.09, 1.38) <.001	1.25 (1.10, 1.42) <.001
Model 3	1.23 (1.09, 1.38) <.001	1.25 (1.10, 1.42) <.001

	DIABETES	NO DIABETES
Unadjusted	1.22 (1.13, 1.33) p<.001	1.31 (1.18, 1.45) p<.001
Model 1	1.22 (1.11, 1.33) p<.001	1.29 (1.16, 1.43) p<.001
Model 2	1.18 (1.07, 1.30) p<.001	1.23 (1.10, 1.37) p<.001
Model 3	1.18 (1.07, 1.30) p<.001	1.22 (1.09, 1.37) p<.001

Appendix, Table 2

Association of 1-SD increase in CML levels with total BMD of the hip.

	Regression coefficient (SE)	95% confidence interval	P-value
Unadjusted	−0.013 (0.005)	−0.022, −0.003	.008
Model 1	−0.013 (0.004)	−0.021, −0.006	<.001
Model 2	−0.007 (0.004)	−0.014, 0.001	.073
Model 3	−0.005 (0.004)	1.05, 1.31	.186

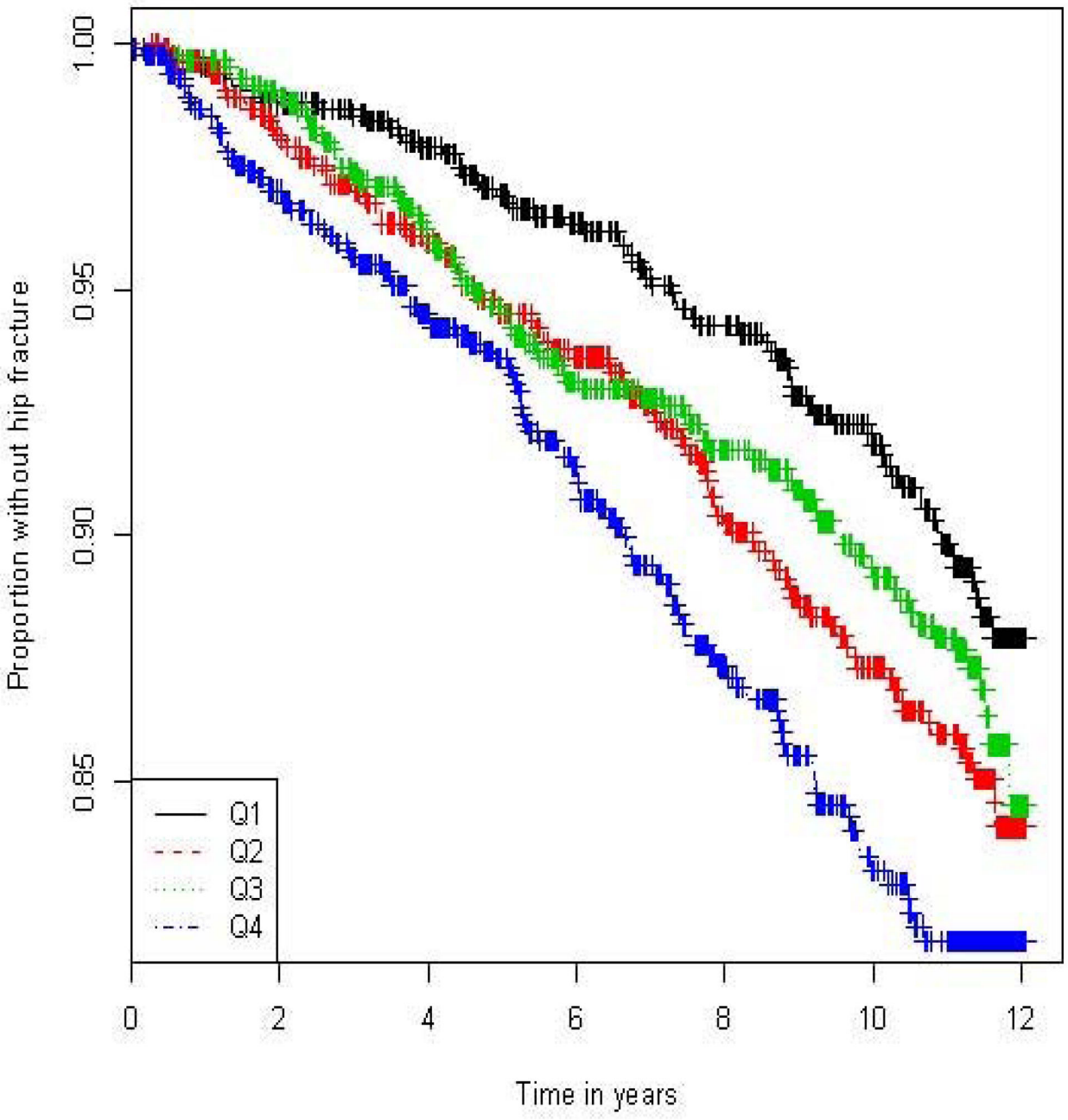

Figure 1.
Survival free of hip fracture by quartile of winsorized CML levels.

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Table 1

Baseline characteristics of the CHS cohort categorized by quartiles of CML.

	Quartile I N=844	Quartile II N=843	Quartile III N=843	Quartile IV N=843	P value
CML Level* (ng/ml)	439.8±47.7	546.7±35.3	636.3±34.2	876.4±182.7	
Clinical					
Male (%)	37.0	41.0	39.1	41.6	.15
Black race (%)	16.8	15.1	17.1	15.1	<.001
Age (years)	77.0±4.3	77.9±4.8	78.5±4.8	78.9±5.2	<.001
BMI (kg/m^2)	28.4±4.7	27.1±4.5	26.4±4.5	25.8±4.5	<.001
SBP (mm Hg)	136.2±19.2	136.2±20.2	136.7±21.6	138.2±21.6	.04
DBP (mm Hg)	69.9±11.2	70.4±10.2	70.2±11.8	69.3±11.8	.25
Smoking (%)					.002
Current	10.2	7.1	7.0	5.7	
Former	44.9	47.6	42.0	43.3	
Never	44.9	45.3	51.0	51.0	
Alcohol (%)					.004
None	51.4	58.2	60.8	57.7	
1–7 drinks/wk	35.6	31.8	30.0	33.1	
>7 drinks/wk	13.0	10.0	9.2	9.2	
Frail (%)					.01
None	38.3	39.9	39.4	33.6	
Pre-frail	51.8	51.3	50.6	52.0	
Frail	9.8	8.8	9.9	14.3	
Time to walk 15 feet (secs)	5.6±2.2	5.8±3.2	5.8±2.7	6.2±4.3	<.001
History					
CHD (%)	22.2	20.9	25.4	28.8	<.001
Fell in last year (%)	15.7	18.5	18.7	21.9	.015
Diabetes (%)	17.4	15.1	14.2	13.0	.07
Energy expenditure/wk (mean, kcal)	1319±1787	1297±1607	1284±1638	1160±1612	.06

	Quartile I N=844	Quartile II N=843	Quartile III N=843	Quartile IV N=843	P value
Education > 12 years (%)	44.5	47.3	49.5	49.6	.12
Laboratory					
CRP (mg/L)	5.2±8.8	5.2±9.2	4.2±5.9	4.4±7.8	.01
eGFR cystatin (ml/min)	73.9±17.3	71.8±18.9	70.2±18.3	65.6±22.2	<.001
Fasting glucose (mg/dl)	105.9±31.8	103.6±33.1	103.1±35.1	99.2±28.3	<.001
Medications (%)					
Beta blocker	16.0	14.7	14.2	15.0	.77
Thiazide	15.9	13.5	18.4	19.2	.007
Loop diuretic	9.5	10.0	10.6	14.4	.005
Thyroid	14.1	12.6	11.7	13.7	.47

* Winsorized at the 99th percentile.

Table 2

Hip fracture rate during a median of 9.2 years of follow up by quartiles of serum CML at baseline.

	Total	Q1	Q2	Q3	Q4
Sample size	3373	844	843	843	843
Hip fracture events	348	69	94	81	104
Person-year follow up	27409	7371	7029	6860	6149
Incidence rate per 100 person-years	1.27	0.94	1.34	1.18	1.69

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Table 3

Hazard ratios of hip fracture by an increase of 1 standard deviation (189 ng/ml) of CML level sequentially adjusted for factors associated with hip fractures.

	Hazard ratio	95% confidence interval	P-value
Unadjusted	1.27	1.16, 1.40	<.001
Model 1	1.25	1.13, 1.38	<.001
Model 2	1.18	1.06, 1.31	.003
Model 3	1.17	1.05, 1.31	.004

Model 1 adjusts for age, sex, race, and clinic site.

Model 2 additionally adjusts for smoking, body-mass index, alcohol consumption, prevalent coronary heart disease, physical activity, and estimated glomerular filtration rate.

Model 3 further adjusts for history of falling.