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The association between dietary vitamin K intake and serum undercarboxylated osteocalcin is modulated by vitamin K epoxide reductase genotype

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Vitamin K acts as a cofactor during the γ-carboxylation of vitamin K-dependent proteins. Undercarboxylated osteocalcin (ucOC) is a suggested biomarker of vitamin K status. The +2255 polymorphism of the vitamin K epoxide reductase gene (*VKORC1*) was shown to be associated with the recycling rate of the active form of vitamin K. We investigated the association between dietary vitamin K intake and serum ucOC and hypothesized that this association might vary by *VKORC1* genotype. ucOC and total intact osteocalcin (iOC) concentrations were quantified using specific ELISA tests in serum samples of 548 male and female participants (aged 18–81 years) of the Bavarian Food Consumption Survey II. ucOC was expressed relative to iOC (ucOC/iOC ratio). Dietary intake of vitamin K (phylloquinone and menaquinones) was estimated from three 24 h dietary recalls using previously published food composition data. The association between dietary vitamin K intake and ucOC/iOC ratio was analysed using linear and non-linear regression models. Median intakes of phylloquinone/menaquinones were 83·4/37·6 μg/d in men and 79·6/29·8 μg/d in women, respectively. As expected, vitamin K intake was significantly inversely associated with the ucOC/iOC ratio. The ucOC/iOC ratio differed significantly across variants of the +2255 polymorphism in the *VKORC1* gene. Stratification by *VKORC1* + 2255 genotype revealed that only in carriers of the GG genotype (39 % of all participants) did the ucOC/iOC ratio significantly decrease with increasing intake of vitamin K. Thus, the results show that the inverse association between dietary vitamin K intake and serum ucOC depends on a functionally relevant allelic variant of the *VKORC1* gene.

Vitamin K: Phylloquinone: Menaquinones: Undercarboxylated osteocalcin: *VKORC1*

Vitamin K acts as a cofactor during the post-translational γ-carboxylation of proteins. The carboxylation of vitamin K-dependent proteins such as blood coagulation factors, osteocalcin and matrix gla-protein confers them with calcium-binding properties that are essential for their biological activity[1]. Although states of severe vitamin K deficiency resulting in bleeding disorders are rare, a poor vitamin K status is considered to be a risk factor for impaired bone health and artery calcification[2].

During the carboxylation process, the active form of vitamin K is converted to vitamin K epoxide. In the so-called vitamin K cycle, the enzyme vitamin K epoxide reductase catalyses the recycling of vitamin K epoxide back to active vitamin K[3]. Both dietary supply with vitamin K and the recycling of vitamin K are expected to determine the carboxylation rate. There are different forms of vitamin K sharing the naphthoquinone-ring but differing in the isoprenoid side-chain. The two K vitamins naturally occurring in the human diet are phylloquinone (vitamin K_1) and the group of menaquinones (vitamin K_2), whereas menadione (vitamin K_3) is a synthetic form of vitamin K. Phylloquinone is abundant in green leafy vegetables[4]. Menaquinones are heterogeneous with respect to the number of C-atoms in the isoprenoid side-chain (denoted by MK-n) and mainly occur in fermented dairy products such as cheese and in meat[5]. Menaquinones with a chain length greater than nine occur in small amounts in certain offal and contribute little to total intake of menaquinones[6]. Despite 10–30 % contribution of menaquinones to total vitamin K intake[1,6], the majority of epidemiological studies on vitamin K took only phylloquinone intake into account[7–11].

Serum undercarboxylated osteocalcin (ucOC) has been proposed as a sensitive biomarker of vitamin K status[12,13] that is inversely associated with vitamin K supply[12]. The responsiveness of the carboxylation status of osteocalcin to vitamin K supplementation has been demonstrated in numerous intervention trials[14–19]. Fewer studies have investigated the association between habitual dietary vitamin K intake and

Abbreviations: iOC, total intact osteocalcin; MK-n, menaquinone-n; ucOC, undercarboxylated osteocalcin.

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serum ucOC concentration, and all of them observed significant inverse associations[8,10,20,21]. Elevated serum ucOC concentrations have been associated with increased hip fracture risk[22–24] and reduced bone mineral density[25,26]. Because the absolute ucOC concentration depends on the endogenous synthesis of osteocalcin, ucOC expressed relative to total osteocalcin is a more reliable measure to describe the vitamin K status[27]. Following the classical determination of ucOC and total osteocalcin concentrations by indirect binding assays (e.g. hydroxy-apatite assay), this relative measure is denoted as %ucOC[28]. Recently, specific assays for ucOC and total intact osteocalcin (iOC) were developed[29]. Using these specific assays the relative measure of ucOC is usually expressed as the ucOC/iOC ratio[20,30].

The blood coagulation inhibitor warfarin binds to the enzyme vitamin K epoxide reductase leading to the inhibition of the recycling of vitamin K[31]. Several single nucleotide polymorphisms of the vitamin K epoxide reductase complex subunit 1 (*VKORC1*) gene were shown to be associated with the warfarin dose required for inhibition of blood coagulation[32]. Most of these polymorphisms are in strong linkage disequilibrium with the only functional single nucleotide polymorphism (rs 9923231), which is associated with reduced activity of vitamin K epoxide reductase due to reduced mRNA expression of the *VKORC1* gene[33].

The aim of the present study was to investigate the association between dietary intake of vitamin K (phylloquinone and menaquinones) and serum ucOC/iOC ratio and to find out if this association is influenced by the +2255 polymorphism of the *VKORC1* gene (rs 2359612), which represents a haplotype determining warfarin dose[34].

Subjects and methods

Study design and population

The Bavarian Food Consumption Survey II is designed as a cross-sectional study representative for the Bavarian population to investigate dietary and lifestyle habits. Between September 2002 and June 2003, 1050 German-speaking subjects aged 13–80 years were recruited following a three-stage random route sampling procedure. During a computer-aided personal interview, data concerning subjects' characteristics, lifestyle, socioeconomic and health status were collected. Within the following 2 weeks, dietary intake was assessed by three 24 h dietary recalls by telephone (two weekdays, one weekend day) conducted by trained interviewers using the software EPIC-Soft. Blood samples were drawn from 568 participants out of 879 invited subjects (inclusion criteria for invitation was age ≥ 18 years and at least one dietary recall completed).

The overall participation rate in the study was 71 %. All study participants gave their written informed consent. The study was approved by the local ethics committee.

Calculation of vitamin K intake

Data from the 24 h recalls were weighted for weekday and weekend day to calculate the average daily food intake. Dietary intakes of phylloquinone and menaquinones were calculated using previously published food content data analysed by the HPLC method. For the calculation of phylloquinone intake, we used published and unpublished data by Bolton-Smith *et al.*[35] including food content values for about 2000 food items. Menaquinone contents of relevant foods were derived from a Dutch publication[5]. For completeness, we supplemented the menaquinone data using menaquinone contents of some offal from a Japanese publication[36]. Phylloquinone and menaquinone contents were assigned to all foods consumed by the study participants according to the 24 h dietary recalls by either direct matching, adaptation of fat content (as described by Bolton-Smith *et al.*[35]) or food similarities.

Blood sampling

Venous blood was drawn into EDTA tubes or serum tubes, chilled at 4°C, and processed subsequently. Serum was separated from blood cells by centrifugation. Samples were cooled for a maximum of 1 d (transportation, aliquoting) until they were stored at −80°C.

Measurement of undercarboxylated and total intact osteocalcin in serum

Two commercially available ELISA tests based on monoclonal antibodies were used for the quantitative analysis of ucOC (Glu-OC EIA Kit, Takara Biomedical Group, Otsu, Shia, Japan) and iOC (Metra Osteocalcin EIA Kit, Quidel Corporation, CA, USA) in serum. iOC corresponds to total osteocalcin (independent of carboxylation status) with the strength of the test to detect only intact osteocalcin molecules and not N- or C-terminal fragments. Intra-assay CV of the ucOC and iOC ELISA were 8·1 and 6·0 %, respectively.

Genotyping

Genomic DNA was extracted from 'buffy coat' using the FlexiGene DNA kit (Qiagen GmbH, Hilden, Germany). The *VKORC1* + 2255 polymorphism was analysed by PCR using the following primers: 5′-CCAAGGGACTGGTCTCTGAA-3′ and 5′-AGGAACCAAGGGAGTGGAAG-3′. The PCR products were subsequently digested with NcoI (site-specific endonuclease from *Nocardia corallina*; New England Biolabs). The resultant DNA fragments were resolved on a 4 % agarose gel, yielding one band (273 bp) for the G allele and two bands for the A allele (109 and 164 bp). Samples (3 %) were repeated for the purpose of quality control of genotyping and concordance was 100 %. In addition, ambiguous samples were repeated. A χ^2 test was used to test for deviation from Hardy–Weinberg equilibrium.

Statistical analysis

Subjects with missing information on diet (*n* 7), iOC (*n* 4), ucOC (*n* 1) or *VKORC1* + 2255 polymorphism (*n* 4) or subjects reporting medication with the vitamin K antagonist Marcumar (*n* 6) were excluded from the present analysis, leaving a total of 548 subjects.

Dietary intake of vitamin K is reported as total vitamin K, phylloquinone, total menaquinones (MK-4 to MK-14) as well as the subgroups of menaquinones MK-4 and MK-5 to MK-9. Menaquinones with a chain length greater than nine

are not presented separately because of the low contribution to total intake of menaquinones and the high proportion of non-consumers of offal (unique food source for very long-chain menaquinones > MK-9).

We expressed ucOC as the ucOC/iOC ratio. Because of the skewed distributions of dietary intakes of vitamin K and its subgroups, on the one hand, and the ucOC/iOC ratio, on the other hand, these variables were log-transformed for the analysis. Mean values of the log-transformed ucOC/iOC ratio were compared across *VKORC1* + 2255 genotypes by ANOVA. Between-group comparisons were performed with the Scheffé test. Geometric means and corresponding CI are presented. For the regression analyses, subjects with missing information on regular sports activities (yes/no) (*n* 45) were assumed to be inactive. Women with missing information on their menopausal status (*n* 33) were assigned pre- or peri/postmenopausal status according to age. The median age at menopause (48 years) reported by the peri/postmenopausal women was used as cut-off, i.e. women below age 48 and with missing information on menopausal status were categorised as premenopausal, women ≥48 years old as peri/postmenopausal.

The association between dietary intake of vitamin K and the ucOC/iOC ratio was assessed by means of linear regression analysis with the ucOC/iOC ratio as the dependent and dietary intake of vitamin K or vitamin K subgroups as the independent variable (both ucOC/iOC ratio and vitamin K intake variables were log-transformed). Models were univariate or multivariate, mutually adjusting vitamin K intake variables and adjusting for potential confounders, including previously identified determinants of ucOC/iOC ratio[27]. Multivariate adjustment variables were sex/menopausal status (males, females premenopausal, females peri/postmenopausal), age (years), total energy intake (kJ/d), smoking status (never, former, current), sports activity (yes/no) and season when blood was collected (spring, summer, autumn, winter). In addition, the linear regression analysis was performed stratified by *VKORC1* + 2255 genotype. A test for multiplicative interaction was performed by entering a product term of the *VKORC1* + 2255 genotype (coded as 0, 1, 2 according to the number of A alleles) and the log-transformed vitamin K intake variable (continuous) along with the main variables of vitamin K intake and *VKORC1* + 2255 genotype into the linear regression model. *P* for interaction was determined by means of a likelihood ratio test comparing the two models with and without the interaction term.

To explore further the shape of the function predicting the ucOC/iOC ratio by dietary intake of total vitamin K or phylloquinone, respectively, we fitted the best non-linear models using fractional polynomials[37]. This was done for all participants as well as for the *VKORC1* + 2255 genotypes separately. The resulting functions are presented graphically.

Statistical analyses were carried out using SAS software package, version 9.1 (SAS Institute, Cary, NC, USA). Non-linear regression analyses using fractional polynomials were conducted using Stata software package, version 7 (Stata Corporation, College Station, TX, USA).

Results

Characteristics of the study population are presented in Table 1. Men were on average older than women, had a higher BMI and were more often current smokers. The frequencies of the GG, AG and AA genotypes of the single nucleotide polymorphism +2255 in the VKORC1 gene were 42·2, 41·7 and 16·1 % in men and 36·8, 45·3 and 17·9 % in women, respectively. Genotype frequencies fulfilled expectations of the Hardy–Weinberg equilibrium ($P=0{\cdot}06$).

Dietary intake of vitamin K and vitamin K subgroups as well as serum osteocalcin variables in men and women are shown in Table 2. Due to the skewed distribution of vitamin K intake variables, median values were substantially lower than arithmetic means. Median intakes of total vitamin K were 128·4 μg/d in men and 112·3 μg/d in women. In both

Table 1. Characteristics of the study population, by sex (Bavarian Food Consumption Survey II)
(Mean values and standard deviations)

	Men (*n* 230)				Women (*n* 318)			
	Mean	SD	*n*	%	Mean	SD	*n*	%
Age	50·6	15·6			46·4	14·5		
Height	174·8	7·0			163·4	6·3		
Weight	83·4	14·0			69·6	13·0		
BMI (kg/m^2)	27·3	4·3			26·1	5·1		
Smoking status								
Never			94	40·9			192	60·6
Past			68	29·6			57	18·0
Current			68	29·6			68	21·5
Sports activity								
Yes			116	50·4			153	48·1
No			96	41·7			138	43·4
Menopausal status in women*								
Premenopausal							166	52·2
Peri/postmenopausal							119	37·4
VKORC1 SNP +2255								
GG			97	42·2			117	36·8
AG			96	41·7			144	45·3
AA			37	16·1			57	17·9

VKORC1 SNP, vitamin K epoxide reductase gene subunit 1 single nucleotide polymorphism.
* Percentages do not sum up to 100 % because of missing values.

Table 2. Description of vitamin K intake (μg/d) and serum osteocalcin variables in the Bavarian Food Consumption Survey II*

(Mean values and standard deviations)

			Percentile			Percentile	
	Mean	SD	5th	25th	Median	75th	95th
Men (*n* 230)							
Total vitamin K intake	158·1	117·8	49·4	88·5	128·4	181·2	403
Phylloquinone	117·9	114·3	24·8	51·2	83·4	143·2	351·3
Menaquinone (MK-4 to MK-14)	40·2	20·7	12·6	24·4	37·6	52·9	78·4
Menaquinone subtypes							
MK-4	18·1	7·8	7·9	12·6	16·7	22·8	32·0
MK-5 to MK-9	22·1	17·7	1·1	8·3	18·6	30·9	56·7
Serum ucOC (ng/ml)	3·03	2·98	0·74	1·46	2·16	3·66	6·82
Serum iOC (ng/ml)	9·17	2·71	5·37	7·42	8·67	10·73	13·79
ucOC/iOC ratio	0·33	0·29	0·09	0·17	0·26	0·42	0·8
Women (*n* 318)							
Total vitamin K inake	146·1	120·0	50·5	80·9	112·3	164·2	387·4
Phylloquinone	114·3	118·8	25·6	50·0	79·6	126·4	354·7
Menaquinone (MK-4 to MK-14)	31·8	15·4	11·2	20·0	29·8	38·7	64·5
Menaquinone subtypes							
MK-4	13·1	5·6	5·0	9·2	12·9	16·1	22·8
MK-5 to MK-9	18·7	13·8	1·2	8·4	16·0	25·8	46·6
Serum ucOC (ng/ml)	2·87	1·95	0·81	1·47	2·52	3·69	6·61
Serum iOC (ng/ml)	8·85	4·73	4·19	6·24	7·87	10·36	15·68
ucOC/iOC ratio	0·36	0·25	0·1	0·18	0·28	0·46	0·89

iOC, total intact osteocalcin; ucOC, undercarboxylated osteocalcin.
* For details of subjects and procedures, see Subjects and methods.

men and women, more than 20 % of total vitamin K intake was provided as menaquinones. Median intakes of phylloquinone and menaquinones were 83·4 and 37·6 μg/d in men and 79·6 and 29·8 μg/d in women, respectively. The proportion of subjects who did not meet the estimated adequate intake of vitamin K as suggested by the German Nutrition Society was 48 % in men (< 80 μg/d) and 38 % in women (< 65 μg/d) considering only phylloquinone as a source of vitamin K. However, repeating this calculation on the basis of phylloquinone and menaquinones, the corresponding proportions of subjects below the recommended intake were 23 % in men and 13 % in women. Phylloquinone was predominantly provided by vegetables (48 % of total phylloquinone intake), especially leafy vegetables such as spinach as well as lettuce and cabbages. The major food source of menaquinones was cheese (all varieties, including fresh cheese), providing 42 % of total intake of menaquinones (mainly MK-5 to MK-9). Meat and meat products (mainly MK-4) contributed another 24 % of total intake of menaquinones. Men had a lower median concentration of ucOC than women (2·16 *v.* 2·52 ng/ml), while the median iOC concentration was higher in men than in women. When ucOC was expressed relative to iOC, mean and median ucOC/iOC ratio was lower in men than in women.

Dietary intake of total vitamin K was significantly inversely associated with the serum ucOC/iOC ratio in all participants (multivariate adjusted $\beta = -0{\cdot}14$, $P=0{\cdot}001$; Table 3). When phylloquinone and menaquinone were entered separately into the model, their influence on ucOC/iOC ratio was of similar magnitude (multivariate adjusted $\beta = -0{\cdot}10$ for phylloquinone and $\beta = -0{\cdot}08$ for menaquinones). Separate evaluation of MK-4 and MK-5 to MK-9 revealed a significant effect on ucOC/iOC ratio only for the long-chain menaquinones MK-5 to MK-9. Multivariate adjusted β estimates differed only slightly from univariate estimates.

Geometric means of the ucOC/iOC ratio differed significantly by genotype of the +2255 polymorphism in the *VKORC1* gene (Fig. 1). The ucOC/iOC ratio decreased with increasing number of A alleles (P for linear trend=0·008). Geometric means in the GG, AG and AA genotypes were 0·30 (95 % CI 0·27, 0·33), 0·28 (95 % CI 0·25, 0·30) and 0·24 (95 % CI 0·21, 0·27), respectively. The ucOC/iOC ratio was significantly higher in carriers of the GG genotype as compared to homozygous carriers of the A allele. Potential confounders of the association between vitamin K intake and ucOC such as sex, age, BMI, sports activity and season of blood collection did not differ significantly across VKORC1 + 2255 genotypes (data not shown). An exception was smoking status, showing the highest proportion of current smokers in the AA genotype group ($P=0{\cdot}02$, χ^2 test).

Linear regression analysis stratified by *VKORC1* genotypes revealed the strongest association between dietary vitamin K intake and ucOC/iOC ratio in homozygous carriers of the G allele (multivariate $\beta = -0{\cdot}23$, $P=0{\cdot}0002$), while the effect levelled off with increasing number of A alleles (multivariate $P_{\text{interaction}} = 0{\cdot}07$) (Table 3). The strong inverse association between dietary vitamin K intake and ucOC/iOC ratio in GG carriers was mainly driven by phylloquinone. Dietary intake of menaquinones was not associated with ucOC/iOC ratio in homozygous carriers of the G allele or A allele, while a significant inverse association was observed in the heterozygous genotype (multivariate $P_{\text{interaction}} = 0{\cdot}43$).

The association between total vitamin K intake and the ucOC/iOC ratio in all participants as well as stratified by genotype is illustrated in Fig. 2. The ucOC/iOC ratio decreases with increasing dietary vitamin K intake in all participants and in

Table 3. Linear regression (β, *P*) for the association between the serum undercarboxylated osteocalcin/total intact osteocalcin (ucOC/iOC) ratio and dietary vitamin K intake by *VKORC1*+2255 genotype (both ratio and vitamin K intake variables were log-transformed)

		VKORC1 SNP +2255									
		All (*n* 548)		GG (*n* 214)		AG (*n* 240)		AA (*n* 94)			
		ucOC/iOC ratio		ucOC/iOC ratio		ucOC/iOC ratio		ucOC/iOC ratio		$P_{interaction}$	
		Uni	Multi*	Uni	Multi*	Uni	Multi*	Uni	Multi*	Uni	Multi*
Total vitamin K	β	−0·16	−0·14	−0·23	−0·23	−0·13	−0·08	0·05	−0·06		
	P	0·001	0·001	0·001	0·0002	0·09	0·25	0·66	0·60	0·05	0·07
Phylloquinone	β	−0·12	−0·10	−0·19	−0·20	−0·08	−0·05	0·02	−0·04		
	P	0·001	0·004	0·0003	0·0003	0·15	0·40	0·82	0·64	0·03	0·05
Total menaquinones (MK-4 to MK-14)	β	−0·11	−0·08	−0·051	−0·03	−0·15	−0·08	0·03	−0·05		
	P	0·03	0·001	0·50	0·48	0·03	0·020	0·82	0·46	0·60	0·43
Menaquinone subtypes											
MK-4	β	−0·03	0·01	−0·02	0·09	−0·08	0·01	0·13	−0·22		
	P	0·56	0·85	0·85	0·38	0·33	0·94	0·38	0·28	0·67	0·81
MK-5 to MK-9	β	−0·07	−0·07	−0·04	−0·03	−0·08	−0·08	−0·05	−0·05		
	P	0·01	0·004	0·32	0·51	0·03	0·03	0·42	0·44	0·63	0·47

Multi, multivariate; Uni, univariate; *VKORC1* SNP, vitamin K epoxide reductase gene subunit 1 single nucleotide polymorphism.
*Adjusted for sex/menopausal status, age, total energy intake, smoking status, sports activity and season when blood was collected, vitamin K intake variables mutually adjusted.

carriers of the GG genotype. The curves are substantially flattened with vitamin K intakes above (approximately) 70 μg/d. In homozygous and heterozygous carriers of the A allele, the ratio of ucOC/iOC is not influenced by dietary intake of vitamin K. Carriers of the AA genotype have the lowest ratio of ucOC/iOC regardless of the dietary vitamin K intake. The picture is similar for dietary intake of phylloquinone (Fig. 3). Only carriers of the GG genotype show a reduction in ucOC/iOC ratio with increasing phylloquinone intakes. No modification of ucOC/iOC ratio with increasing phylloquinone intakes is seen in subjects with the AG and AA genotype. The fractional polynomial models were calculated unadjusted. However, when the fractional polynomial approach was repeated with fully adjusted models, the resulting best models had the same shape as in the unadjusted approach.

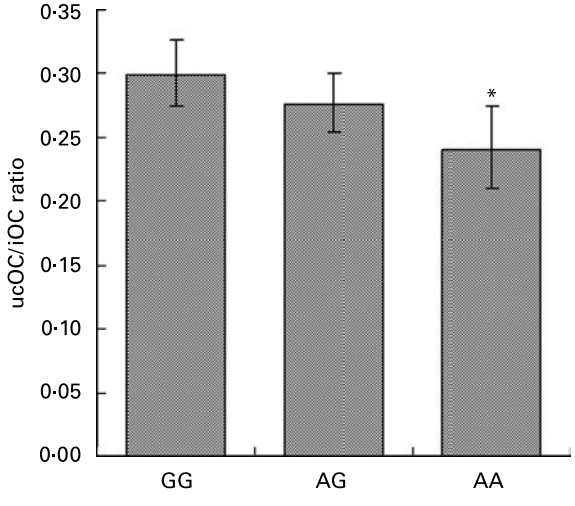

Fig. 1. Mean undercarboxylated osteocalcin/total intact osteocalcin (ucOC/iOC) ratio by genotypes of *VKORC1* + 2255. Values are geometric means with 95% CI depicted by vertical bars. *Mean value was significantly different from that of the GG group (ANOVA, Scheffé test; $P<0·05$). *P* for linear trend=0·008.

Discussion

In the present paper, we report dietary intakes of vitamin K as assessed by three 24 h dietary recalls in a representative sample of the Bavarian population. The ucOC/iOC ratio was significantly inversely associated with dietary intakes of phylloquinone and menaquinones. The ucOC/iOC ratio differed significantly by *VKORC1* genotype, showing highest values in subjects carrying the GG genotype (found in 39% of participants) and we observed the strongest dependency of the ucOC/iOC ratio on vitamin K intake in carriers of the GG

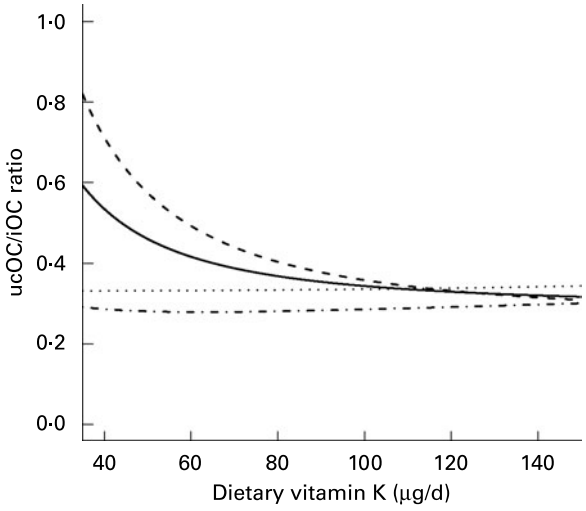

Fig. 2. Association between dietary vitamin K (phylloquinone and menaquinones) intake and the serum undercarboxylated osteocalcin/total intact osteocalcin (ucOC/iOC) ratio, in all participants (—) and by genotype of *VKORC1* + 2255 (- - -, GG; ·····, AG; ·–·–·, AA). The obtained functions are $y = 0·29 - 164·58x^{-2} + 150·59x^{-2}\ln(x)$ for all participants, $y = 0·26 - 310·17x^{-2} + 280·23x^{-2}\ln(x)$ for GG, $y = 0·33 + (1·50 \times 10^{-8}x^{3}) - 2·26 \times 10^{-9}x^{3}\ln(x)$ for AG, $y = -0·53 + 2·09x^{-0·5} + 0·13\ln(x)$ for AA genotype, where *y* denotes the ucOC/iOC ratio and *x* denotes dietary vitamin K (μg/d).

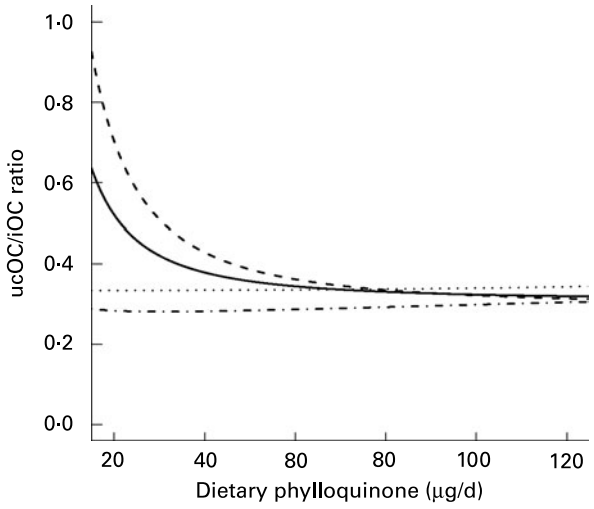

Fig. 3. Association between dietary phylloquinone intake and the serum undercarboxylated osteocalcin/total intact osteocalcin (ucOC/iOC) ratio, in all participants (—) and by genotype of *VKORC1* + 2255 (- - -, GG; ·····, AG; -·-·-, AA). The obtained functions are $y = 0{\cdot}31 - 29{\cdot}98x^{-2} + 38{\cdot}26x^{-2}\ln(x)$ for all participants, $y = 0{\cdot}29 - 61{\cdot}61x^{-2} + 75{\cdot}36x^{-2}\ln(x)$ for GG, $y = 0{\cdot}33 + (1{\cdot}92 \times 10^{-8}x^{3}) - 2{\cdot}89 \times 10^{-9}x^{3}\ln(x)$ for AG, $y = 0{\cdot}43 - 0{\cdot}087\ln(x) + 0{\cdot}01\ln(x)^{2}$ for AA genotype, where y denotes the ucOC/iOC ratio and x denotes dietary phylloquinone (μg/d).

genotype, while in carriers of the AG and AA genotype, the ucOC/iOC ratio was nearly unaffected by vitamin K intake.

The dietary intake of phylloquinone observed in our sample is well comparable to other studies[7,9,10,21] using the same database[35] for calculation of phylloquinone intake. Also the observation that the majority of phylloquinone intake was derived from vegetables was consistent with other studies[7,9–11]. However, phylloquinone intakes in the Rotterdam Study (dietary assessment by FFQ, using a Dutch database on phylloquinone contents of common foods) were more than two-fold higher than in the present study[38]. This discrepancy may be attributable to dietary assessment by FFQ, on the one hand, and country-specific nutritional habits, on the other hand. Menaquinone intake results from the Rotterdam Study (using the same database for menaquinone contents of foods as in the present study), however, were comparable to the present results. Inaccuracies of dietary vitamin K intake estimation due to the use of food composition databases form a potential source of error in the present study. The phylloquinone database used here has been successfully applied for the estimation of phylloquinone intake in previous epidemiological studies[7,10,21,39]. In addition, phylloquinone intake data calculated by means of this database showed significant correlations with plasma phylloquinone concentrations[39]. Compared to phylloquinone, the currently available food composition data for menaquinones is less comprehensive. Apart from the Rotterdam Study and the data in the present paper, figures on the habitual dietary intake of menaquinones from population-based studies in Western countries are scarce.

Serum ucOC and iOC concentrations were measured using specific ELISA tests. The ucOC ELISA has been shown to have a higher sensitivity and specificity as compared to the frequently used hydroxy-apatite binding assay, in which a significant fraction of ucOC is bound non-specifically by hydroxy-apatite and, therefore, not analysed as ucOC[24,40]. We observed a significant inverse association between total vitamin K intake and the ucOC/iOC ratio. This is consistent with a study from Japan, where dietary intake of total vitamin K was significantly inversely associated with the ucOC/iOC ratio[20]. Furthermore, inverse associations between phylloquinone intake and serum ucOC concentration or %ucOC were observed in an Irish study[10] and in the Framingham Offspring Study[8], respectively. According to the present data, phylloquinone and menaquinone intakes were associated similarly with the ratio of ucOC/iOC. Due to their lower contribution to total vitamin K intake menaquinones were neglected in the majority of epidemiological studies on vitamin K. The present results, however, indicate that despite contributing less to total vitamin K intake than phylloquinone, menaquinones may have a considerable impact on vitamin K status as reflected by the ucOC/iOC ratio. This seems plausible considering the higher bioavailability and longer half-life in the blood circulation of menaquinones as compared to phylloquinone[5]. Among the menaquinones, only the higher subtypes, MK-5 to MK-9, which are almost exclusively found in fermented dairy products, were significantly inversely associated with ucOC/iOC ratio. This observation may be related to the longer half-life of higher menaquinones as compared to MK-4[41]. The observed inverse association between dietary intake of vitamin K and serum ucOC/iOC ratio is in agreement with studies examining the association between plasma vitamin K concentrations and serum ucOC/iOC ratio[20,30] or %ucOC[8,18], respectively.

In the present study, ucOC/iOC ratio was chosen as the biomarker of vitamin K status because it reflects supply with both phylloquinone and menaquinones. Direct measurement of vitamin K in serum poses an alternative biomarker of vitamin K status. However, while serum phylloquinone measurement was applied in a number of epidemiological studies, measurement of circulating levels of menaquinones has been rarely done. Due to the low menaquinone concentrations in plasma, very sensitive methods of analysis are required, and to date, it is only possible to detect certain menaquinones such as MK-4 and MK-7 as representatives for total menaquinones[42].

The fractional polynomial approach revealed that the inverse association between total vitamin K intake and ucOC/iOC ratio was strongest in low intake ranges below approximately 70 μg/d. In contrast, supplementation studies have shown that supra-dietary doses of 200–1000 μg/d phylloquinone[14,18,43] or 45 mg/d MK-4[15–17] reduce ucOC/iOC ratio or %ucOC. This discrepancy may be related to differences in the absorption efficiency of vitamin K from foods as compared to supplemental vitamin K[44].

Polymorphisms of the *VKORC1* gene have been investigated in the context of warfarin sensitivity[31–33,45–48] or CVD[34,49]. In the present study, the +2255 polymorphism located on the second intron of the *VKORC1* gene was selected for analysis, because it has been previously shown that ucOC concentrations vary by genotype of this single nucleotide polymorphism[34]. Due to high linkage disequilibrium, variation in other potential single nucleotide polymorphisms is likely to be sufficiently covered by the

analysed polymorphism. The +2255 polymorphism has been reported to be significantly associated with required warfarin dose[32] as well as the risk of stroke, CHD and aortic dissection[34]. Carriers of the GG genotype were shown to be most warfarin-sensitive, i.e. these subjects require the lowest warfarin dose for inhibition of blood coagulation. The warfarin sensitivity decreases from GG to AG to AA genotype[32]. A low requirement of warfarin for inhibition of blood coagulation mirrors low vitamin K epoxide reductase activity, i.e. low recycling rates of vitamin K. The present observation of highest ucOC/iOC ratios in carriers of the GG genotype can be explained by reduced epoxide reductase activity and consequently low vitamin K recycling rate resulting in low carboxylation rate. Lower ucOC concentrations in AG and AA genotypes as compared to the GG genotype of the +2255 *VKORC1* polymorphism have also been observed in a Chinese study[34]. We observed only in carriers of the GG genotype a strong association between the ucOC/iOC ratio and dietary intake of vitamin K. This homozygous genotype may therefore be characterized not only as warfarin-sensitive but also as vitamin K-sensitive. It seems plausible that subjects with a low activity of the vitamin K cycle can enhance the carboxylation of vitamin K-dependent proteins by increased intakes of vitamin K. Whereas in subjects with a high activity of the vitamin K recycling (AA genotype), carboxylation activity is not as much affected by high vitamin K intakes. The present observations point to the activity of vitamin K epoxide reductase as the limiting factor in the interplay of vitamin K supply and recycling of vitamin K. The separate evaluation of phylloquinone and menaquinones revealed that the strong association between vitamin K intake and ucOC/iOC ratio in GG-genotype subjects was predominantly driven by phylloquinone intake. No striking differences regarding the activity of phylloquinone versus menaquinones as a cofactor for γ-glutamyl carboxylase have been observed[50]. Thus, it is conceivable that the stronger effect of phylloquinone on ucOC/iOC ratio in GG carriers may be related to differences in the affinity of phylloquinone versus menaquinones to vitamin K epoxide reductase. However, so far no studies comparing the affinity of phylloquinone and menaquinones to vitamin K epoxide reductase have been conducted that could resolve this speculation. The observation that menaquinones, especially MK-5 to MK-9, were significantly inversely associated with the ucOC/iOC ratio in AG, but not in GG subjects, was unexpected and remains unexplained. An analysis of the modification of the association between vitamin K and ucOC by genetic variation in the *VKORC1* gene has not been reported in the literature so far, and, thus, replication of the here observed findings in other population-based studies would be desirable. In a Japanese study in young males, the association between serum menaquinones (MK-7) and ucOC/iOC ratio was modified by a polymorphism of the γ-glutamyl carboxylase gene[20].

The present observations have shown that in all participants, the benefit of vitamin K intakes above 70 μg/d is minor with respect to further reduction of serum ucOC/iOC ratio. However, a substantial proportion of subjects do not meet the estimated adequate vitamin K intakes of 65 μg/d in women and 80 μg/d in men, even when both phylloquinone and menaquinones are considered. As we showed, also intake of menaquinones can contribute to the reduction of the ratio ucOC/iOC. In the present study, stratification by *VKORC1* + 2255 genotype suggested for the first time that subjects may differ with respect to vitamin K sensitivity, i.e. the magnitude to which the ratio of ucOC/iOC can be influenced by dietary vitamin K intake.

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