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ORIGINAL COMMUNICATION

Ethnic differences in osteocalcin γ-carboxylation, plasma phylloquinone (vitamin K_1) and apolipoprotein E genotype

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Objective: To investigate plasma osteocalcin γ-carboxylation and its relationship to plasma phylloquinone concentration and apolipoprotein E (apoE) genotype in women from three ethnic groups with differing osteoporotic fracture risk.
Design and subjects: Fasted blood samples were collected from postmenopausal Gambian ($n=50$), British ($n=31$) and Chinese women ($n=23$), and 11 premenopausal women in each group from three cross-sectional studies.
Results: After adjustment for total osteocalcin, plasma undercarboxylated osteocalcin (adjusted ucOC) was lowest in Chinese and highest in British women postmenopause (British *vs* Chinese 103% higher, $P<0.0001$; Gambian *vs* Chinese 66% higher, $P<0.01$). No differences were observed premenopause. Within each ethnic group, adjusted ucOC was similar pre- and postmenopause. Postmenopause, plasma phylloquinone was higher in Chinese women (1.0 ng/ml) than in British (0.31 ng/ml) and Gambian women (0.36 ng/ml) ($P<0.0001$). Premenopause, plasma phylloquinone was higher in Gambian and Chinese women (0.6 ng/ml) than in British women (0.3 ng/ml; $P=0.01$). Plasma phylloquinone and adjusted ucOC were inversely related in postmenopausal British women ($R^2=32.4\%$; $P=0.0008$). ApoE4 frequency was Gambian 32.6%, British 13.8% and Chinese 6%. A lower adjusted ucOC was associated with apoE2 genotype in British and Chinese women. Ethnic differences in adjusted ucOC persisted after adjustment for phylloquinone and apoE genotype.
Conclusion: These preliminary data indicate suboptimal vitamin K status in postmenopausal British compared to Chinese and Gambian women. Ethnic differences in apoE genotype may also influence osteocalcin γ-carboxylation status. The study highlights the need for larger epidemiological investigations of ethnic differences in vitamin K status and the possible implications to bone health.
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Introduction

Osteoporotic fractures are major public heath problems in Caucasian populations while age-adjusted fracture incidence in Africa and Asia is substantially lower (Solomon, 1968; Adebajo *et al*, 1991; Lau & Cooper, 1996; Yan *et al*, 1999). The aetiology of ethnic differences in osteoporotic fracture incidence is not known and cannot be explained by variations in dietary calcium intake or bone mineral density (BMD) (Prentice *et al*, 1991; Cummings *et al*, 1994; Aspray *et al*, 1996; Lau & Cooper, 1996).

Subclinical vitamin K deficiency, indicated by undercarboxylation of plasma osteocalcin, has been implicated in the pathogenesis of osteoporotic fracture in most investigations (Szulc *et al*, 1993; Binkley & Suttie 1995;

Vergnaud *et al*, 1997; Shiraki *et al*, 2000). Osteocalcin is the most abundant noncollagenous protein of bone. Nanomolar concentrations enter the blood to provide an index of osteoblast activity and bone turnover. During synthesis, osteocalcin undergoes a vitamin K-dependent post-translational modification in which three glutamic acid residues are γ-carboxylated to produce carboxyglutamic acid residues (Dowd *et al*, 1995). The precise role of osteocalcin and importance of Gla in bone physiology is not understood.

The predominant dietary and circulating form of vitamin K is phylloquinone (vitamin K_1). Previous studies have established that the degree of osteocalcin γ-carboxylation is responsive to changes in dietary intake of phylloquinone and reflects plasma phylloquinone concentration (Sokoll & Sadowski, 1996; Sokoll *et al*, 1997; Booth *et al*, 1999). Phylloquinone is mainly transported in the circulation by triacylglycerol-rich lipoproteins (TRL) (Lamon-Fava *et al*, 1998). It is likely that the availability of phylloquinone to bone is affected by factors influencing lipoprotein metabolism. One of these factors is apolipoprotein E (apoE) that acts as a ligand for TRL cellular uptake in general (Mahley & Hussain, 1991) and for osteoblast uptake of TRL-associated phylloquinone in particular (Newman *et al*, 2002). The apoE gene is polymorphic, with three common alleles resulting in six genotypes (E2/2, E2/3, E2/4, E3/3, E3/4 and E4/4) (Davignon *et al*, 1988). ApoE genotype has been linked to the TRL-mediated transport of phylloquinone to bone (Saupe *et al*, 1993; Kohlmeier *et al*, 1997) and to osteoporotic fracture risk (Kohlmeier *et al*, 1998; Cauley *et al*, 1999) and low BMD (Shiraki *et al*, 1997), although not all studies find associations between apoE genotype and BMD or fracture risk (Booth *et al*, 2000, Heikkinen *et al*, 2000).

To date, it is not known whether there are ethnic differences in vitamin K status, and if so, whether these may be implicated in ethnic variations in osteoporotic fracture risk. We have performed a preliminary investigation of vitamin K status of bone by measuring plasma osteocalcin γ-carboxylation in premenopausal and postmenopausal women from Britain, The Gambia (West Africa) and north-eastern People's Republic of China. We also studied the inter-relationship between osteocalcin carboxylation, plasma phylloquinone concentration and apoE genotype.

Subjects and Methods

Subjects

Approval was granted by the ethics committees responsible for the research in each country (MRC Dunn Nutritional Ethical Committee, MRC Gambia Ethics Committee and Institutional Committee for Shenyang Medical College). British women were recruited in Cambridge, UK. Recruitment was based on volunteers responding to poster displays. ApoE genotyping was performed on 152 subjects and plasma phylloquinone and osteocalcin measurements were performed on a subgroup of 11 premenopausal women (mean ± s.d. age 35.6 ± 11.0 y, weight 58.5 ± 6.2 kg, height 163.8 ± 5.2 cm) and 31 postmenopausal women (mean ± s.d. age 67.4 ± 7.1 y, weight 68.6 ± 10.4 kg, height 163.0 ± 7.1 cm). Blood analyses were performed following apoE genotyping to ensure each allele was represented. All of the British subjects were Caucasians.

The Gambian women were recruited from Keneba, a remote rural village in the West Kiang district. It is inhabited by approximately 3 000 people, predominantly of the Mandinka ethnic group. All women in the village between the ages of 55 and 75 y and women who were premenopausal nonpregnant and nonlactating were approached to participate in the study. Recruitment was based on willingness to participate. A total of 50 postmenopausal (mean ± s.d. age 66.7 ± 7.8 y, weight 48.8 ± 6.6 kg, height 156.4 ± 4.8 cm) and 11 premenopausal (mean ± s.d. age 37.2 ± 4.3 y, weight 58.5 ± 9.3 kg, height 159.8 ± 8.3 cm) women were recruited into the study (apoE genotyping, plasma phylloquinone and osteocalcin measurements).

To obtain more accurate information about apoE genotype distribution in Gambian subjects from Keneba, additional blood samples for genotyping were obtained from 77 subjects participating in studies for which approval for genotyping had been obtained. They represented a cross-section of the local community and included male and female children 8–12 y of age and women >20 weeks pregnant. In common with other areas of West Africa, hip fracture incidence is low in rural areas of The Gambia, although accurate statistics are not available (Prentice *et al*, 1991; Aspray *et al*, 1996).

Chinese women were recruited from the Huanggu district of Shenyang, the capital city of Liaoning Province in the north-east of the Peoples Republic of China. Recruitment was made through residences and workshops of a large factory. The subjects were all Han Chinese and represented staff or retired female employees who volunteered to participate in the study. ApoE genotyping was performed on 116 volunteers. Owing to cost limitations, plasma phylloquinone and osteocalcin measurements were performed on 23 postmenopausal (mean ± s.d. age 67.6 ± 3.0 y, weight 58.7 ± 7.0 kg, height 152.5 ± 4.3 cm) and 11 premenopausal women (mean ± s.d. age 30.9 ± 11.0 y, weight 56.0 ± 6.2 kg, height 159.6 ± 4.7 cm) based upon their apoE status to ensure that each allele was included. Hospital admission data of people over 50 y in Shenyang show the annual age-adjusted hip fracture incidence in women is 67 per 100 000. This is eight-times lower than in women from USA and North European countries (Yan *et al*, 1999).

In each country, the exclusion criteria were pathological disorders or medications (such as thyroid disorders or cancer) known to alter calcium or bone metabolism. Postmenopausal women had not menstruated within the last 2 y. Premenopausal women were either never pregnant or had not been pregnant/lactating for the last 2 y. Blood samples were collected into EDTA (for phylloquinone) or lithium

heparin (for osteocalcin) anticoagulant in the morning, following an overnight fast and immediately placed on ice. Samples were processed within 90 min of collection with a refrigerated centrifuge at each study site. The plasma, plus an aliquot of whole blood (for apoE genotyping) were stored at −70°C immediately. Samples were collected over a period of 2 y. British and Gambian samples were collected during different seasons, whereas Chinese samples were collected in the spring.

Plasma osteocalcin γ-carboxylation

Assessment of plasma osteocalcin γ-carboxylation was based on the differential binding of Glu and Gla residues to hydroxyapatite (Price *et al*, 1981; Knapen *et al*, 1989; Merle & Delmas, 1990). Amounts of 5 mg hydroxyapatite (calcium phosphate tribasic, Sigma, Poole, UK) and 250 μl of heparinised plasma were incubated for 1 h at 4°C and centrifuged (2000 *g*, 15 min, 4°C). An aliquot of plasma was treated similarly but in the absence of hydroxyapatite. The osteocalcin concentration in the plasma or supernatant following hydroxyapatite incubation was assayed immediately in duplicate by a radioimmunoassay which measures intact and fragmental osteocalcin (Incstar Corporation, Stillwater, MN, USA). Owing to limited sample volume, hydroxyapatite binding was performed in singleton for each sample. The concentration of osteocalcin after hydroxyapatite incubation represented undercarboxylated osteocalcin (ucOC). Assay performance was monitored by kit and in-house controls. The in-house control was a pooled plasma sample with intermediate concentrations of tOC, as determined by repeat measurements. Limit of sensitivity was defined as the concentration 3 s.d. from counts per minute at maximal binding (0.2 ng/ml). For all subjects, tOC concentration was greater than this value. Posthydroxyapatite binding, ucOC concentrations below this limit were found in four postmenopausal and five premenopausal British, four postmenopausal and three premenopausal Gambian, and 10 postmenopausal Chinese and nine premenopausal Chinese subjects, and were assigned an intermediate concentration of 0.1 ng/ml. Samples from each study site were included in each assay. The inter- and intra-assay coefficients of variation (CV) were 7.6 and 2.0% for tOC, and were 16.9 and 6.2% for ucOC, respectively.

Different methods were used to analyse and compare osteocalcin γ-carboxylation data between ethnic groups. An ANCOVA model was constructed for ucOC using ethnic group and tOC as independent variables. This permitted comparison of ethnic groups after adjustment for differing concentrations of tOC, without making assumptions about the nature of the relationship between tOC and ucOC. Carboxylation was also compared between ethnic groups when expressed as the conventional term %ucOC (ucOC as a percentage of tOC concentration). This makes the assumption that ucOC is directly proportional to tOC throughout the range of observed values.

Plasma phylloquinone and triacylglycerol

Plasma phylloquinone concentrations were determined by a multistage procedure using an internal standard of menaquinone-6 (MK-6) and HPLC with redox-mode electrochemical (ECD) (McCarthy *et al*, 1997) and/or spectrofluorimetric detection after post-column zinc reduction (Davidson & Sadowski, 1997). Plasma phylloquinone measurements made by either detection method were highly correlated ($r = 0.95$; $P < 0.001$), with no significant bias as shown by Bland and Altman analysis. Samples from each ethnic group were assayed using both detection methods. The lower limit of detection from 0.5 ml plasma was 0.05 ng/ml. The interassay coefficients of variance by ECD and by fluorescence detection were 7.4 and 9.8% respectively.

A known amount (ca. 0.8 ng) of the MK-6 internal standard (in 0.2 ml of ethanol) was added to EDTA plasma (0.5 ml) and mixed with a further 3.8 ml of ethanol and 12 ml of hexane. The mixture was thoroughly vortex-mixed and centrifuged to obtain an upper hexane phase. The hexane extract was used for subsequent chromatographic analysis. For the ECD method, a semipreparative normal-phase HPLC stage was used to isolate a vitamin K-rich fraction; this comprised a Spherisorb cyanopropyl modified silica column (Phase Separations, Clywd, UK), a mobile phase of 3–10% (v/v) dichloromethane in hexane and a UV detector. For the spectrofluorometric method, a solid-phase extraction stage using Sep-Pak silica cartridge (Waters Associates, Watford, UK) and 3% (v/v) diethylether in hexane as eluting solvent was used as a prepurification procedure. Phylloquinone and MK-6 were resolved by RP-HPLC. The HPLC system for ECD comprised a 5 μm (250 × 4.6 mm) Exsil octyl column (Hichrom, Reading, UK), and a mobile phase of 3% (v/v) 0.05 M acetate buffer (containing 0.1 mM EDTA) in methanol. The electrochemical detector comprised a Coulochem cell (model 5011, ESA Inc., Aylesbury, UK) with two porous graphite coulometric electrodes (set at −1.2 V to act as postcolumn reducer) joined in series to an amperometric wall jet electrode (set at +0.40 V for detection), all electrodes being controlled by a DECADE detector (Antec, Leyden, The Netherlands). The fluorescence HPLC system comprised a 5 μm (150 × 3.2 mm) Hypersil H3BDS octadecyl column (Hichrom), and a mobile phase of 20% (v/v) acetonitrile in methanol, which contained 0.5% (v/v) 'zinc acid' solution (aqueous solution of 2 M zinc chloride, 1 M sodium actetate, 2 M acetic acid). The postcolumn reducer was a zinc-packed column (30 mm × 2.1 mm) that was connected to a fluorescence detector (model 474 from Waters Associates, Herts, UK) set at an excitation wavelength of 330 nm and an emission wavelength of 430 nm). Chemicals and solvents were purchased from Sigma, Poole, UK, and BDH, Poole, UK.

Since plasma concentrations of phylloquinone are positively correlated with triacylglycerol (Sadowski *et al*, 1989; Saupe *et al*, 1993), we also measured plasma triacylglycerol concentrations using a COBAS analyser and enzymatic colorimetric kit method according to the manufacturer's

instructions (Unimate 5, Roche, Lewes, UK). Duplicate measurements were made with kit and in-house controls.

Apolipoprotein E genotyping

ApoE genotyping was based on a previously described method (Wenham *et al*, 1991). DNA extracted from whole blood (Nucleon II; Scotlab, Coatbridge, UK) was amplified using AmpliTaq (PE Biosystems, Warrington, UK), 1.5 mM $MgCl_2$, 10% DMSO, 0.2 mM dNTPs, 2.5 μM primers (5′TCCAAGGAGCTGCAGGCGGCGCA3′, 5′ACAGAATTCGC CCCGGCCTGGTACACTGCCA3′) and 30 cycles (94°C for 30 s; 65°C for 30 s; 72°C for 30 s). The 227 base-pair (bp) product encompassing the polymorphic nucleotides 3745 and 3883 was restricted overnight at 37°C with 20 U of Cfo (Promega, Southampton, UK). Genotype was determined using a 5% Metaphor agarose gel (Flowgen, Ashby de la Zouch, UK). Two bands (91 and 81 bp) were observed with E2/2. An additional band of 48 bp found with E2/3. E3/3 had two bands of 91 and 48 bp. E3/4 showed an additional band of 72 bp. Two bands (72 and 48 bp) were observed with E4/4 and all four bands were associated with E2/4 genotype. For analysis of possible genotype effect on osteocalcin carboxylation, postmenopausal subjects were grouped according to the presence of one or more copies of E2 allele (E2/2 and E2/3), one or more copies of E4 allele (E3/4 and E4/4) or two copies of E3 allele (E3/3). One postmenopausal Gambian subject of E2/4 genotype was excluded from analyses.

Statistical analyses

Statistical analyses were conducted using the Data Desk 5.0 software (Data Description Inc, Ithaca, NY, USA). The 95% level of significance ($P<0.05$) was taken as evidence against the null hypothesis. Continuous variables were transformed to a natural logarithm scale to allow expression of proportional differences between groups (Prentice *et al*, 1994). In all cases, the distributions of the logged variables approximated normality. Ethnic differences were examined by ANOVA and comparisons between pairs of ethnic groups made with Scheffé *post hoc* tests. Regression analyses described the relationships between dependent and independent predictor variables. ANCOVA was used to investigate the influence of phylloquinone and apoE genotype on osteocalcin carboxylation. Comparisons of genotype distributions were made by χ^2 analysis.

Results

Osteocalcin concentrations

Scheffé *post hoc* analyses revealed ethnic differences in plasma concentrations of tOC and ucOC (Table 1). Postmenopause, tOC in the Gambian subjects was significantly higher than in both British and Chinese women, but there was no significant difference in tOC between British and Chinese subjects. Gambian and British postmenopausal women did not differ significantly in the concentration of ucOC, but both had a higher concentration than that of Chinese women postmenopause. Premenopause, mean tOC and ucOC concentrations in the Gambians were significantly higher than that of Chinese subjects. The intermediate concentrations present in premenopausal British women were not significantly different from those of the Gambian or Chinese subjects. Within each ethnic group, tOC and ucOC concentrations were all significantly higher in postmenopausal than in premenopausal women.

Relative concentrations of undercarboxylated osteocalcin

When expressed as %ucOC, interethnic differences were observed in the relative proportions of plasma ucOC to tOC (Table 1), which indicated that Chinese women had a significantly lower %ucOC than British and Gambian women postmenopause. No interethnic differences were observed in premenopausal women. Intraethnic comparisons

Table 1 Concentrations of tOC and percentage ucOC

		British		*Gambian*		*Chinese*	
		Premenopause (n = 11)	*Postmenopause (n = 31)*	*Premenopause (n = 11)*	*Postmenopause (n = 50)*	*Premenopause (n = 11)*	*Postmenopause (n = 23)*
tOC (ng/ml)*	Geometric mean	2.18[a]	3.42	3.10[a,b]	5.36[c,d]	1.53[a]	3.04
	ln mean ± s.e.	0.78 ± 0.09	1.23 ± 0.07	1.13 ± 0.09	1.68 ± 0.05	0.43 ± 0.12	1.11 ± 0.08
ucOC (ng/ml)*	Geometric mean	0.19[a]	0.61[c]	0.26[a,b]	0.99[c]	0.10[a]	0.17
	ln mean ± s.e.	−1.66 ± 0.25	−0.50 ± 0.19	−1.34 ± 0.32	−0.01 ± 0.16	−2.21 ± 0.06	−1.75 ± 0.16
%ucOC	Geometric mean	8.7[a]	17.2[c]	8.4[a]	18.6[c]	6.5	5.7
	ln mean ± s.e.	2.16 ± 0.20	2.84 ± 0.14	2.13 ± 0.28	2.92 ± 0.13	1.87 ± 0.12	1.74 ± 0.13

Total osteocalcin (tOC), undercarboxylated osteocalcin (ucOC), percentage undercarboxylated osteocalcin (%ucOC).
*To convert ng/ml to nmol/l divide by 5.94. Variables were transformed to natural logarithms and comparisons made by ANOVA with Scheffé *post hoc* analysis.
[a]Significantly different from postmenopausal women in same ethnic group, $P<0.01$.
[b]Significantly different from premenopausal Chinese women, $P<0.05$.
[c]Significantly different from postmenopausal Chinese women, $P<0.01$.
[d]Significantly different from postmenopausal British women, $P<0.01$.

revealed that %ucOC was higher in postmenopausal British and Gambian subjects than in their premenopausal counterparts, but there was no difference in Chinese women.

The conventional term '%ucOC' relies upon the assumption that ucOC and tOC concentrations are directly proportional, such that a 1% increase in ucOC is matched by a 1% increase in tOC concentration throughout the concentration range of compared samples. The validity of this assumption was examined by regression analysis of logged variables (Table 2). A coefficient of 1 for the postmenopausal Chinese women confirmed a linear relationship between tOC and ucOC, but this was not evident in either the British and Gambian postmenopausal data. Coefficient values close to 2 demonstrated that ucOC and tOC were related in an approximately squared manner, which means that 2% increase in ucOC was matched by 1% increase in tOC (Prentice *et al*, 1994).

In order to fully adjust for tOC, ucOC was compared between ethnic groups with tOC included in the ANCOVA model (adjusted ucOC). This showed that relative proportions of ucOC decreased in the order Britain > Gambia > China, with postmenopausal British and Gambian women having levels that were 103% ($P \leq 0.0001$) and 66% ($P = 0.01$) higher respectively than their Chinese counterparts. The 38% higher adjusted ucOC in the British compared to Gambian postmenopausal subjects was not significant by ANCOVA. The higher adjusted ucOC concentration of British postmenopausal women is further supported by calculations of ucOC concentration at the mean tOC concentration of each ethnic group. At the mean tOC concentration of British women, the predicted ucOC concentration was 35 and 101% lower in Gambian and Chinese women respectively (Figure 1).

The ANCOVA model showed no significant ethnic difference in osteocalcin carboxylation in the premenopausal subjects. Similar adjusted ucOC concentrations were present between premenopausal and postmenopausal subjects within each ethnic group.

The above relationships were not altered when samples with ucOC concentration below the detection limit of the RIA (assigned value 0.1 ng/ml) were excluded from the analysis.

Plasma phylloquinone concentration

Plasma phylloquinone and triacylglycerol concentrations are shown in Table 3. Postmenopausal Chinese women had significantly higher phylloquinone concentrations compared to their British and Gambian counterparts. In the

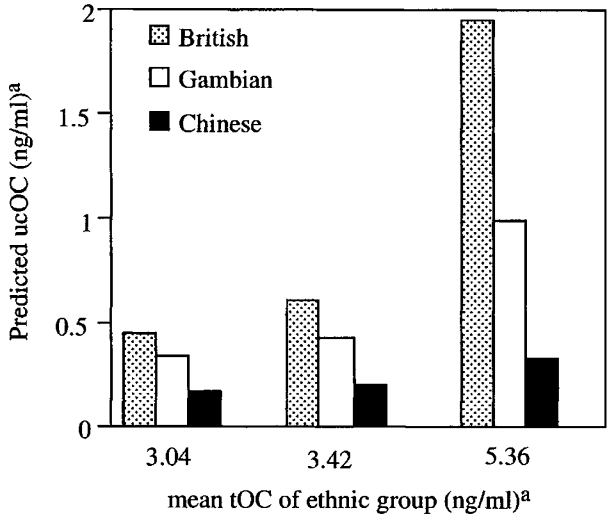

Figure 1 Predicated undercarboxylated osteocalcin concentrations (ucOC) at the mean total osteocalcin concentrations (tOC) observed in postmenopausal Chinese (3.04 ng/ml), British (3.42 ng/ml) and Gambian (5.36 ng/ml) women. Values were calculated using the following regression equations: British: ln ucOC = −3.70 + 2.60 × ln- × ln total osteocalcin; Gambian: ln ucOC = −3.18 + 1.89 × ln total osteocalcin; Chinese: ln ucOC = −3.02 + 1.14 × ln total osteocalcin. [a]To convert ng/ml to nmol/l divide by 5.94.

Table 2 Regression analyses describing the relationships between ucOC and tOC concentrations

	Constant (intercept)		*Coefficient (slope)*		
Subjects	*Constant*	*s.e.*	*Coefficient*	*s.e.*	t-*Value*
Postmenopausal British ($n = 31$)	−3.70[a]	0.29	2.60[c]	0.23	7.0[e]
Postmenopausal Gambian ($n = 50$)	−3.18[a]	0.55	1.89[c]	0.32	2.8[e]
Postmenopausal Chinese ($n = 23$)	−3.02[a]	0.42	1.14	0.35	0.4
Premenopausal British ($n = 11$)	−3.02[b]	0.56	1.75[d]	0.67	1.12
Premenopausal Gambian ($n = 11$)	−4.00[b]	1.11	2.35[d]	0.95	1.42

Continuous variables were transformed to natural logarithms total osteocalcin (tOC) undercarboxylated osteocalcin (ucOC) $y = \ln[\text{ucOC}]$, $x = \ln[\text{tOC}]$; *t*-value = (regression coefficient − 1)/s.e.
[a]$P \leq 0.0001$ (intercept $\neq 0$).
[b]$P \leq 0.006$ (intercept $\neq 0$).
[c]$P \leq 0.004$ (slope $\neq 0$).
[d]$P \leq 0.04$ (slope $\neq 0$).
[e]$P \leq 0.001$ (slope $\neq 1$).
Analysis of premenopausal Chinese subjects was inappropriate because ucOC was below the limit of detection in nine subjects. *t*-value slope $\neq 1$.

Table 3 Plasma phylloquinone and triacylglycerol concentrations

		British		*Gambian*		*Chinese*	
		Premenopause (n = 11)	*Postmenopause (n = 31)*	*Premenopause (n = 11)*	*Postmenopause (n = 50)*	*Premenopause (n = 11)*	*Postmenopause (n = 23)*
Phylloquinone (ng/ml)	Geometric mean	0.30[a]	0.31[b]	0.59	0.36[b]	0.62[c]	1.00
	ln mean ± s.e.	−1.22 ± 0.17	−1.17 ± 0.10	−0.52 ± 0.20	−1.02 ± 0.11	−0.47 ± 0.15	0.00 ± 0.13
Triacylglycerol (mmol/l)	Geometric mean	0.64	1.30	0.54	1.22	0.61	1.55
	ln mean ± s.e.	−0.45 ± 0.07	0.26 ± 0.07	−0.61 ± 0.10	0.20 ± 0.05	−0.49 ± 0.10	0.44 ± 0.09

Variables were transformed to natural logarithms and comparisons made by ANOVA with Scheffé *post hoc* analysis.
[a]Significantly different from premenopausal Chinese, $P = 0.03$.
[b]Significantly different from postmenopausal Chinese, $P \leq 0.0001$.
[c]Significantly different from postmenopausal Chinese, $P = 0.03$.

Table 4 Frequency of apoE genotypes in all subjects

	ApoE genotype											
	E2/2		*E2/3*		*E3/3*		*E3/4*		*E4/4*		*E2/4*	
	n	%	n	%	n	%	n	%	n	%	n	%
British ($n = 152$)	2	1.3	21	13.8	92	60.5	31	20.4	5	3.3	1	0.7
Gambian ($n = 138$)	1	0.7	19	13.8	43	31.2	53	38.4	15	9.8	7	5.1
Chinese ($n = 116$)	0	0	20	17.2	82	70.7	13	11.2	0	0	1	0.9

All distributions were in Hardy–Weinberg equilibrium. Comparisons made by χ^2 analysis. British, Gambian, Chinese: $P < 0.001$; British, Gambian: $P < 0.001$; Gambian, Chinese: $P < 0.001$; British, Chinese: $P > 0.05$.

premenopausal groups, the similar phylloquinone concentrations seen in Gambian and Chinese women were significantly higher than in the British women. Comparisons between pre- and postmenopausal women within each ethnic group showed significantly higher plasma phylloquinone postmenopause in Chinese subjects, a nonsignificant reverse trend in the Gambian women (post−premenopause difference = −50%, $P = 0.07$), but no difference in British women. There was no correlation between plasma phylloquinone and triacylglycerol concentrations in any of the three ethnic groups. The ethnic differences in plasma phylloquinone concentrations persisted when triacylglycerol concentration was included in the ANCOVA model.

ApoE genotype

Significant differences in apoE genotype distribution were found (Table 4). Within each ethnic group, there was no significant difference in genotype distribution between the postmenopausal and other subjects. Data for all subjects within each ethnic group were therefore combined. Frequencies of the E2 allele were similar (British = 8.6%, Gambian = 10.1%, Chinese = 9.1%). The E4 allele was more common, and the E3 allele was less common in the Gambian subjects (E4 Gambian = 32.6%, E3 Gambian = 57.2%, E4 British = 13.8%, E3 British = 77.6%, E4 Chinese = 6.0%, E3 Chinese = 84.9%). There was a nonsignificant trend for a higher proportion of the E4 allele and consequently a lower proportion of the E3 allele in British than in Chinese subjects.

Plasma phylloquinone, apoE genotype and osteocalcin γ-carboxylation postmenopause

In the British postmenopausal women, there was an inverse relationship between plasma adjusted ucOC and plasma phylloquinone concentrations (Figure 2). This persisted when plasma triacylglycerol concentration was included in the regression model. There was no association between plasma phylloquinone concentration and adjusted ucOC concentration in the Gambian or Chinese women.

The relationship between osteocalcin γ-carboxylation and apoE genotype in postmenopausal subjects is shown in Table 5. In the British subjects, the mean adjusted ucOC was significantly lower in women with apoE2/3 genotype than in those of apoE3/3 genotype, and in those of apoE3/4 genotype. A similar finding was observed in the subset of postmenopausal Chinese women ($n = 23$) selected according to their apoE genotype. There was no significant effect of apoE genotype on osteocalcin γ-carboxylation in postmenopausal Gambian women. Exclusion of subjects with estimated ucOC concentrations did not alter these results.

Although osteocalcin γ-carboxylation was influenced by apoE genotype and plasma phylloquinone concentration in

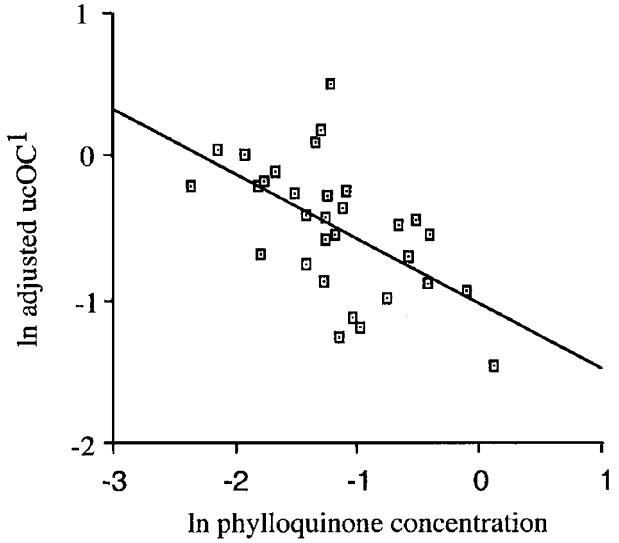

Figure 2 Plasma phylloquinone and osteocalcin γ-carboxylation in postmenopausal British women. [1]ucOC adjusted for tOC and normalised against mean tOC in postmenopausal British women (ln ucOC−2.60 × ln tOC + 2.60 × 1.23). $\gamma = -1.03 - 0.45x$; $r^2 = 32.4\%$; $P = 0.0008$.

Table 5 ApoE genotype and adjusted ucOC in postmenopausal subjects

		Adjusted ucOC (ng/ml)*[†]		
		British	*Gambian*	*Chinese*
E2/3	Geometric mean	0.41[a]	0.96	0.12[b]
	ln mean ± s.e.	−0.88 ± 0.13	−0.05 ± 0.27	−2.10 ± 0.14
	n	8	8	10
E3/3	Geometric mean	0.77	0.75	0.25
	ln mean ± s.e.	−0.27 + 0.09	−0.29 + 0.26	−1.39 + 0.18
	n	16	17	8
E3/4 & E4/4	Geometric mean	0.56	1.22	0.19
	ln mean ± s.e.	−0.58 ± 0.17	0.22 ± 0.13	−1.67 ± 0.35
	n	7	24	5

*Adjusted ucOC = ucOC adjusted for tOC, normalised against mean tOC for postmenopausal British/Gambian/Chinese.
[†]To convert ng/ml to nmol/l divide by 5.94. E2/2 genotype was not present in these subjects.
Comparisons within each ethnic group made by ANOVA with Scheffé *post hoc* analysis.
[a]Significantly different from E3/3, $P = 0.003$.
[b]Significantly different from E3/3, $P = 0.04$.

one or more groups of postmenopausal women, adjustment for these factors did not remove ethnic differences in osteocalcin γ-carboxylation. After inclusion of each factor in ANCOVA models, relative ucOC in postmenopausal British women remained approximately double that in Chinese women ($P = 0.0004$), and was 40% higher than in Gambian women, although this was not significant ($P = 0.09$). Relative ucOC concentrations in Gambian women remained approximately two-thirds higher than in Chinese women ($P = 0.04$).

The slopes of the relationships between ucOC and tOC concentration measured in each ethnic group (Table 2) were unaltered when apoE genotype and plasma phylloquinone were included in the ANCOVA model as independent factors. No significant relationship was observed between apoE genotype and plasma phylloquinone in any ethnic group.

Discussion

This study reports considerable ethnic differences in circulating concentrations of osteocalcin and its γ-carboxylation in a preliminary investigation of pre- and postmenopausal British, Gambian and Chinese women. Absolute plasma concentrations of tOC and ucOC were highest in Gambian and lowest in Chinese subjects. After adjustment for plasma tOC concentration, ucOC was highest in British and lowest in Chinese subjects.

Our data also suggest that '%ucOC', the conventional term of expressing osteocalcin γ-carboxylation, may often be inappropriate because it assumes that the relationship between ucOC and tOC across different subjects is directly proportional. This was observed in Chinese women, but an approximate squared relationship between ucOC and tOC was found in British and Gambian women. When the relationship between ucOC and tOC is not linear, %ucOC may under- or overcorrect ucOC for tOC. Therefore, the appropriate comparisons of ucOC between individuals with differing tOC could be made using an ANCOVA model in which tOC was included as an independent factor. This permitted adjustment for differing tOC concentrations without making assumptions about the relationship with ucOC (adjusted ucOC).

The method of expressing osteocalcin γ-carboxylation data affected some conclusions. For pre- and postmenopausal comparisons in British and Gambian women, expression of the results as %ucOC suggested that relative ucOC was higher in postmenopausal women, whereas the ANCOVA model found no difference in adjusted ucOC pre- and postmenopause. This shows that the higher ucOC concentration in older subjects may be fully accounted for by the higher tOC. Our finding that tOC was higher in postmenopausal women in all three countries is in agreement with most other studies (Plantalech & Guillamont, 1991; Knapen *et al*, 1993; Sokoll & Sadowski, 1996). We noted that plasma tOC concentration was significantly higher in the Gambian than in the British and Chinese postmenopausal women. This could be explained by their high plasma parathyroid hormone (PTH) compared with British and Chinese counterparts (Prentice *et al*, 2001). The effect of a high circulating PTH on bone metabolism and risk of fracture in the Gambian population is currently under investigation.

Previous comparisons of %ucOC between pre- and postmenopausal women have been equivocal; either suggesting increased relative ucOC after the menopause (Knapen *et al*, 1989; Plantalech and Guillamont, 1991) or no change (Knapen *et al*, 1993; Sokoll & Sadowski, 1996). These discrepancies may be due to incomplete adjustment for differences in tOC. The method of comparing osteocalcin γ-carboxylation data also affected interethnic comparisons. When relative ucOC was expressed as %ucOC, there was little difference between British and Gambian postmenopausal women, whereas after appropriate adjustment for tOC by ANCOVA, relative ucOC was about 40% higher in the British cohort. Differences in osteocalcin γ-carboxylation between the ethnic groups are also suggested by the proportion of ucOC measurements below the sensitivity limit (0.2 ng/ml) of the RIA, being approximately three-fold greater in Chinese compared to British postmenopausal women.

Physiological factors may contribute to the nonlinear relationship between ucOC and tOC. An increased production of osteocalcin, as occurs postmenopause, may place greater demands on the osteoblast carboxylase system, and therefore may result in greater relative synthesis of ucOC. This is consistent with our findings that the proportion of ucOC increases as tOC increases in the British and Gambian women. In addition there are methodological problems associated with osteocalcin carboxylation binding assays. As recently reviewed by Gundberg *et al* (1998), ucOC measurements are dependent upon several factors, including the tOC concentration. In future, immunoassays specific for ucOC may provide an improvement to binding studies.

Our results also suggest that there are ethnic differences in circulating phylloquinone concentrations, which may reflect ethnic differences in vitamin K status of bone. The plasma phylloquinone concentrations in this British cohort were comparable to previous studies (Hart *et al*, 1985; Bitensky *et al*, 1988; Roberts *et al*, 1996). Studies in the USA have reported higher absolute phylloquinone levels in elderly compared to younger subjects (Sadowski *et al*, 1989; Sokoll & Sadowski, 1996). This is in contrast to the similar phylloquinone concentrations pre- and postmenopause in the cohort of British women in this study. However, after consideration of plasma triacylglycerol concentration, the trend for lower phylloquinone concentration postmenopause is consistent with studies in the USA (Sadowski *et al*, 1989; Sokoll & Sadowski, 1996). To our knowledge, this is the first report of plasma phylloquinone concentrations in Chinese and African subjects. The high phylloquinone concentrations in the cohort of Chinese women from Shenyang where osteoporotic fractures are rare (Yan *et al*, 1999), is compatible with the association of low circulating phylloquinone with a high incidence of osteoporotic fracture in Britain and North America (Hart *et al*, 1985; Bitensky *et al*, 1988; Roberts *et al*, 1996; Booth *et al*, 2000). Plasma phylloquinone concentration is readily affected by dietary intake and rapidly raises blood concentrations, which then fall within a few hours (Suttie *et al*, 1988; Ferland *et al*, 1993; Sokoll *et al*, 1997; Booth *et al*, 1999). Plasma phylloquinone also correlates with vitamin K intake in population studies (Booth *et al*, 1995, 1997). Consequently, it is likely that the high plasma phylloquinone concentration in the fasted Chinese subjects reflects usual dietary intake. Dark green leafy vegetables and some vegetable oils are rich sources (Booth *et al*, 1995, 1996), and these foods are common in the diet of north-east China (Yan *et al*, 2002). Plasma phylloquinone concentration was higher in the Gambian premenopausal subjects than in their postmenopausal counterparts. We were unable to identify a possible dietary explanation for this other than the fact that leaves and oil are relatively recent introductions to the rural diet in this part of The Gambia and the older women may not have access to or preferred not to eat these phylloquinone-rich foods. Further interpretation of plasma phylloquinone in this cohort of Chinese and Gambian women is limited by the absence of data of phylloquinone content of foods consumed in these countries, and demonstrates a need for investigation of the phylloquinone content of foods consumed outside of UK and USA.

The direct relationship between phylloquinone concentration and osteocalcin γ-carboxylation in British women in this study is consistent with other reports (Sokoll & Sadowski, 1996; Sokoll *et al*, 1997). The lack of this association in Gambian or Chinese women may reflect higher circulating phylloquinone concentrations and/or a greater degree of osteocalcin γ-carboxylation in these subjects, and may indicate suboptimal vitamin K status in British postmenopausal women. In the postmenopausal British and the subset of Chinese women selected according to apoE genotypes, apoE2 allele was associated with the lowest adjusted ucOC for tOC. Previous studies have shown that circulating phylloquinone concentrations in hemodialysis patients followed the order of apoE2 > E3 > E4 (Saupe *et al*, 1993). It was postulated that this reflected apoE isoform-dependent clearance of phylloquinone-rich remnant lipoproteins by the liver in the order apoE4 > E3 > E2, which has been well established for retinyl ester-labelled remnants and may result in more phylloquinone rich-lipoprotein being available for uptake by bone in subjects with apoE2 (Saupe *et al*, 1993; Kohlmeier *et al*, 1998). However, in our study, apoE4 allele was not associated with an increase in adjusted ucOC. Direct evidence that apoE plays an important role in the uptake of lipoprotein-borne phylloquinone into osteoblasts was recently obtained by Newman *et al* (2002). Their findings suggest that the osteoblast uptake of vitamin K is mediated by apo E in TRL-rich lipoproteins and heparan sulphate proteoglycans on the osteoblast surface. Interestingly, apoE4 seems to stimulate cellular binding (Cullen *et al*, 1998) and uptake (Newman *et al*, 2002) to a greater degree than other isoforms. Since *in vivo*, newly absorbed vitamin K is predominately taken up by the liver, it would still be expected that this apoE4 effect would manifest in an enhanced hepatic

clearance of TRL-vitamin K leaving lower vitamin K concentrations available for uptake by bone cells (Newman *et al*, 2002).

In our study, there were no associations between apoE genotype and plasma phylloquinone, in contrast to that previously described in hemodialysis patients (Saupe *et al*, 1993; Kohlmeier *et al*, 1998). This difference may be due to several factors including the greater range and lower intrasubject variance of plasma phylloquinone in these patients, their more controlled diet and presence of hyperlipidaemia in some patients (Booth *et al*, 1997; Kohlmeier *et al*, 1998). Our study does not demonstrate that the influence of apoE on osteocalcin γ-carboxylation was mediated via its influence on plasma phylloquinone. This may be due to small subject numbers and reduced statistical power after subdivision into apoE genotype groups, and also to known intraindividual variability in plasma phylloquinone (Booth *et al*, 1997). Another possibility is that apoE may affect vitamin K-dependent γ-carboxylation of osteocalcin in ways that are independent of its postulated influence on lipoprotein-mediated transport of vitamin K to bone. The combined influence of plasma phylloquinone and apoE genotype on osteocalcin γ-carboxylation did not reduce the variation between ethnic groups, with adjusted ucOC remaining highest in British and lowest in Chinese postmenopausal women. This may indicate that other factors yet to be identified are more important determinants of osteocalcin γ-carboxylation. However, this interpretation is confused by a clustering effect in which a high concentration of phylloquinone, a predictor variable, was not independent of ethnicity.

In summary, this preliminary investigation demonstrated ethnic differences in plasma ucOC and vitamin K, suggesting suboptimal vitamin K status in postmenopausal British women. This is consistent with the rarity of osteoporotic fractures in The Gambia and China and the reported association between high ucOC and osteoporotic fracture risk in Caucasian women. This study highlights the need for larger epidemiological investigations of ethnic differences in vitamin K status and whether variations in vitamin K status are causally associated with ethnic differences in osteoporotic fracture risk.

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