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Circulating amino acids are associated with bone mineral density decline and ten-year major osteoporotic fracture risk in older community-dwelling adults

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ABSTRACT

With aging, poor bone mineral density (BMD) and accelerated decrease in BMD are strong risk factors for fracture. Reports of the associations of dietary protein intake with bone strength are inconsistent, possibly owing to differences in protein sources and amino acid (AA) composition. We examined the associations of serum AA with 4-year hip BMD loss and subsequent fracture risk within 10 years in older community-dwelling adults, and further addressed whether lifestyle, dietary protein intake and its source, and body composition would affect the associations. In 1424 men and 1573 women (mean age 72 years), using binary logistic regression, higher serum valine, leucine, isoleucine and tryptophan concentrations were associated (or approaching a borderline significance in case of the last three ones) with less hip BMD decline (defined as BMD loss ≥ 2.8 times the precision error of the BMD measurement at femoral neck) in 4 years later, with the OR (95%CI) /SD of AA increase, ranging from 0.83 (0.75, 0.91) to 0.92 (0.87, 0.98) after multiple adjustments for baseline age, gender, BMI, BMD, estimated glomerular filtration rate (eGFR), dietary protein intake (animal- and plant-derived protein intakes), calcium intake, established lifestyles (physical activity level, smoking and alcohol drinking status), osteoporosis medications, and changes of body fat and lean muscle mass. Higher serum total homocysteine (tHcy) concentration was independently associated with BMD decline 4 years later (OR (95%CI) /SD of 1.16 (1.05, 1.27)). Using multivariate Cox regression, higher serum tryptophan concentration potentially predicted low risk of incident major osteoporotic fractures (MOFs) (HR/SD (95%CI) = 0.86 (0.75, 0.98)) after multiple adjustments. Higher serum tHcy was associated with MOFs (HR/SD (95%CI) = 1.29 (1.12, 1.50)) risk after multiple adjustments in men. These findings suggest that a specific AA profile correlates with greater BMD and lower subsequent fracture risk, independent of diet and lifestyle factors.

1. Introduction

One of the largest musculoskeletal disease burdens is attributable to osteoporotic fractures, the incidence of which increases exponentially with age [1]. Recently, a large scale GWAS meta-analysis identified a series of genetic determinants of fractures, all of which are related to bone mineral density (BMD) [2]. With increasing age, accelerated decrease in BMD is a strong risk factor for fracture [3]. Besides osteoporosis therapy, many ways have been proposed to maintain bone strength or to improve it. Calcium intake from dietary sources or from supplements was found to have only minor effects on BMD [4,5]. On the other hand, several large studies and meta-analyses have revealed a benefit of higher protein intakes in attenuating age-related bone loss and reducing hip fracture risk [6–10]. Conflictingly, however, some non-significant associations have also been observed [9,11–13], suggesting a negligible net anabolic effect of dietary protein on BMD. An alternative explanation is that differences in the dietary protein sources and their amino acid (AA) composition may partly underlie these discrepant findings [14,15].

Mechanistic evidence suggests that specific AAs are beneficial for bone health, principally through promoting osteoblast growth and differentiation [16–18], improving collagen formation [19,20], and

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selectively acting as signalling molecules in bone cells [21,22]. Additionally, the potential function of the branched-chain AAs (BCAAs) is thought to take effect through increasing muscle protein synthesis [23,24]. Increasing intake of aromatic AAs (AAAs) might stimulate a rise in circulating level of insulin-like growth factor-1 (IGF-1) and influence calcium homeostasis [18], which are involved in the stimulation of mature osteoblasts [25] and regulate skeletal growth [26]. However, the endogenous acid load imposed by the metabolism of sulphur-containing AAs (SAAs) [27,28], may implicate SAAs in acid-mediated impairment of osteoblast function and stimulation osteoclast activity, thus increasing bone resorption and decreasing bone mass [27,29,30].

A study of men with idiopathic osteoporosis found specific changes in their plasma free AA profiles. [31] Recent metabolomics studies showed potential cross-sectional correlations of some circulating AAs and their metabolites with BMD in women (young, menopausal and post-menopausal), mainly reported AAs included cysteine [32–34], taurine [33], tryptophan [35], glutamine [36], etc. Further, a study in monozygotic twins demonstrated the genetic independent benefit of several specific AA (leucine in particular) intakes for bone health [24]. To date, there is scant data on the relationship of circulating AAs with bone health in a longitudinal perspective, as well as in later adult life. Given that circulating AAs are affected by dietary factors and body mass [37–39], we examined the association of serum BCAAs, AAAs and SAAs with hip BMD loss and incident osteoporotic fracture risk in older community-dwelling adults in Hong Kong, and further assessed whether lifestyle, dietary protein intake and its source, and body composition would influence these relationships.

2. Materials and methods

2.1. Study participants

The evaluations were conducted in the Mr. OS and MS. OS Hong Kong study which is the first large-scale cohort study to examine the determinants of osteoporotic fractures in older Chinese men and women; and the methodology has been described previously [40,41]. Two thousand Chinese men and 2000 Chinese women aged ≥ 65 years were recruited from local communities from August 2001 to March 2003 by recruitment notices and talks in community centres and housing estates. Those who [1] were unable to walk without assistance of another person [2] had a bilateral hip replacement [3] were not competent to give informed consent were excluded. A stratified sampling method was adopted so that approximately 33% would be in each of these age groups: 65–69 y, 70–74 y, ≥75 y. Participants were followed up by a visit to the research centre at the 4th year. Only those who were followed up at 4th year and had frozen serum available were selected for assay of serum AAs. For the present study, serum AA data was available for N = 1424 men (71.2% of men) and N = 1573 women (78.7% of women). Informed consent was obtained from all participants. The study was approved by the Clinical Research Ethics Committee of The Chinese University of Hong Kong.

2.2. Lifestyle data

The baseline assessment included an interview using a standardized, structured questionnaire. Data on medication and supplement use, lifestyle (smoking and alcohol consumption), and demographics were collected. Physical activity level was assessed using the Physical Activity Scale of the Elderly (PASE). [42] Body weight (kilograms) was measured wearing an examination gown using the Physician Beam Balance Scale (Healthometer, IL, USA). Body height (centimeters) was measured with a Holtain Harpenden stadiometer (Holtain Ltd., Crosswell, UK). Body mass index (BMI) (kg/m^2) was calculated.

Assessment of BMD, body fat and lean muscle mass

Areal hip BMD, body fat and lean muscle mass were measured with dual energy X-ray absorptiometry (DXA) using a Hologic QDR 4500 W device (Waltham, MA, USA). Centralized quality control procedures, certificated DXA operators, and standardized procedures for scanning were used to ensure measurement reproducibility [43]. The coefficients of variation (CVs) of the scanners estimated using a central phantom was 1.3%, 1.5%, and 0.8% for femoral neck BMD, fat and lean mass, respectively. Least significant difference (LSD) is calculated as 2.8 times the precision error of the test on a specific machine and site of measurement [44]. So, the difference in repeated measurement of femoral neck BMD ≥ 3.6% is assumed to be clinically significant in the present study. Each participant had the same hip (> 99.7% was right) scanned at both visits unless there was a fracture, implant hardware, or other problem preventing the scan, then the other hip was scanned. If a different hip side was used during the follow-up visits, scans were set to missing for longitudinal analyses. Repeat BMD measures were not available in 438 (21.9%) men and 434 (21.7%) women at the year-4 visit, due to death, refusal, or unqualified scans. The osteoporosis category was defined using a femoral neck BMD T-score calculated based on the NHANES III reference database for femoral neck measurements in women aged 20–29 years. [45,46]. Change of BMD (fat mass or lean muscle mass) was determined by the subtraction of femoral neck BMD (fat mass or lean muscle mass) at baseline from femoral neck BMD (fat mass or lean muscle mass) at year-4. Negative values equal to or less than minus one LSD indicate a decline in BMD during the period of 4 years, while the others are considered to be a maintained BMD.

2.3. Amino acid assays

At the baseline visit, blood was taken after an overnight fast. Serum was separated within 3 h and stored at −80 °C. AAs were measured in the stored serum by liquid chromatography–tandem mass spectrometry (LC–MS/MS) using a modified version of a previously described method [47]. Briefly, isotopically-labelled internal standards were added to serum, followed by reduction of disulphides using dithioerythritol and then protein precipitation using 5-sulfosalicyclic acid. The extracts were diluted with an aqueous solution of formic acid [0.5%] and heptafluorobutyric acid (HFBA) [0.3%] prior to analysis. LC–MS/MS was carried out using a Shimadzu LC-20ADXR Prominence LC system (Kyoto, Japan) coupled to a Sciex QTRAP5500 mass spectrometer with a Turbo V ion source and TurboIonspray probe (Framingham, MA, USA). Chromatographic separation was achieved on a Phenomenex Kinetex Core Shell C18 (100 x 4.6 mm, 2.6 μm) LC column (Torrance, CA, USA) with an aqueous solution of formic acid [0.5%] and HFBA acid [0.3%] and acetonitrile gradient mobile phase. Positive mode multiple reaction monitoring was used for detection. Linear calibration curves of the peak area ratios of analyte and internal standard were used for quantification. Coefficient of variation for the analytes were 3–7%. The method was validated for 16 of the 19 analytes using spiked serum QA samples from an external quality assurance scheme ERNDIM (www.erndim.org). Measured serum AAs included BCAAs (valine, leucine, and isoleucine), aromatic AAs (phenylalanine, tryptophan, and tyrosine), and SAAs (methionine, total homocysteine (tHcy), cystathionine, total cysteine (tCys), taurine, and total glutathione (tGSH)). The estimated glomerular filtration rate (eGFR) was calculated using the Modification of Diet in Renal Disease study equation based on standardized serum creatinine, gender and age [48].

2.4. Dietary data

Daily and weekly dietary intakes in the past year were assessed at baseline using a validated semi-quantitative FFQ [49]. Mean nutrient quantitation per day was calculated using food tables derived from McCance and Widdowson [50] and the Chinese Medical Sciences Institute [51]. Average intakes of total protein, animal- and plant-derived protein per day, and calcium per day were determined [52,53].

Table 1
The differences in characteristics, dietary intakes, and serum amino acid level of men and women between osteoporosis status.

Variables	Men (BMD status [a])		P value [b]	Women (BMD status [a])		P value [b]
	Non-osteoporosis (n = 1777) Mean (SD)	Osteoporosis (n = 223) Mean (SD)		Non-osteoporosis (n = 1175) Mean (SD)	Osteoporosis (n = 825) Mean (SD)	
Age (year)	72.1(4.8)	74.4(5.8)	< 0.001	71.5(4.8)	74.1(5.8)	< 0.001
Body mass index (BMI, kg/m^2)	23.8(3)	20.6(3.1)	< 0.001	24.9(3.2)	22.6(3.3)	< 0.001
Femoral neck BMD (g/cm^2)	0.71(0.1)	0.51(0.04)	< 0.001	0.65(0.07)	0.49(0.05)	< 0.001
Protein (% kcal) per day	16.5(3.8)	16.1(3.9)	0.053[c]	16.4(3.7)	16.0(3.8)	0.013 [c]
Animal protein (% kcal) per day	9.44(3.68)	9.04(3.98)	0.053 [c]	8.67(3.56)	8.33(3.88)	0.012 [c]
Plant protein (% kcal) per day	7.05(2.47)	7.00(2.45)	0.676 [c]	7.73(2.5)	7.68(2.44)	0.701 [c]
Calcium (mg/1000kcal) per day	301(116)	287(115)	0.050 [c]	365(135)	355(140)	0.062 [c]
Branched chain amino acids						
Valine (uM)	308(45)	280(41)	< 0.001	284(44)	272(44)	< 0.001
Leucine (uM)	159(24)	144(24)	< 0.001	143(22)	137(23)	< 0.001
Isoleucine (uM)	85.8(14.8)	77.0(14.0)	< 0.001	77.0(13.4)	74.0(14.2)	< 0.001
Aromatic amino acids						
Phenylalanine (uM)	98.7(13.1)	93.5(13.6)	< 0.001	97.1(13.8)	95.5(14.7)	0.033
Tryptophan (uM)	61.5(11.1)	58.9(10.3)	0.008	57.2(10)	55.6(11.1)	0.003
Tyrosine (uM)	84.9(14.6)	79.9(13.8)	< 0.001	83.2(14.5)	81.3(14.5)	0.012
Sulfur amino acids						
Methionine (uM)	31.1(4.7)	30.0(4.7)	0.008	24.0(4.3)	23.8(4.6)	0.621
tHcy (uM)	15.5(5.3)	15.5(4.8)	0.991 [c]	13.0(4.2)	13.2(4.4)	0.381 [c]
Cystathionine (nM)	287(193)	288(182)	0.585 [c]	232(168)	243(229)	0.635 [c]
tCys (uM)	362(45)	354(44)	0.066	353(43)	351(46)	0.416
Taurine (uM)	172(33)	167(33)	0.052 [c]	172(33)	169(32)	0.091 [c]
tGSH (uM)	4.15(1.37)	3.93(1.24)	0.074	4.15(1.31)	4.37(1.41)	0.002

SD: standard deviation; tHcy: total homocysteine; tCys; total cysteine; tGSH: total glutathione.
[a] BMD status defined using the femoral neck BMD T-score that was calculated based on the NHANES III reference database for femoral neck measurements in women aged 20–29 years: Non-osteoporosis: femoral neck BMD T score > −2.5, Osteoporosis: femoral neck BMD T score ≤ −2.5.
[b] P value for difference by Student's t test; 1424 men and 1573 women are available for serum amino acids assessment.
[c] P value for difference using log-transformed value for variables with skewed distribution.

2.5. Follow-up for major osteoporotic fractures (MOFs)

Fracture occurrence was mainly determined by carrying out a search of the Hospital Authority electronic database, which includes all visits to Accident and Emergency Departments and outpatient clinics and covers all publicly funded hospitals in Hong Kong from baseline to October 2013 [54]. A MOF was defined as a fracture of the hip, clinical spine, wrist or humerus.

2.6. Statistical analysis

The differences in anthropometric characteristics and serum AAs by BMD status (non-osteoporosis: femoral neck BMD T-score > -2.5, and osteoporosis: femoral neck BMD T-score ≤ −2.5) were evaluated using Student's *t*-test Log-transformed values were used for variables with skewed distribution. Spearman partial correlation co-efficient (r_s) adjusted for baseline age and gender was calculated for the correlations between baseline BMI, femoral neck BMD and individual AAs, and between dietary intakes of total protein, animal- and plant-derived protein and individual AAs, respectively.

Binary logistic regression was used to estimate the odds ratio (OR) with 95% confidence intervals (CI) per standard deviation (SD) in AA increase for 4-year change in femoral neck BMD (0: maintained; 1: decline). The basic models were adjusted for baseline age and femoral neck BMD. The full models were additionally adjusted for baseline BMI, eGFR, dietary protein intake (or animal- and plant-derived protein intakes), and calcium intake. Baseline current smoking and alcohol drinking status, baseline physical activity level, and osteoporosis medications at baseline or during follow-up, and change of body composition in 4 years were further adjusted for in the models. Cox regression model was used to estimate the hazards ratio (HR) with 95% CI for incident MOFs risk during a 10-year follow-up period (0: no fracture; 1: at least one fracture) per 1-SD higher concentrations of individual AAs. Since the association of tHcy with BMD decline was in opposite direction to the other SAAs, and since all SAAs are positively correlated, we added a model in which the associations of methionine, tCys, cystathionine, taurine and tGSH with BMD decline or fracture risk were adjusted for tHcy. P value for interaction between genders was calculated by introducing a product term in the models. The log-transformed values of dietary protein intake (or animal- and plant-derived protein intakes) and concentrations of specific AAs with skewed distribution were used to estimate their associations with BMD decline and fracture risk in the sensitivity analyses.

All statistical tests were two-tailed with P < 0.05 considered significant. To correct for multiple testing (of 12 individual AAs), Bonferroni adjustment was further used for AA-BMD (status or decline) and AA-fracture associations: a lower P value threshold of 0.004 (0.05/12) was set for significance. P values that were < 0.05 but did not meet the Bonferroni threshold of 0.004 are discussed as a trend. Statistical analyses were performed using SAS 9.4 (SAS Institute, Inc., Cary, NC, USA).

3. Results

3.1. Baseline population characteristics and associations

The average age of participants was 72.4 (SD 5.0) years in men and 72.6 (SD 5.4) years in women. At baseline, 223 (11.2%) men and 825 (41.3%) women had osteoporosis. Compared to subjects without osteoporosis, participants with osteoporosis were more likely to be older and have lower BMI. There were modest differences in dietary intakes between the groups, including lower intake of total and animal-derived protein in those with osteoporosis, relative to those without, and a trend for calcium intake to be lower in men and women with osteoporosis (P = 0.050 and 0.062, respectively). Serum BCAAs (valine, leucine, isoleucine) and AAAs (phenylalanine, tryptophan and tyrosine) were significantly lower in osteoporotic subjects, while serum methionine was lower in osteoporotic men (P = 0.008) and tGSH was higher in osteoporotic women (P = 0.002; Table 1) Although these statistical significances were observed, the absolute declines in the dietary intakes

and serum AAs aforementioned were actually small (< 10% in men and < 6% in women). When a Bonferroni-adjusted p value threshold of significance was applied (P < 0.004), serum BCAAs in both genders, phenylalanine and tyrosine in men, and tryptophan and tGSH in women were still significantly different by osteoporosis status.

Cross-sectional age- and gender- adjusted association of serum AAs with baseline measures of BMI, BMD and dietary intakes were examined. Serum BCAAs and AAAs showed positive correlations with baseline femoral neck BMD (r_s from 0.07 to 0.20; Supplementary Table 1). Among SAAs, only tHcy, tCys, cystathionine (r_s from 0.04 to 0.13) and tGSH (r_s = -0.05) had a minor but significant correlation with baseline femoral neck BMD. Baseline femoral neck BMD was correlated with BMI (r_s = 0.42; P < 0.001). Serum BCAAs and AAAs also showed positive correlations with baseline BMI (r_s from 0.11 to 0.34; Supplementary Table 1). Most SAAs were positively correlated with baseline BMI (r_s from 0.04 to 0.21), except tGSH, which was negatively correlated (r_s = -0.11; Supplementary Table 1). The associations of dietary intakes of total, animal- and plant-derived protein with serum AA concentrations were overall minor and not consistent, apart from positive correlations between animal protein intake and serum BCAAs, AAAs (apart from tyrosine) and taurine (Supplementary Table 2). Neither methionine nor tCys, the 2 SAAs ingested in diet, showed any correlations with dietary protein quantity or type.

3.2. AA predictors of BMD decline

The mean (SD) of BMD change over 4 years was -0.005 (0.031) g/cm^2 in men and -0.013 (0.032) g/cm^2 in women. According to the LSD of 3.6% as assumed previously, those who had a 4-year BMD decrease ≥ 3.6% of baseline BMD were defined as having a decline in BMD. Four years later, 319 (22.5%) men and 604 (38.9%) women had a decline in their BMD. These men and women had a mean 4-year BMD change of -0.045 (0.022) g/cm [2] and -0.042 (0.018) g/cm^2 respectively, whereas men and women with maintained BMD had a mean change of 0.007 (0.022) g/cm [2] and 0.006 (0.024) g/cm^2 respectively. Multivariate logistic regression models evaluated the AA predictors of BMD decline. In the basic model, adjusted for baseline measures of age, BMD and gender, higher serum valine, tryptophan and tGSH concentrations predicted lower risk of BMD decline (OR (95%CI) = 0.88 (0.81, 0.95), 0.88 (0.81, 0.96) and 0.92 (0.85, 0.99) per 1 SD increase in concentration of the respective AA). High serum tHcy predicted greater BMD decline (OR/SD (95%CI) = 1.17 (1.08, 1.27)). The associations in relation to serum valine, tryptophan and tHcy largely persisted after further adjustment for baseline measures of BMI, eGFR, dietary protein intake (or animal- and plant-derived protein intakes), calcium intake, smoking, alcohol consumption, and baseline physical activity, osteoporosis medication at baseline or during follow-up, as well as change of body fat mass and lean muscle mass within first 4 years (Table 2). After full adjustments, higher serum leucine and isoleucine were significantly associated with BMD decline (OR (95%CI) = 0.92 (0.87, 0.98) and 0.87 (0.79, 0.96) per 1 SD increase in concentration of the respective AA), while none of the other SAAs apart from tHcy were associated with BMD decline. No significant difference between genders was observed for the AA-BMD decline association with P values for interaction all above 0.05 (from 0.064 to 0.968). Serum tCys, taurine and tGSH predicted lower risk of BMD decline only after controlling for tHcy in a basic model that included gender and baseline femoral neck BMD (OR/SD = 0.89 – 0.91; Supplementary Table 3). The associations became non-significant after further adjustment for BMI and other confounders. In the full model, the associations of valine (inverse) and tHcy (positive) with BMD decline in the total population remained significant after applying the Bonferroni-corrected P value threshold of 0.004. The P value for the association of tryptophan with BMD decline was 0.003 in the basic model and 0.005 in the full model.

3.3. Predictors of fractures

The subjects were followed up for fractures for a median of 9.6 years (range 4.0–12.2). In those who had measurements for AAs (N = 2997), 252 (8.8%) had at least one incident MOF. The incidence rate of MOFs was 10.1/1,000 person-years. Multivariate cox regression models evaluated the 4-year BMD decline, AA and dietary protein/calcium intake predictors of incident fracture risk, respectively. BMD decline over the first 4 years was significantly associated with incident MOFs (HR/SD (95%CI) = 1.76 (1.35, 2.29)) risk, without gender difference (P-interaction > 0.05). As shown in Table 3,1 SD-higher serum tryptophan and taurine predicted a 14% lower risk of MOFs (HR/SD (95%CI) = 0.86 (0.75, 0.98) and 0.86 (0.76, 0.99), respectively) after adjustment for baseline measures of age, gender, femoral neck BMD, BMI, eGFR, dietary protein intake (or animal- and plant-derived protein intakes), calcium intake, smoking, alcohol consumption, and baseline physical activity, osteoporosis medication at baseline or during follow-up, as well as change of body fat mass and lean muscle mass within first 4 years. Conversely, higher serum tHcy was associated with incident MOFs risk in men (HR/SD (95%CI) = 1.29 (1.12, 1.50)), but not in women (HR/SD (95%CI) = 0.92 (0.76, 1.11), P-interaction = 0.007). No significant association of serum BCAAs (valine, leucine, and isoleucine) with fracture risk was observed. Higher dietary total and animal-protein intakes were associated with protection against incident MOFs risk in men (HR/SD (95%CI) = 0.65 (0.49, 0.86) and 0.64 (0.50, 0.82), respectively), but not in women (HR/SD (95%CI) = 1.04 (0.88, 1.22) and 1.04 (0.90, 1.19), P-interaction = 0.052 and 0.002, respectively). No significant association of dietary vegetable-protein and calcium intakes with fracture risk was observed. (Table 3 and Fig. 1) Using a Bonferroni-corrected P value threshold of 0.004, only serum tHcy significantly predicted 10-year fracture risk (P < 0.001). There was no notable difference in HR/SD in the basic model when only baseline age and femoral neck BMD were adjusted for (data not shown). In the sensitivity analyses, the associations of log-transformed total protein intake (or animal- and plant-protein intakes), calcium intake, and concentrations of tHcy, cystathionine and taurine with BMD decline and fracture risk were not essentially changed (Supplemental Tables 4 and 5).

4. Discussion

Dietary and/or circulating concentrations of BCAAs, AAAs, SAAs have emerged as correlates of bone health [24,31,34,36]. However, their associations with longitudinal change of BMD and fracture risk have not been systematically investigated in older adults. We evaluated the associations of serum BCAAs, AAAs, SAAs with 4-year decline of femoral neck BMD and 10-year MOFs risk in older community-dwelling adults. Higher serum valine was independently associated with lower risk of BMD decline over 4 years (17% lower risk per SD), with similar but weaker associations observed for leucine and isoleucine. Tryptophan, but not other aromatic AAs, also predicted lower risk of BMD decline (12% lower risk per SD), and a trend towards lower 10-year fracture risk (14% per SD), independent of dietary and lifestyle factors. Conversely, higher tHcy was associated with significant BMD decline, and was independently associated with higher risk of MOFs in men. Subjects without osteoporosis at baseline had modestly higher dietary total and animal-protein intakes, but the longitudinal dietary protein benefits on fracture risk were only significant in older men.

Consistent with previous studies, the mean change of hip BMD was close to the estimates reported for older Caucasian men in the US, and significant BMD decline was a strong and independent risk factor for incident MOFs [3]. The potential benefit of BCAAs in bone health, may be linked to their associations with greater muscle mass [23] and fat mass [55], both of which are thought to be critical for the maintenance of bone strength and density [56,57] (also as shown in the present study; Supplemental Table 6). However, the associations of BCAAs with

Table 2
The association of anthropometric characteristics, dietary intakes, and serum amino acid with the decline of femoral neck BMD over 4 years.

	BMD Decline		
	No (n = 2167)	Yes (n = 961)	
Variable	OR/SD (95%CI)[a, b]	OR/SD (95%CI)[a]	OR/SD (95%CI)[b]
Age (year)	Reference (1.00)	1.15 (1.06, 1.25) [c]	1.05 (0.95, 1.15)
Body mass index (BMI, kg/m^2)	Reference (1.00)	0.98 (0.90, 1.07)	0.90 (0.82, 0.99) [c]
Femoral neck BMD (g/cm^2)	Reference (1.00)	1.00 (0.91, 1.10)	0.97 (0.87, 1.09)
Protein (% kcal) per day	Reference (1.00)	0.94 (0.87, 1.01)	0.95 (0.86, 1.04)
Animal protein (% kcal) per day	Reference (1.00)	0.97 (0.90, 1.05)	0.97 (0.89, 1.06)
Plant protein (% kcal) per day	Reference (1.00)	0.95 (0.88, 1.03)	0.97 (0.88, 1.07)
Calcium (mg/1000kcal) per day	Reference (1.00)	0.95 (0.88, 1.03)	1.02 (0.92, 1.13)
Branched chain amino acids			
Valine (uM)	Reference (1.00)	0.88 (0.81, 0.95) [c, d]	0.83 (0.75, 0.91) [c, d]
Leucine (uM)	Reference (1.00)	0.92 (0.84, 1.00)	0.92 (0.87, 0.98) [c]
Isoleucine (uM)	Reference (1.00)	0.93 (0.85, 1.01)	0.87 (0.79, 0.96) [c]
Aromatic amino acids			
Phenylalanine (uM)	Reference (1.00)	0.98 (0.91, 1.07)	1.00 (0.91, 1.09)
Tryptophan (uM)	Reference (1.00)	0.88 (0.81, 0.96) [c, d]	0.88 (0.80, 0.96) [c]
Tyrosine (uM)	Reference (1.00)	0.97 (0.89, 1.05)	0.97 (0.89, 1.06)
Sulfur amino acids			
Methionine (uM)	Reference (1.00)	1.03 (0.93, 1.14)	1.02 (0.91, 1.13)
tHcy (uM)	Reference (1.00)	1.17 (1.08, 1.27) [c, d]	1.16 (1.05, 1.27) [c, d]
Cystathionine (nM)	Reference (1.00)	1.03 (0.96, 1.11)	1.00 (0.92, 1.09)
tCys (uM)	Reference (1.00)	0.97 (0.89, 1.05)	0.94 (0.85, 1.04)
Taurine (uM)	Reference (1.00)	0.94 (0.86, 1.02)	0.93 (0.85, 1.01)
tGSH (uM)	Reference (1.00)	0.92 (0.85, 0.99) [c]	0.93 (0.85, 1.01)

OR: odd ratio; SD: standardized deviation. CI: confidence interval. tHcy: total homocysteine; tCys; total cysteine; tGSH: total glutathione. 1420 men and 1552 women are available for serum amino acids assessment.
[a] adjusted for baseline age, femoral neck BMD and gender.
[b] adjusted for baseline age, femoral neck BMD, gender, BMI, eGFR, dietary protein intake (/and animal- and plant-derived protein intakes), calcium intake, smoking and alcohol drinking status, physical activity level, osteoporosis medications, and change of body fat and lean muscle mass during 4-y follow-up.
[c] indicates for significant value ($P < 0.05$).
[d] indicates for significant value ($P < 0.004$) for AAs.

Table 3
The associations of dietary protein and calcium intakes and serum amino acids with the incident major osteoporotic fracture risk during 10-year follow-up.

Predictors	Major osteoporotic fractures, HR/SD (95%CI) [a]			
	Men, n = 84 (6.1%)	Women, n = 168 (11.3%)	All, n = 252 (8.8%)	P for interaction [b]
Dietary intakes				
Protein (% kcal) per day	0.65 (0.49, 0.86) [c]	1.04 (0.88, 1.22)	0.91 (0.79, 1.05)	0.052
Animal protein (% kcal) per day	0.64 (0.50, 0.82) [c]	1.04 (0.90, 1.19)	0.90 (0.79, 1.03)	0.002
Plant protein (% kcal) per day	1.19 (0.95, 1.50)	0.98 (0.81, 1.17)	1.06 (0.92, 1.22)	0.139
Calcium (mg/1000kcal) per day	1.30 (0.97, 1.73)	1.02 (0.84, 1.24)	1.09 (0.93, 1.27)	0.529
Branched chain amino acids				
Valine (uM)	0.95 (0.74, 1.22)	0.88 (0.74, 1.04)	0.89 (0.77, 1.02)	0.599
Leucine (uM)	1.00 (0.79, 1.28)	0.90 (0.76, 1.06)	0.92 (0.79, 1.07)	0.449
Isoleucine (uM)	0.97 (0.76, 1.24)	0.94 (0.79, 1.11)	0.94 (0.81, 1.09)	0.721
Aromatic amino acids				
Phenylalanine (uM)	1.02 (0.81, 1.28)	0.94 (0.80, 1.11)	0.97 (0.85, 1.10)	0.486
Tryptophan (uM)	0.92 (0.74, 1.16)	0.83 (0.71, 0.98) [c]	0.86 (0.75, 0.98) [c]	0.524
Tyrosine (uM)	0.96 (0.76, 1.20)	0.96 (0.81, 1.12)	0.96 (0.84, 1.09)	0.813
Sulfur amino acids				
Methionine (uM)	0.95 (0.75, 1.20)	0.86 (0.73, 1.01)	0.86 (0.72, 1.01)	0.475
tHcy (uM)	1.29 (1.12, 1.50) [c, d]	0.92 (0.76, 1.11)	1.10 (0.98, 1.23)	0.007
Cystathionine (nM)	1.00 (0.82, 1.22)	0.85 (0.65, 1.11)	0.93 (0.78, 1.11)	0.238
tCys (uM)	1.09 (0.85, 1.40)	0.99 (0.83, 1.18)	1.01 (0.88, 1.17)	0.416
Taurine (uM)	0.75 (0.59, 0.95) [c]	0.92 (0.79, 1.09)	0.86 (0.76, 0.99) [c]	0.133
tGSH (uM)	1.04 (0.84, 1.29)	1.03 (0.88, 1.19)	1.03 (0.91, 1.17)	0.953
BMD decline over 4 years (Yes/No)	1.98 (1.25, 3.13) [c]	1.68 (1.22, 2.31) [c]	1.76 (1.35, 2.29) [c]	0.424

HR: hazard ratio; SD: standardized deviation. CI: confidence interval; tHcy: total homocysteine; tCys; total cysteine; tGSH: total glutathione.
[a] Adjusted for baseline age (/and gender), femoral neck BMD, BMI, eGFR, dietary protein intake (/and animal- and plant-derived protein intakes), calcium intake, smoking and alcohol drinking status, physical activity level, osteoporosis medications, and change of body fat and lean muscle mass during 4-y follow-up.
[b] P value for interaction between genders.
[c] Indicates for significant value ($P < 0.05$). [d] indicates for significant value ($P < 0.004$) for AAs.

bone loss were robust to the adjustments for BMI and longitudinal change of body fat and muscle mass. Unexpectedly, the association of BCAAs with fracture risk was not significant, irrespective of its benefit on BMD maintaining over first 4 years. It is possible that falls related factors had been involved during the follow-up which had attenuated the effect related to bone density. More studies are needed to clarify these associations.

A consistent inverse association of serum tryptophan concentration

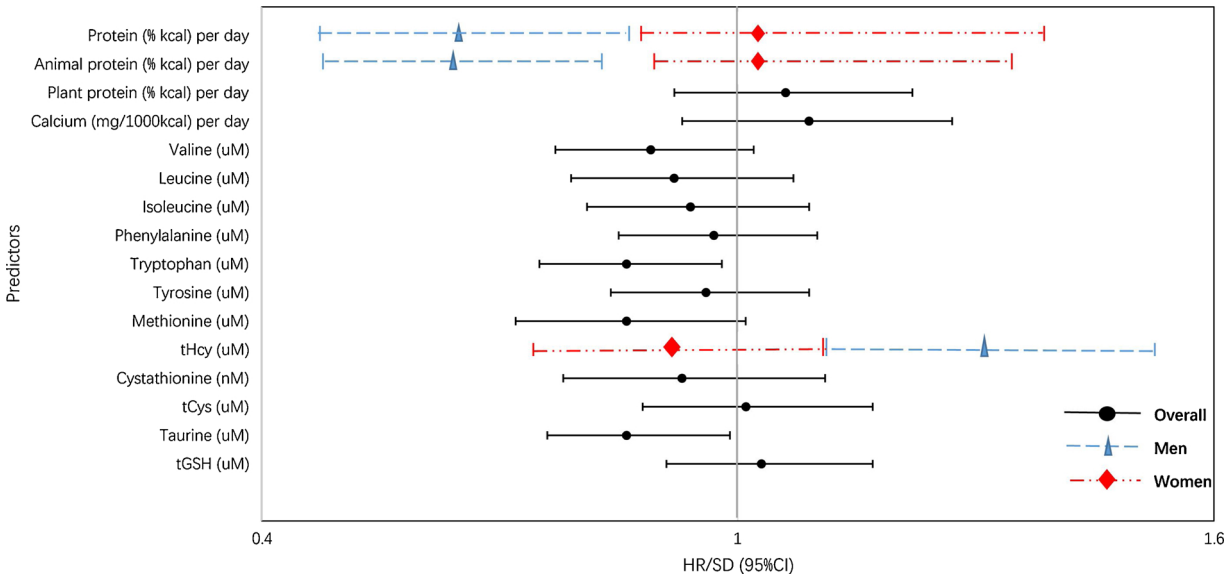

Fig. 1. Hazard ratios (HRs) for incident major osteoporotic fracture risk during a 10-year follow-up period for each SD higher concentrations of serum amino acids at baseline or per SD-higher of dietary protein or calcium intakes. Where there was a significant interaction by gender ($P < 0.05$), the HRs were separately shown for men and women.

with BMD decline and fracture risk was observed in the present study, although they became marginal- or non-significant after Bonferroni adjustment. A particularly strong correlation of circulating tryptophan concentrations with lean mass has been observed previously [58]. However, in the present study tryptophan predicted lower risk of BMD decline and fractures independent of the change in lean mass and fat mass. Although tryptophan is an essential AA, suggesting that it may be acting as a diet biomarker, the associations were also independent of dietary protein quality and quantity. A potential role of tryptophan and its metabolites in bone metabolism has been reviewed [59]. In vitro, tryptophan, enhanced calcium uptake by bone marrow stromal cells, which are key targets for bone anabolism [21]. Aromatic AAs including tryptophan were found to inhibit osteoclastic differentiation [60], and when supplemented in mouse diet, were able to rescue the decline in femoral BMD caused by low protein intake [21]. In men with idiopathic osteoporosis erythrocyte (albeit not plasma) tryptophan concentrations were decreased, and they correlated with BMD and histomorphometric indicators of bone formation [31]. Further, plasma tryptophan was shown to correlate with BMD in a cross-sectional study of older men and women from the Hordaland Health Study [61]. The present study using prospective clinical outcomes extends this diverse body of evidence suggesting a prospective role of tryptophan in bone health. In contrast, high serum concentrations of the tryptophan products kynurenine and serotonin were recently linked to bone loss in mice [62] and fracture risk in older men [63], respectively. It would be interesting to test whether serum kynurenine, serotonin and tryptophan are correlated, and how they simultaneously link to longitudinal change of BMD and fracture risk.

The observed association of tHcy with BMD decline (and increased fracture risk in men) is in line with a large body of evidence [64]. Homocysteine levels are greatly impacted by folate and B-vitamin, and high tHcy concentrations are thought to enhance bone resorption by simulating osteoclastic activity and disturbing collagen cross-linking [65], although tHcy-lowering by B-vitamin therapy did not lower fracture risk [66]. SAAs also were suggested to be associated with increased acid production and hence increased bone resorption and subsequent bone loss, at least in certain population subgroups [30,67]. Yet no detrimental effect was observed for the other SAAs besides tHcy, including the 2 proteinogenic SAAs ingested in diet, methionine and tCys. In fact, consistent with previous observations in a Caucasian population [68], serum tCys, as well as taurine, were associated with a trend towards lower risk of BMD decline once tHcy was adjusted for. The associations were modest and appeared to be mediated via BMI [69]. In addition, serum higher taurine was independently associated with a trend towards lower fracture risk. Overall, these data point to a modest protective effect of higher taurine and tCys in relation to bone health, and a detrimental effect of elevated tHcy on BMD and fracture risk.

Inconsistent with the acid-load hypothesis, subjects with osteoporosis had slightly lower dietary total and animal-protein intakes. Further, higher intake of total and animal-derived protein predicted a 35% lower fracture risk in men, but not in women. The beneficial role of dietary protein may be related to improving calcium absorption efficiency and altering the bioavailability of IGF-1 in relation to longitudinal bone health [6,17]. The gender difference in 10-year fracture risk prediction may be due to older men having a higher protein intake than older women in the present study [52] (median of 1.32 g/kg body weight in men and 1.11 g/kg body weight in women, respectively). The overall weak associations of dietary intakes of total, animal- and plant-derived protein with serum AA concentrations suggest that circulating level of AAs are a function of metabolism and homoeostatic control in addition to diet [70,71]. The Epic-Oxford study similarly noted that large differences in dietary protein sources were not necessarily associated with large or consistent differences in circulating amino acid concentrations [72].

Strengths of the present study include the large sample of well-characterized participants with approximately 4-year follow-up for BMD and 10-year follow-up for fracture events, and comprehensive data on lifestyle and dietary confounders. However, our study only investigated serum BCAAs, AAAs, and SAAs, without considering their metabolites, which hinders mechanistically relevant pathway analyses, possibly achievable in metabolomics studies.

In conclusion, high circulating valine significantly predicted a 17% lower risk of BMD decline after 4 years, independent of diet and lifestyle factors, with similar but weaker associations for the other BCAAs. Tryptophan, but not other aromatic AAs, also predicted a trend towards lower risk of BMD decline (12% per SD), as well as lower 10-year fracture risk. Among the SAAs, high circulating tHcy predicted greater bone loss and, in men, higher fracture risk, independent of diet and lifestyle factors. These findings support a role for specific AAs in bone health and fracture prevention in older adults. Further evaluations in larger cohorts of older adults of different ethnicities are required.

Ethical approval

All procedures performed in the studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Declaration of Competing Interest

Yi Su, Amany Elshorbagy, Cheryl Turner, Helga Refsum, Ruth Chan and Timothy Kwok declare that they have no conflict of interest.

Data described in the manuscript, code book, and analytic code will be made available upon application and approval.

Author contributions

Study concept and design: TK and YS; Acquisition of data: TK, HR and CT; Analysis and interpretation of data: YS, AE and CT; Drafting of the manuscript: YS and AE; Revision of the manuscript: TK, HR, RC and CT. Approving final version of manuscript: YS, AE, CT, HR, RC and TK. YS takes responsibility for the integrity of the data analysis.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bone.2019.115082.

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