

Short Communication

Vitamin D–vitamin K interaction: effect of vitamin D supplementation on serum percentage undercarboxylated osteocalcin, a sensitive measure of vitamin K status, in Danish girls

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There is some evidence for a nutritional interaction between vitamin D and vitamin K status. We have recently reported that serum percentage undercarboxylated osteocalcin (%ucOC; a marker of vitamin K status) was inversely correlated with serum 25-hydroxyvitamin D (25(OH)D) concentration (reflective of vitamin D status) in healthy Danish girls (aged 11–12 years), in line with a similar relationship reported in elderly women. While the causal nature of the relationship between vitamin D status and serum %ucOC has been tested in studies of elderly women, it has not been investigated in children. The objective of the present study was to test the hypothesis that improving vitamin D status significantly lowers serum %ucOC. Serum samples from sixty-seven healthy Danish girls (aged 11–12 years), who participated in a 12-month double-blind, placebo-controlled, vitamin D₃ intervention trial were used for the present study. These girls were a subset of subjects which began and finished the intervention during wintertime, thus avoiding the influence of seasonality on vitamin D status. A total of thirty-three and thirty-four of the girls had been randomised to treatment with 10 µg vitamin D₃ per d and placebo, respectively, for 12 months. Total osteocalcin and the fraction of ucOC in serum (via enzyme-immunoassay) as well as serum 25(OH)D (via HPLC) were assessed at baseline and end-point. Vitamin D₃ supplementation significantly increased serum 25(OH)D (21.6%; $P < 0.002$) but had no effect on serum %ucOC ($P > 0.8$). In conclusion, the findings of the present intervention study in young girls suggest that vitamin D supplementation does not affect serum %ucOC, a marker of vitamin K status.

Vitamin D: Vitamin K: Undercarboxylated osteocalcin: Girls: Interactions

Vitamin K is a cofactor for the vitamin K-dependent carboxylase, a microsomal enzyme that facilitates the post-translational conversion of glutamyl to γ -carboxyglutamyl (Gla) residues⁽¹⁾. Its classic role in this respect involves the synthesis of several coagulation factors^(2–4). More recently, the identification of Gla-containing proteins in bone, notably osteocalcin and matrix Gla protein, has generated much interest in the role of vitamin K in bone metabolism and bone health^(4–7). The circulating concentration of under- γ -carboxylated osteocalcin (ucOC), a sensitive marker of vitamin K nutritional status^(8,9), has been reported to be a marker of hip fracture risk and a predictor of bone mineral density in adults^(6,10–17). More recently, higher serum ucOC has been associated with increased bone turnover⁽¹⁸⁾ and lower bone mineral content⁽¹⁹⁾ in young girls.

The synthesis of functional osteocalcin depends on both vitamin D and vitamin K⁽¹⁰⁾. Vitamin D (as 1,25-dihydroxyvitamin D₃) induces the synthesis of osteocalcin by

promoting the transcription of its gene⁽²⁰⁾, while vitamin K is needed for its γ -carboxylation, as mentioned already. There is some *in vitro* evidence, however, that vitamin D might stimulate the γ -carboxylation of osteocalcin and other Gla-containing proteins^(21,22). Szulc *et al.*⁽¹⁰⁾ have suggested that these observations could be relevant to the age-related impairment of the γ -carboxylation of osteocalcin, in that vitamin D deficiency is a common feature of elderly populations. However, vitamin D deficiency is also very common during adolescence, a period of rapid bone development (for a review, see Cashman⁽²³⁾).

We have recently reported that serum %ucOC was inversely correlated with serum 25-hydroxyvitamin D (25(OH)D) levels, the most widely used marker of vitamin D status, in healthy Danish girls (aged 11–12 years)⁽¹⁹⁾. This is in line with a similar relationship reported in elderly women⁽¹⁰⁾. In both studies, serum %ucOC as well as serum 25(OH)D

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; ucOC, undercarboxylated osteocalcin.

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concentrations exhibited clear seasonal variation (with lowest and highest values, respectively, evident in late summer/early autumn)^(10,19). Furthermore, serum %ucOC was correlated with sun exposure in the study by Szulc *et al.*⁽¹⁰⁾. In a longitudinal 1-year follow-up study, Szulc *et al.*⁽¹⁰⁾ showed that vitamin D plus Ca supplementation significantly reduced the %ucOC in elderly women. However, Bolton-Smith *et al.*⁽²⁴⁾ in a 2-year vitamin K and/or vitamin D plus Ca randomised control intervention trial in elderly women showed that vitamin D plus Ca had no effect on %ucOC. Whether the relationship between vitamin D status and serum %ucOC is causal in children has not been investigated.

Therefore, the objective of the present study was to test the hypothesis that improving vitamin D status in young adolescent girls would significantly lower serum %ucOC. This was possible by using data and samples available from a randomised, double-blind, placebo-controlled, 12-month vitamin D₃ intervention study in Danish girls, aged 11–12 years⁽²⁵⁾.

Subjects and methods

Subjects

Our initial study investigated the relationship between vitamin K status and bone health indices in 223 Danish girls, aged between 10.9 and 11.9 years⁽¹⁹⁾. These girls were recruited for participation in a 12-month vitamin D₃ intervention trial on bone health (as part of the Optimal Strategy for Vitamin D Fortification (OPTIFORD) project; <http://www.optiford.org>), details of which have been described previously^(19,25). Of the 221 girls who completed the double-blind, placebo-controlled vitamin D intervention trial, sixty-seven subjects were selected as a subset who began and finished the intervention during wintertime (November to April), and who were randomised to receive either placebo (*n* 34) or 10 µg vitamin D₃ (*n* 33) for 12 months. Selection of this wintertime subgroup avoided the influence of seasonality on vitamin D status. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Research Ethical Committee of Copenhagen and Frederiksberg (J.nr (KF) 01-129/01). Written informed consent was obtained from the parent or guardian of each participant.

Design

This was a double-blind, placebo-controlled vitamin D₃ intervention trial, which in the context of the present work examined the effect of improving vitamin D status on serum %ucOC, as a widely used marker of vitamin K status⁽²⁶⁾, in a cohort of healthy young Danish girls, aged 10.9–11.9 years. After an overnight fast, a blood sample (10 ml) was taken between 08.00 and 10.00 hours from each subject. On the same day as blood sampling, each girl, with the help of their parent or guardian and a trained researcher, completed various questionnaires, including a general health and lifestyle questionnaire, a FFQ, a physical activity questionnaire, and a pubertal status questionnaire, as described previously^(19,25). Anthropometric measurements (weight and height) were also taken at this time. Pubertal status was assessed and physical activity recorded in each subject, as described previously⁽¹⁹⁾.

Collection and preparation of samples

Fasting blood was collected by venepuncture into vacutainer tubes with no additive and was processed to serum, which was immediately stored at –80°C until required for analysis.

Experimental techniques

Details of the analytical methodology for serum total osteocalcin and ucOC as well as 25(OH)D have been described in detail elsewhere^(19,27). In brief, total (intact) osteocalcin levels were measured in serum samples using an ELISA (Metra™ Osteocalcin EIA Kit; Quidel Corporation, San Diego, CA, USA). The intra- and inter-assay CV was 6.0 and 7.6 %, respectively. ucOC was measured by treating 60 µl of serum samples with 30 µl of hydroxyapatite (15 mg/ml) (Calbiochem, Merck Biosciences, Bestor, Nottingham, UK). Samples were shaken for 1 h at room temperature and then centrifuged for 5 min. ucOC in the supernatant fractions was quantified using the Metra™ Osteocalcin EIA Kit. ucOC was expressed as the percentage of total osteocalcin (%ucOC). The intra- and inter-assay CV was 9.5 and 12.8 %, respectively. Serum 25(OH)D levels were measured by an HPLC-based method.

Statistical methods

We have previously shown that %ucOC was significantly lower (14 %) during summer compared with wintertime in these girls, coinciding with better vitamin D status in summer also⁽¹⁹⁾. Therefore, using the mean and standard deviation of serum %ucOC in the girls in the present study at baseline and the percentage difference between summer and winter, possibly arising from improved vitamin D status (our hypothesis), we calculated we needed thirty subjects per group. Serum 25(OH)D and %ucOC were normally distributed as determined by Kolmogorov–Smirnov tests and allowed for parametric statistical analysis. Data are presented as means and standard deviations. Baseline characteristics were compared between the placebo and vitamin D-supplemented group by unpaired Student's *t* tests and χ^2 where appropriate. The effect of vitamin D₃ supplementation on serum 25(OH)D concentration, %ucOC and total osteocalcin in the placebo and 10 µg vitamin D-supplemented groups was evaluated by repeated-measures ANOVA. Paired Student's *t* tests were used to compare serum 25(OH)D concentrations at baseline *v.* end-point in both the placebo and vitamin D-supplemented groups. *P* < 0.05 was considered significant.

Results

The mean age, height, weight, BMI, serum 25(OH)D and serum %ucOC of the girls were 11.3 (SD 0.2) years, 148.6 (SD 6.4) cm, 40.1 (SD 7.3) kg, 18.1 (SD 2.8) kg/m², 36.0 (SD 12.1) nmol/l and 23.2 (SD 4.4) %, respectively. There were no significant differences in these baseline characteristics between the placebo and vitamin D-supplemented group (data not shown).

The effect of vitamin D₃ supplementation on biochemical indices of vitamin D and vitamin K status in the Danish girls is shown in Table 1. There was no significant difference

Table 1. Serum 25-hydroxyvitamin D (25(OH)D) (nmol/l) and serum percentage undercarboxylated osteocalcin (%ucOC) pre- and post-vitamin D (10 µg/d) intervention (Mean values and standard deviations)

	Pre-intervention				Post-intervention			
	Placebo (n 34)		Vitamin D ₃ (n 33)		Placebo (n 34)		Vitamin D ₃ (n 33)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Serum 25(OH)D	47.8	18.4	47.6	15.8	39.7†	17.7	57.9*	14.3
Serum %ucOC	24.0	5.2	22.5	3.4	27.8*	5.7	26.7*	7.0

* Mean value was significantly increased relative to pre-intervention concentration within a group ($P < 0.05$).

† Mean value was significantly decreased relative to pre-intervention concentration within placebo group ($P < 0.003$).

in serum 25(OH)D concentration or %ucOC between the placebo and vitamin D-supplemented groups at baseline. Repeated-measures ANOVA showed a significant time \times treatment group interaction ($P < 0.0001$) in serum 25(OH)D concentration. While serum 25(OH)D concentration decreased (-16.9% ; $P < 0.003$) from baseline to end-point in the placebo group, it significantly increased (21.6% ; $P < 0.002$) in the supplemented group. There was no significant time \times treatment group interaction ($P > 0.79$) in serum %ucOC. There was, however, a significant change over time (from baseline to end-point) in serum %ucOC, with similar increases in the placebo and vitamin D-supplemented groups (15.8 and 18.6% , respectively). There was no significant time \times treatment group interaction ($P > 0.65$) in serum total osteocalcin (data not shown).

Discussion

Interest in the role of vitamin K nutritive status in childhood bone health has heightened in recent times with the findings that better vitamin K status was associated with decreased bone turnover⁽¹⁰⁾ and greater bone mineral content of total body and lumbar spine⁽¹⁹⁾ in healthy young girls. Of concern, there is evidence that a high proportion of girls have suboptimal dietary vitamin K intake and/or vitamin K status^(18,19,28,29). Thus, with this in mind, strategies for improving vitamin K status during childhood and adolescence need to be developed. Various lines of epidemiological evidence point towards a nutritional interaction between markers of vitamin D and vitamin K status^(10,19,20–22). A causal relationship between vitamin D and vitamin K, should it exist, would offer a possible indirect benefit of increasing vitamin D intake to bone health. The findings of the present study, however, which had the possibility that improving vitamin D status of young adolescent girls would decrease serum %ucOC as its central hypothesis, do not support this notion. While vitamin D status, as reflected by serum 25(OH)D concentration, was significantly increased by vitamin D₃ supplementation in the girls, serum %ucOC was not reduced.

The lack of effect of vitamin D supplementation on serum %ucOC in adolescent girls in the present study, which to our knowledge is the first to investigate this directly, is in line with recently reported lack of effect of vitamin D₃ supplementation (plus Ca) on serum ucOC in postmenopausal women⁽²⁴⁾. In contrast, Szulc *et al.*⁽¹⁰⁾ showed that vitamin D₃ plus Ca supplementation significantly reduced the %ucOC in elderly women. There is no other report of vitamin D

supplementation alone on serum %ucOC. Takahashi *et al.*⁽³⁰⁾ reported no effect of 1 µg of 1 α -hydroxyvitamin D₃ (a synthetic pro-drug of the active form of vitamin D) on ucOC in elderly osteoporotic patients with vertebral fractures. Furthermore, Bolton-Smith *et al.*⁽²⁴⁾ and Takahashi *et al.*⁽³⁰⁾ failed to find any evidence of an additive effect of supplementation with vitamin K and vitamin D (alone or plus Ca) on %ucOC. It is possible that the inverse associations observed between serum 25(OH)D and %ucOC in our young girls at baseline⁽¹⁹⁾, as well as in older women⁽¹⁰⁾, may simply reflect the fact that the two biochemical measures track healthy diet and/or lifestyles. However, given that the dietary and other sources of vitamin D and vitamin K are so different, this area requires more investigation.

The mean increment in serum 25(OH)D concentration (10.3 nmol/l) over 12 months following daily supplementation with 10 µg vitamin D₃ was similar to that reported recently in similarly-aged Finnish girls supplemented for 12 months with an equivalent dose of vitamin D₃⁽³¹⁾. There was a significant decrease in mean serum 25(OH)D (-8.1 nmol/l) in the placebo group over the 12-month intervention period. Viljakainen *et al.*⁽³¹⁾ also showed a decline in serum 25(OH)D concentration over 12 months in a group of 11-year-old Finnish girls randomised to a placebo treatment and sampled during wintertime. The reasons for this decrease in serum 25(OH)D over time are not clear, but may relate to the advancing sexual maturity of the girls over the 12 months. For example, Tanner stage has been shown to negatively relate to serum 25(OH)D in children, aged 10–16 years⁽³²⁾, although Ginty *et al.*⁽³³⁾, while reporting a decrease in serum 25(OH)D with advancing Tanner stage in boys (aged 11–16 years), did not see this relationship in girls. The increased rate of bone accretion during the pubertal spurt requires adequate serum Ca levels, and 25(OH)D may be more rapidly converted to 1,25-dihydroxyvitamin D₃ to support the bone metabolic activity at this life-stage. Alternatively, the decrease in serum 25(OH)D may reflect differences in vitamin D stores achieved in these girls during the two preceding summers arising from differences in sun exposure and/or vitamin D intake during these times. Serum %ucOC increased in both groups over time. This, again, may have been related to the advancing sexual maturity of the girls over the 12-month intervention period. For example, Van Summeren *et al.*⁽²⁹⁾ recently reported that the ratio between ucOC and carboxylated osteocalcin (i.e. %ucOC) was highest at the end of puberty (Tanner stage V; 2.7) and during puberty (Tanner stages II–IV; 2.6) compared with pre-puberty (Tanner stage I; 2.0), although

these were not statistically compared. The authors suggest that the high %ucOC in children compared with that seen in adults in their study may be suggestive of subclinical vitamin K deficiency during high bone turnover in children⁽²⁹⁾. Whether a high %ucOC has a detrimental effect on bone health outcomes in children requires further investigation. Data from available randomised controlled trials with vitamin K (alone or in combination with other micronutrients) in older adults have produced mixed findings in relation to its effect on bone mineral density even though it significantly reduced %ucOC (or ucOC where %ucOC was not reported) in all studies^(24,34–37). However, vitamin K may lower the risk of osteoporotic fractures by other mechanisms, such as through effects on bone quality parameters, an area which would also require more research^(29,38).

In conclusion, improving vitamin D status did not reduce serum %ucOC in adolescent girls. Other strategies for improving vitamin K status need to be explored, especially as a high percentage of adolescent girls appear to have low vitamin K status^(18,19,28,29). While achieving dietary recommendations for vitamin K would seem the best way forward in terms of improving status, there is evidence that phylloquinone intakes in many children and adolescents are below the recommended level^(18,27,39–42). For example, in a representative sample of Irish children aged 5–12 years about half of all boys and girls had suboptimal phylloquinone intakes⁽⁴²⁾. Further research is needed to explore strategies of promoting vitamin K intake during childhood.

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