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Clinical Research Article

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Serum Glycine Levels Are Associated With Cortical Bone Properties and Fracture Risk in Men

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Abbreviations: BMD, bone mineral density; BMI, body mass index; CV, coefficient of variation; DXA, dual-energy X-ray absorptiometry; FHS, Framingham Heart Study; FN, femoral neck; HKOS, Hong Kong Osteoporosis Study; HR, hazard ratio; HR-pQCT, high-resolution peripheral quantitative computed tomography; MrOS, Osteoporotic Fractures in Men study; vBMD, volumetric bone mineral density.

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Abstract

Context: In a recent study a pattern of 27 metabolites, including serum glycine, associated with bone mineral density (BMD).

Objective: To investigate associations for serum and urinary glycine levels with BMD, bone microstructure, and fracture risk in men.

Methods: In the population-based Osteoporotic Fractures in Men (MrOS) Sweden study (men, 69-81 years) serum glycine and BMD were measured at baseline (n = 965) and 5-year follow-up (n = 546). Cortical and trabecular bone parameters of the distal tibia were measured at follow-up using high-resolution peripheral quantitative computed

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tomography. Urinary (n = 2682) glycine was analyzed at baseline. X-ray-validated fractures (n = 594) were ascertained during a median follow-up of 9.6 years. Associations were evaluated using linear regression (bone parameters) or Cox regression (fractures).
Results: Circulating glycine levels were inversely associated with femoral neck (FN)-BMD. A meta-analysis (n = 7543) combining MrOS Sweden data with data from 3 other cohorts confirmed a robust inverse association between serum glycine levels and FN-BMD ($P = 7.7 \times 10^{-9}$). Serum glycine was inversely associated with the bone strength parameter failure load in the distal tibia ($P = 0.002$), mainly as a consequence of an inverse association with cortical cross-sectional area and a direct association with cortical porosity. Both serum and urinary glycine levels predicted major osteoporotic fractures (serum: hazard ratio [HR] per SD increase = 1.22, 95% CI, 1.05-1.43; urine: HR = 1.13, 95% CI, 1.02-1.24). These fracture associations were only marginally reduced in models adjusted by FRAX with BMD.
Conclusions: Serum and urinary glycine are indirectly associated with FN-BMD and cortical bone strength, and directly associated with fracture risk in men.

Key Words: glycine, BMD, HR-pQCT, fracture, men

Environmental as well as genetic factors and their interactions contribute to osteoporosis and increased fracture risk. Metabolomics can be used to identify markers of disease and some recent non-hypothesis-driven metabolomics studies evaluating multiple metabolites have suggested that serum glycine is associated with bone health parameters.

In a small early study (n = 22 men with osteoporosis and 20 controls) from 2010, men with idiopathic osteoporosis had higher plasma glycine levels than control subjects (1). Two recent non-hypothesis-driven metabolomics studies have evaluated the association between multiple metabolites and bone mineral density (BMD). In the first study using the TWINS-UK study, 280 blood metabolites were tested for associations with BMD. Glycine did not pass the Bonferroni corrected threshold for statistical significance in that study, but data on associations between serum glycine and femoral neck (FN)-BMD were released in a supplementary appendix, revealing a nominally significant inverse association between glycine and BMD (2). In a second study, 209 blood metabolites were evaluated in the Framingham Heart Study (FHS) Offspring cohort and machine learning procedures revealed that a pattern of 27 metabolites, including serum glycine, collectively associated with BMD. None of these 27 metabolites were individually associated with BMD after correcting for multiple testing, but some metabolites including glycine were nominally associated with FN-BMD (3). Furthermore, Mendelian randomization analyses indicated that there was a causal inverse association between circulating glycine levels and FN-BMD (2, 3)}. However, it remains to be established if there is a reproducible significant observational association between circulating glycine levels and FN-BMD. In addition, it has not been investigated if glycine levels are associated with cortical and/or trabecular bone microstructure parameters. Moreover, it is unknown if serum or urinary glycine is associated with incident fractures.

Glycine is a nonessential amino acid involved in a wide range of metabolic pathways, including the biosynthesis of heme, glutathione, creatine, and nucleic acids, and the conjugation of bile acids. Glycine is also a building block for proteins, and every third residue of the collagen triple helix is a glycine residue (4). Because collagen is the major protein in bone matrix, it is possible that glycine metabolism may influence or reflect bone metabolism.

Urine has several advantages over blood; it is abundant, sterile, and easy to collect. However, urine is a complementary body fluid that also reflects kidney function and the detoxification capabilities of the human body. Moreover, urine is a waste product that limits the need for the tight metabolic control exercised in blood. Associations between metabolite levels and phenotypes are thus not necessarily the same in urine as in blood (5, 6).

The aim of the present study was to investigate the observational associations of serum and urinary glycine with BMD, bone microstructure and fracture risk in men. Previous studies have measured bone using dual-energy X-ray absorptiometry (DXA), but here we also used high-resolution peripheral quantitative computed tomography (HR-pQCT) to investigate if glycine is associated with calculated bone strength, cortical bone microstructure, or trabecular bone microstructure.

Materials and Methods

Study Sample

The study population consisted of 3014 older men in Gothenburg (n = 1010), Malmö (n = 1005), and Uppsala

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(n = 999), Sweden. These men constituted the Swedish cohort of the cross-sectional and prospective multicenter Osteoporotic Fractures in Men (MrOS) study. Study subjects were randomly selected from national population registers, contacted, and asked to participate. To be eligible for the study, the participants had to be able to walk without assistance, provide self-reported data, and sign an informed consent. The participant rate was 45%. The MrOS Sweden Study was approved by the ethics committees at the Universities of Gothenburg, Lund, and Uppsala. Informed consent was obtained from all study participants (7, 8).

Five-year follow-up assessments were performed in the MrOS Gothenburg cohort. A total of 600 men (59.4%) attended the 5-year follow up. The last 479 of these men underwent HR-pQCT imaging on the distal tibia. Ultimately, 450 men had acceptable image quality of the tibia.

Serum and urinary samples were collected at baseline following an overnight fast in the MrOS Gothenburg cohort. In the MrOS Malmö and Uppsala cohorts, only urinary samples, fasting or spontaneous, were available. Fasting serum samples were also collected at follow-up in the MrOS Gothenburg cohort. All samples were frozen and stored at -80°C.

Assessment of Covariates

A standardized questionnaire was used to gather information about self-reported previous fractures after age 50 years, smoking, alcohol use, rheumatoid arthritis and other prevalent major diseases that are known to induce secondary osteoporosis, use of glucocorticoids, and parental hip fractures. Height was measured using a Harpender stadiometer, and weight was measured with an electric scale. Body mass index (BMI) was calculated by dividing the weight in kilograms by the height in meters squared.

The country-specific FRAX tool was used to assess a participant's calculated 10-year probability of a major osteoporotic fracture (clinical spine, distal radius, proximal humerus, or hip) or a hip fracture (http://www.shef.ac.uk.ezproxy.ub.gu.se/FRAX/). The FRAX tool integrates the fracture risks associated with clinical risk factors, which were retrieved from the questionnaires, as well as FN-BMD (9).

Assessment of BMD

Areal BMD (g/cm^2) of the femoral neck and the lumbar spine (vertebrae L_1 to L_4) was assessed at baseline by DXA using the Lunar Prodigy DXA (n = 2004 from the Uppsala and Malmö cohorts; GE Lunar Corp., Madison, WI, USA) or Hologic QDR 4500/A-Delphi (n = 1010 from the Gothenburg cohort; Hologic, Waltham, MA, USA). The coefficients of variation (CVs) for the BMD measurements ranged from 0.5% to 3%. To be able to use DXA measurements performed with equipment from 2 different manufacturers, a standardized BMD was calculated, as described (7). All follow-up measurements were performed using a Hologic QDR 4500/A-Delphi DXA.

HR-pQCT Measurements of Cortical and Trabecular Bone Parameters Separately

Following a previously described protocol (10), an HR-pQCT device (XtremeCT; ScancoMedical AG, Brüttisellen, Switzerland) was used to measure cortical and trabecular bone parameters separately at the left distal tibia (or nonfractured leg if prior fracture). Using a grading scale supported by the manufacturer, each image was ranked on a scale of 1 (optimum quality) to 5 (unacceptable). Images with a quality of 4 or 5 were discarded.

All scans were analyzed with the manufacturer's standard in vivo analysis protocol and processed according to Laib et al (11). The following parameters were analyzed in this study, presented with their corresponding CVs: cortical area (mm^2, 0.4%), cortical volumetric BMD (vBMD; mg/cm^3, 0.1%), trabecular number (mm^{-1}, 1.6%), trabecular thickness (µm, 0.7%), trabecular separation (mm, 1.4%), and trabecular vBMD (mg/cm^3). With an extended cortical bone analysis, incorporated in a customized version of the manufacturer's Image Processing Language (IPL v5.08b Scanco Medical AG), cortical porosity was assessed according to a previously described method (12). The CV for cortical porosity measured at the tibia was 5.5%.

Microfinite element models of the tibia were created directly from the segmented HR-pQCT images to estimate failure load in compression. This was made by a finite-element software (version V5.11/FE-V01.15), incorporated in the analysis software provided by Scanco. To estimate failure load, each bone voxel tissue was converted into an equally sized brick element (13) and all bone materials were given a Young modulus of 10 GPa and a Poisson ratio of 0.3 as reported by Pistoia et al (14). The estimated failure load (N) was computed as earlier described (14), and the failure load is calculated based on the assumption that fracture occurs when 2% of the bone elements surpass the critical limit of 7000 microstrains. The CV for failure load was 0.2%.

Assessment of Incident Fractures

The prospective study period for assessing incident fractures was calculated from each study participant's study inclusion date until

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either an end point (i.e., fracture or death), the end of follow-up period, or emigration. The median follow-up time was 9.6 years. Date of death was retrieved from the regional administrative patient registry. Fracture events and dates of the events were obtained using a digital radiology database consisting of regional patient data (i.e., radiology reports and corresponding images) from all radiology examinations. We did not include asymptomatic fractures. The occurrence of fractures was cataloged by type into a main group, consisting of men with any fracture, and 2 subgroups; men with major osteoporotic fractures and men with hip fractures, according to the FRAX definition (15).

Analysis of Serum Samples

Fasting morning serum samples were available from 969 individuals at baseline and 599 individuals at follow-up in the MrOS Gothenburg cohort. Glycine was quantifiable in all samples. High-throughput nuclear magnetic resonance (NMR) metabolomics (Nightingale Health Ltd, Helsinki, Finland) was used for quantifying glycine in serum (16).

Preparation and Analysis of Urinary Levels of Glycine

Urinary samples were available from 2683 study subjects. Urinary glycine and creatinine were analyzed using a Bruker DRX-400 NMR spectrometer (17). The analysis of urinary creatinine failed in 1 sample, leaving 2682 study subjects available for analysis of glycine normalized to urinary creatinine. Glycine was below the detection limit in the samples from 2 individuals.

For spectroscopic analysis, 450 µL of urine was mixed with 50 µL of phosphate buffer to stabilize urinary pH at 7.0 (±0.35). The buffer was prepared with D2O and contained sodium 3-trimethylsilyl-(2,2,3,3-D4)-1-propionate as the reference to annotate NMR signals. Urine spectra were acquired according to a previously published protocol (17). In brief, 1-dimensional spectra were recorded by a NOESYPRESAT pulse sequence with suppression of the water peak on a Bruker DRX-400 NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) operating at a 1H frequency of 400.13 MHz.

Statistical Analysis

Correlations between glycine parameters measured at different time points or between levels in serum and urine were analyzed using Spearman's rank-order correlation. Association analyses between glycine levels and bone parameters were performed on inverse normal transformed glycine levels. Urinary glycine levels were normalized to urinary creatinine before inverse normal transformation. For metabolite-BMD trait associations, linear regression models adjusted for age, BMI, smoking status, and (for urine) MrOS Sweden study site were used. Beta values are expressed per SD increase in inverse normal transformed glycine (serum) or inverse normal transformed glycine normalized to urinary creatinine (urine). Because the units and assays for serum glycine measurements differed between the 4 cohorts included in a meta-analysis of the association between serum glycine and FN-BMD, a *z*-scored meta-analysis was performed. Cox proportional hazard models were used to evaluate associations between glycine and incident fractures. In predefined analyses, the following validated fracture types were analyzed: all fractures, major osteoporotic fractures (defined as hip, clinical vertebral, distal radius, and proximal humerus fractures), and hip fractures. Hazard ratios (HRs) and 95% CIs were estimated from the models and expressed as 1 SD increase in inverse normal transformed glycine. Fracture estimates were adjusted for (1) age and MrOS Sweden study site or (2) FRAX without BMD and MrOS Sweden study site or (3) FRAX with BMD and MrOS Sweden study site. The 10-year risk of a major osteoporotic fracture was used as a FRAX parameter in the models with all fractures or major osteoporotic fracture as the dependent variable. The 10-year risk of a hip fracture was used as a FRAX parameter in the models with hip fracture as the dependent variable.

Results

Serum Levels of Glycine Are Inversely Associated With FN-BMD

Characteristics of the MrOS Sweden cohort (men aged 69-81 years) are presented in Table 1. There was a moderate correlation between baseline and follow-up serum levels of glycine ($r_s = 0.59$, $P = 3.2 \times 10^{-55}$). Serum levels of glycine in MrOS Sweden were inversely associated with FN-BMD both at the baseline visit (ß = -0.084, $P = 8.4 \times 10^{-3}$) and at the follow-up visit five years later (ß = -0.12, $P = 5.3 \times 10^{-3}$, Table 2) in linear regression models adjusted for age, BMI, and current smoking. Serum glycine levels were not significantly associated with lumbar spine BMD (Table 2).

We next performed a *z*-scored meta-analysis of the association between circulating glycine and FN-BMD using the results from the present study together with previously reported data from TWINS-UK, FHS Offspring, and Hong Kong Osteoporosis Study (HKOS), not passing the significant level after adjustment for multiple testing in these previous studies (2, 3). This large-scale meta-analysis, including a total of 7543 subjects, provided compelling statistical evidence ($P = 7.7 \times 10^{-9}$) that serum/plasma glycine levels are inversely associated with FN-BMD (Table 3).

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Table 1. Characteristics of the MrOS Sweden Cohort

	Baseline	Follow-up
	n = 3014	n = 600
Age, y	75.4 ± 3.2	79.9 ± 3.4
BMI, kg/m^2	26.4 ± 3.6	26.0 ± 3.4
Current smokers, n (%)	252 (8.4)	30 (5.0)
Femur neck BMD, mg/cm^2	831 ± 132	777 ± 133
Lumbar spine BMD, mg/cm^2	1142 ± 201	1068 ± 194
FRAX major osteoporotic fracture without BMD (%)	13.1 ± 5.6	
FRAX major osteoporotic fracture with BMD (%)	11.1 ± 6.3	
FRAX hip fracture without BMD (%)	7.0 ± 4.8	
FRAX hip fracture with BMD (%)	5.2 ± 5.5	

Values are given as mean ± SD or n (%). FRAX is the country-specific calculated estimate of the 10-year risk of a major osteoporotic fracture or a hip fracture.
Abbreviations: BMD, bone mineral density; BMI, body mass index; MrOS, Osteoporotic Fractures in Men study.

Table 2. Associations between serum glycine levels and BMD

	N	Beta (SE)	*P* value
FN-BMD			
Baseline	958	-0.084 (0.032)	8.4×10^{-3}
Follow-up	524	-0.12 (0.042)	5.3×10^{-3}
LS-BMD			
Baseline	965	-0.036 (0.031)	0.25
Follow-up	546	-0.044 (0.042)	0.29

Beta values are given as standard deviation (SD) BMD per SD increase of inverse normal transformed glycine. Models are adjusted for age, BMI, and current smoking.
Abbreviations: FN-BMD, femoral neck bone mineral density; LS-BMD, lumbar spine bone mineral density.

Serum Levels of Glycine Are Inversely Associated With Cortical Bone Microstructure Parameters

HR-pQCT analyses in the distal tibia region (Table 4), separating the cortical and trabecular bone microstructure compartments from each other, revealed that serum glycine levels were inversely associated with cortical bone area and cortical vBMD and directly associated with cortical porosity (Table 5). Although serum glycine levels were indirectly associated with trabecular thickness, no association was observed for other trabecular bone parameters, including trabecular vBMD, trabecular separation, and trabecular number. A robust inverse association between serum glycine levels and the calculated bone strength parameter failure load was observed (ß = -0.14, *P* = 0.002; Table 5).

Table 3. Meta-analysis of the association between serum/plasma glycine levels and FN BMD

Study	N	Direction of association	*P* value
MrOS Sweden	958	inverse	8.4×10^{-3}
TWINS-UK	4399	inverse	7.3×10^{-5}
FHS Offspring	1552	inverse	0.014
HKOS	634	inverse	0.048
Combined	**7543**	**inverse**	$\mathbf{7.7 \times 10^{-9}}$

Because the units and assays for serum glycine measurements differed between the 4 cohorts included in the present meta-analysis of the associations between serum glycine and FN-BMD, a *z*-scored meta-analysis was performed. Data from TWINS-UK (Supplemental Data S1 (2)), FHS Offspring (Supplemental Table 3 (3)), and HKOS (Supplemental Table 3 (3)) are from the indicated supplemental tables from previous publications.
Data models are adjusted for the following parameters in the different cohorts included in the meta-analyses; MrOS Sweden: age, BMI, current smoking; TWINS-UK: family relatedness and zygosity (random effects variables), and age, height, weight, and duration of hormone replacement therapy (confounders); FHS and HKOS: sex, age, BMI, current smoking, and menopausal status.
Abbreviations: BMI, body mass index; FHS, Framingham Heart Study; FN-BMD, femoral neck bone mineral density; HKOS, Hong Kong Osteoporosis Study; MrOS, Osteoporotic Fractures in Men study.

Urinary Glycine Levels Are Inversely Associated With BMD

There was a modest correlation between urinary and serum levels of glycine at baseline in MrOS Sweden ($r_s = 0.26$, $P = 5.4 \times 10^{-14}$). Urinary levels of glycine were inversely associated not only with FN-BMD (ß = -0.066 [SE, 0.018], $P = 2.9 \times 10^{-4}$), but also with lumbar spine-BMD (ß = -0.068 [SE, 0.019], $P = 2.8 \times 10^{-4}$; Table 6) in MrOS Sweden.

Both Serum and Urinary Glycine Levels Predict Incident Fractures

Given the findings of associations between high serum glycine levels and several indicators of reduced bone strength including reduced FN-BMD, reduced cortical bone area, increased cortical porosity, and reduced calculated failure load, we next investigated if serum or urinary glycine levels predict fracture risk in the MrOS Sweden cohort. Cox proportional hazards models adjusted for age demonstrated that high serum glycine levels (n = 969) were associated with an increased risk of fractures at any bone site (HR per SD increase, 1.17; 95% CI, 1.03-1.33) and major osteoporotic fractures (HR per SD increase, 1.22; 95% CI, 1.05-1.43; Table 7). More well-powered studies (n = 2682) revealed that urinary glycine levels were significantly directly associated not only with fractures at any bone site and major osteoporotic fractures, but also with hip fractures (HR per SD increase, 1.18; 95% CI, 1.01-1.36; Table 7). To evaluate the impact of other risk factors

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Table 4. Descriptive data of the HR-pQCT analyses in tibia

	N	
Cortical area (mm^2)	450	120 ± 35
Cortical vBMD (mg/cm^3)	449	804 ± 81
Cortical porosity (%)	450	11.8 ± 4.1
Trabecular vBMD (mg/cm^3)	450	179 ± 34
Trabecular number (mm^{-1})	450	1.97 ± 0.30
Trabecular thickness (µm)	450	76.1 ± 11.6
Trabecular separation (mm)	450	0.44 ± 0.08
Failure load (kN)	444	12.2 ± 2.2

Values are given as mean ± SD.
Abbreviations: HR-pQCT, high-resolution peripheral quantitative computed tomography; vBMD, volumetric bone mineral density.

Table 5. Association between serum glycine levels and HR-pQCT parameters in the distal tibia

	N	Beta (SE)	*P* value
Cortical area (mm^2)	450	-0.12 (0.047)	0.011
Cortical vBMD (mg/cm^3)	449	-0.10 (0.048)	0.037
Cortical porosity (%)	450	0.10 (0.048)	0.035
Trabecular vBMD (mg/cm^3)	450	-0.070 (0.048)	0.14
Trabecular number (mm^{-1})	450	0.037 (0.045)	0.41
Trabecular thickness (µm)	450	-0.12 (0.048)	0.015
Trabecular separation (mm)	450	-0.020 (0.046)	0.66
Failure load (kN)	444	-0.14 (0.046)	0.002

Beta values are given as SD bone parameter per SD increase of inverse normal transformed glycine. Models are adjusted for age, BMI, and current smoking.
Abbreviations: BMI, body mass index; HR-pQCT, high-resolution peripheral quantitative computed tomography; vBMD, volumetric bone mineral density.

Table 6. Association between urinary glycine levels and BMD

	N	Beta (SE)	*P* value
FN-BMD	2648	-0.066 (0.018)	2.9×10^{-4}
LS-BMD	2654	-0.068 (0.019)	2.8×10^{-4}

Beta values are given as SD BMD per SD increase of inverse normal transformed glycine normalized to urinary creatinine. Models are adjusted for age, BMI, current smoking, study site.
Abbreviations: BMI, body mass index; FN-BMD, femoral neck bone mineral density; LS-BMD, lumbar spine bone mineral density.

on the observed associations between glycine and incident fractures, we performed additional Cox regression models adjusted for FRAX estimates with or without BMD. The associations between both urinary and serum glycine and fracture risk at the different bone sites remained essentially unchanged after adjustments for FRAX and the estimates were only marginally reduced in models adjusted for FRAX with BMD (Table 7), indicating that glycine levels may contribute to fracture prediction beyond FRAX.

Discussion

In this study, we establish glycine as a robust predictor of FN-BMD. The meta-analysis with a large number of study subjects provides compelling statistical evidence of an inverse association between circulating glycine levels and FN-BMD. We also demonstrate that serum glycine is robustly inversely associate with the calculated bone strength parameter failure load, mainly as a consequence of an inverse association with cortical cross-sectional bone area and a direct association with cortical porosity. Finally, we demonstrate that both serum and urinary glycine levels predict incident fractures in men.

Previous studies evaluating the association between multiple metabolites and BMD have indicated that there might be an association between circulating glycine levels and BMD (2, 3). However, those studies were not designed to investigate glycine levels specifically in relation to BMD and in none of the studies did the association between glycine levels and BMD pass the predefined significance level adjusted for multiple testing. In the present study, we show significant associations between not only serum, but also urinary levels of glycine and FN-BMD. The association between glycine levels and FN-BMD also appears to be stable over time, as indicated by the significant association between serum glycine and FN-BMD both at the baseline visit and at the 5-year follow-up visit in the MrOS Sweden cohort.

We next performed a meta-analysis of the association between circulating glycine levels and FN-BMD using the significant results from the present study together with previously reported data from TWINS-UK, FHS Offspring, and HKOS, not passing the significant level after adjustment for multiple testing in any of these previous studies (2, 3). This meta-analysis, which included as many as 7543 study subjects, provided compelling statistical evidence for an inverse association between circulating glycine levels and FN-BMD. The MrOS cohort included only men. The largest cohort in the meta-analysis (TWINS-UK, n = 4399) consisted mainly of women. In the remaining 2 cohorts combined (FHS Offspring, n = 1552; HKOS, n = 634), women constituted 62% of the participants. Thus, glycine appears to be associated with FN-BMD in both men and women.

The HR-pQCT assessments in combination with glycine measurements make the MrOS Sweden cohort unique. Whereas DXA is a 2-dimensional technique that gives information about areal BMD, HR-pQCT produces a 3-dimensional image that permits separate analyses of the cortical and trabecular bone microstructure parameters. Previous human genetic studies using CT-based techniques have strongly suggested that different cortical and

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Table 7. Glycine levels and incident fractures

Fracture trait	Adjustment	N	Fractures	HR	95% CI	*P* value
All fractures						
S-Glycine	Age	969	238	1.17	1.03-1.33	0.02
	FRAX without BMD	969	238	1.17	1.03-1.34	0.01
	FRAX with BMD	959	233	1.16	1.02-1.32	0.02
U-Glycine	Age	2682	594	1.12	1.03-1.21	0.01
	FRAX without BMD	2682	594	1.11	1.02-1.21	0.01
	FRAX with BMD	2657	587	1.10	1.02-1.20	0.02
MOF						
S-Glycine	Age	969	170	1.22	1.05-1.43	0.01
	FRAX without BMD	969	170	1.23	1.05-1.43	0.01
	FRAX with BMD	959	169	1.21	1.04-1.41	0.01
U-Glycine	Age	2682	425	1.13	1.02-1.24	0.01
	FRAX without BMD	2682	425	1.12	1.02-1.24	0.02
	FRAX with BMD	2657	423	1.11	1.01-1.23	0.03
Hip fractures						
S-Glycine	Age	968	61	1.22	0.94-1.57	0.14
	FRAX without BMD	968	61	1.23	0.95-1.59	0.11
	FRAX with BMD	958	61	1.21	0.94-1.57	0.14
U-Glycine	Age	2681	177	1.18	1.01-1.36	0.03
	FRAX without BMD	2681	177	1.17	1.01-1.36	0.04
	FRAX with BMD	2656	176	1.17	1.00-1.36	0.05

Cox proportional hazards regression models adjusted for (1) age (and MrOS Sweden study site for urinary glycine), (2) FRAX without BMD (and MrOS Sweden study site for urinary glycine), or (3) FRAX with BMD (and MrOS Sweden study site for urinary glycine). Hazard ratios (HR), given with 95% CIs, are expressed per 1 SD increase in inverse normal transformed glycine (serum) or inverse normal transformed glycine normalized to urinary creatinine (urine). FRAX is the country-specific calculated estimate of the 10-year fracture risk. The FRAX estimated risk of a major osteoporotic fracture was used as covariate in models with fractures at any bone site (= all fractures) or MOF as the dependent variable. The FRAX estimate of risk of a hip fracture was used in the models with hip fractures as the dependent variable.

Abbreviations: BMD, bone mineral density; MOF, major osteoporotic fracture.

trabecular bone components are separately regulated (18, 19). In the present study of glycine levels, we found associations mainly with cortical bone parameters. This is interesting because a large proportion of fractures occur at sites containing mostly cortical bone (20). Moreover, cortical area and cortical density, and the calculated parameter failure load that intrinsically includes information on both structure and density, were all inversely associated with glycine levels, whereas cortical porosity was directly associated with glycine levels. All of these parameters have previously been shown to predict fracture risk independently of DXA (8, 21). Taken together, our HRpQCT data indicate that the association between glycine levels and DXA BMD are caused by an association between glycine and cortical bone microstructure parameters with a true impact on fracture risk.

Our study is the first to demonstrate that glycine levels predict incident fractures. In a recent study, circulating glycine was included in a panel of 27 metabolites belonging to different classes of compounds, which collectively improved fracture prediction. However, there were no data in that study on the associations for individual metabolites and fracture risk (3). The large number of fractures in our data set enabled us to study the predictive role of glycine for all fractures as well as for the subgroups of major osteoporotic and hip fractures. Both serum and urinary glycine were significantly associated with all fractures and major osteoporotic fractures. In the models with serum glycine, the point estimates for hip fractures were essentially the same as the point estimates for major osteoporotic fractures, but the associations did not reach statistical significance. This was most likely caused by the relatively low number of hip fractures (n = 61) in the data set with serum glycine available. However, in the more well-powered analyses (n = 177 hip fractures) using urinary glycine, the association with hip fractures risk was significant.

The association between glycine levels and fracture risk remained largely unchanged after adjustments for FRAX estimates without BMD. This indicates that glycine levels add information beyond well-established clinical risk factors. Although glycine levels were associated with FN-BMD in our study, inclusion of FRAX estimates with DXA-derived FN-BMD in the Cox regression models did only marginally affect the association between glycine levels and fracture risk. However, as previously mentioned, bone microstructure as assessed by HR-pQCT adds information on fracture

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risk beyond DXA measurements that are limited to the assessment of areal BMD (8, 21). Our findings suggest that there are associations between glycine levels and fracture risk mediated via bone properties that cannot be captured by 2-dimensional DXA measurements only. In addition, glycine may associate with non-bone factors including risk of falls contributing to fracture risk.

Our study is the first to provide data on the association between urinary glycine levels and bone parameters. Because urinary glycine was available in almost 3 times as many study subjects as serum glycine, this also substantially increased our sample size. Glycine is filtered through the glomerulus and reabsorbed by solute transporters in the proximal tubule. In a urine metabolomics study, the single nucleotide polymorphism rs3846710 in the *SLC36A2* gene, which encodes one of these transporters, was associated with glycine levels, but for this single nucleotide polymorphism, no association has so far been reported with serum glycine (22). This suggests that the regulation of urinary glycine is at least partly independent of the regulation of serum glycine. Consequently, associations with phenotypes could also differ between glycine measured in the 2 bio-mediums.

Plasma concentrations of amino acids reflect the combination of their rate of appearance (intake, tissue release from proteolysis, and de novo synthesis) and their rate of disappearance (metabolism, incorporation in proteins, and loss in urine and feces) (23). Glycine is involved in a wide range of biological functions and metabolic pathways that makes interpretation of associations intriguing. However, given that 90% of the bone matrix proteins are collagen, with every third amino acid residue being glycine, it is possible that increased bone resorption leads to higher circulating and urinary glycine levels. This is in line with the inverse association between glycine levels and BMD in our study. Hydroxyproline is another residue of the collagen triple helix. It has previously been used as a marker of bone resorption but has now been superseded by more bone specific markers (24). Interestingly, hydroxyproline can be converted to glycine in the kidney (25). Indeed, hydroxyproline has been suggested to be a major precursor for the synthesis of glycine in animals (26). One could thus speculate that glycine is a marker of bone resorption also as a product of hydroxyproline degradation.

A limitation of our study, however, is the lack of direct mechanistic studies evaluating the nature of the association for glycine with bone metabolism. Further studies are warranted to determine the role of glycine for bone metabolism.

In conclusion, we establish high glycine levels as a risk factor for low FN-BMD and osteoporotic fractures. This association was observed both for serum and urinary glycine and was observed at 2 different time points for serum glycine. We propose that high glycine levels are biomarkers of reduced cortical bone strength and increased risk of fractures.

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