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Vitamin K-Induced Changes in Markers for Osteoblast Activity and Urinary Calcium Loss

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Summary. The objective of this study was to identify subjects in whom vitamin K has an effect on markers for calcium and bone metabolism and to detect hitherto-unnoticed correlations between vitamin K–induced changes in these markers. Participants in our studies were apparently healthy women, in whom we measured serum-immunoreactive osteocalcin (irOC) before and after adsorption to hydroxylapatite; total serum alkaline phosphatase (T-AP) and bone-specific alkaline phosphatase (B-AP); and fasting urinary calcium and creatinine. We describe a trial among 145 women who were treated with vitamin K (1 mg/day) for 2 weeks, and a prospective placebo-controlled trial among two groups each of 70 postmenopausal women with a treatment period of 3 months. It turned out that in elderly women vitamin K induced increased levels of serum irOC with a high affinity for hydroxylapatite ($irOC_{bound}$), whereas that with low affinity ($irOC_{free}$) remained unaffected. In placebo-treated women the ratio $irOC_{free}/irOC_{bound}$ shifted from 0.38 to 0.65 around the 50th year of age. This shift was not found in vitamin K–treated women. After 3 months of treatment the vitamin K–induced changes in $irOC_{bound}$ were correlated with changes in B-AP, whereas $irOC_{free}$ was correlated to urinary calcium excretion. In fast losers of urinary calcium vitamin K induced a 30% decrease of calcium excretion. The hypothesis is put forward that $irOC_{bound}$ may be a marker for bone formation, that serum $irOC_{free}$ may be a marker for bone resorption, and that the serum $irOC_{free}/irOC_{bound}$ ratio may become a marker for skeletal remodeling. It is concluded that vitamin K administration may help to reduce urinary calcium loss in postmenopausal women, notably in the fast losers of calcium. The ratio $irOC_{free}/irOC_{bound}$ provides more information than total irOC and may become a practical marker for bone metabolism.

Key words: Vitamin K – Osteocalcin – γ-carboxyglutamic acid – Bone – Calcium excretion

Vitamin K functions as the coenzyme for γ-glutamylcarboxylase, a microsomal enzyme mediating the posttranslational carboxylation of protein-bound glutamate into γ-carboxyglutamate (Gla) residues [1–3]. Gla-containing proteins are found in body fluids (plasma, urine) [4, 5] and in calcified tissues (bone, hardened atherosclerotic plaques) [6–8]; well-known examples are the vitamin K–dependent blood coagulation factors and the bone Gla proteins osteocalcin (OC) and matrix Gla protein (MGP). In all these proteins the only known function of Gla is the binding of calcium ions. Blood coagulation is the only process in which the role of Gla proteins has been well established and in which their function has been described to a molecular level. Accumulating evidence indicates, however, that calcium metabolism is a second physiological process in which Gla proteins are involved [9].

In a previous paper we have reported that in a selected group of women (nuns between 55 and 75 years of age) the hydroxylapatite binding capacity of the circulating immunoreactive osteocalcin (irOC) was abnormally low compared to premenopausal women [10]. Explanations for this phenomenon are that either the Gla content of the irOC is suboptimal or the immunoreactive material contains a partly degraded form of OC, from which the Gla residues have been split off. Administration of vitamin K (1 mg daily for 14 days) resulted in an increase of both the concentration and the hydroxylapatite binding capacity of circulating irOC. In parallel, the treatment induced a significant decrease in the fasting urinary calcium excretion, notably in the fast losers of calcium (calcium/creatinine ratio > 0.5). To test the universality of these findings we have extended our studies to a group of civilian women. In a first trial we investigated the occurrence and age dependency of the vitamin K–induced effects on serum irOC. Second, a longer study in a selected age group was undertaken to assess the duration of the vitamin K–induced effects. To obtain more insight into the value of serum irOC as a marker for bone metabolism, the irOC fractions with high affinity for hydroxylapatite ($irOC_{bound}$) and that with low affinity ($irOC_{free}$) were quantified separately in all cases.

Materials and Methods

Subjects

Apparently healthy, physically active women were recruited via a local newspaper call. All had normal serum calcium levels, as well as normal liver and renal functions. Exclusion criteria were malnutrition, a history of recent fractures or metabolic bone disease, and medication known to affect calcium and bone metabolism or vitamin K status. Solubilized vitamin K_1 (Konakion) was obtained from Hoffmann-La Roche (Basle, Switzerland) and distributed among the participants in drop-bottles containing 20 mg/ml; the placebo was constituted of the same solvent as the vitamin K preparation. One drop of the vitamin (1 mg) or placebo was taken orally by mixing it

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with tap water immediately before drinking it. The study was approved by the local ethics committee, and informed consent was obtained from all subjects, according to the institutional guidelines.

Tests

Fasting blood samples were collected by venipuncture between 8 and 10 AM. The samples were left at room temperature for 2 hours and after a short centrifugation step serum was immediately frozen at −80°C until use. Serum irOC concentrations were assessed by radioimmunoassay (Incstar, Stillwater, MI) before (total irOC) and after ($irOC_{free}$) extraction with 100 mg/ml hydroxylapatite [10], and $irOC_{bound}$ was calculated from the difference between these two figures. The inter- and intraassay coefficients of variation of serum irOC were 10 and 5%, respectively. Analysis of total alkaline phosphatase activity (T-AP) were performed in a Cobas centrifugal analyzer (Roche) with a commercial kit (Roche). The inter- and intraassay variation coefficients were 2 and 1.5%, respectively. Bone-specific alkaline phosphatase activity (B-AP) was measured according to Behr and Barnert [11], with inter- and intraassay variation coefficients of 7 and 5%.

To exclude dietary influences, urine was collected during the last 2 hours of a 16-hour fasting period, as recommended by Nordin [12]. The samples were acidified (pH 1) and stored at −30°C until serial testing. Urinary calcium concentrations were determined with an atomic absorption spectrophotometer (Perkin Elmer, Norwalk, CN), and creatinine with a commercial kit (Roche). The urinary calcium excretion is expressed throughout this paper as the molar ratio between calcium and creatinine in the fasting urine samples. Fast losers of urinary calcium are defined as those with a calcium/creatinine ratio >0.5, normal losers as those with a ratio between 0.15 and 0.5.

Data Analysis

For both trials reported, statistical analysis was performed with the software package SPSS/PC+ version 3.1 (SPSS Inc., Chicago, IL). Changes between groups and within one group (second trial) were detected with the two-sided unpaired and paired Student's t-test, respectively; differences were considered to be significant if $P < 0.05$. Relations between variables were tested by least-square linear regression. All data are expressed as the mean ± SE. Only those subjects who completed the protocol without omissions were included in the analysis of correlation between the different parameters tested.

Results

Effects of Vitamin K on Circulating Osteocalcin: Relation with Age

In a first study among 145 women between 20 and 85 years of age we measured whether the vitamin K–induced effects on serum irOC are related to age. Participants were assembled in age groups differing from each other by 5 years. Each group consisted of at least 10 women. Blood was taken before and after a 2-week period during which the participants received vitamin K (1 mg/day).

Before vitamin K treatment the mean level of total irOC in this group was 3.47 (±0.10) ng/ml, but as can be seen in Figure 1A, this marker varied in the different age groups. In the age groups between 20 and 50 there was a gradual increase of serum irOC with age. The increase seemed to accelerate around the 50th year of life, but irOC levels sharply dropped after the age of 60. Treatment of the participants with vitamin K for 2 weeks resulted in an increase of the circulating total irOC levels in all groups, but this increase was only statistically significant for those older than 45.

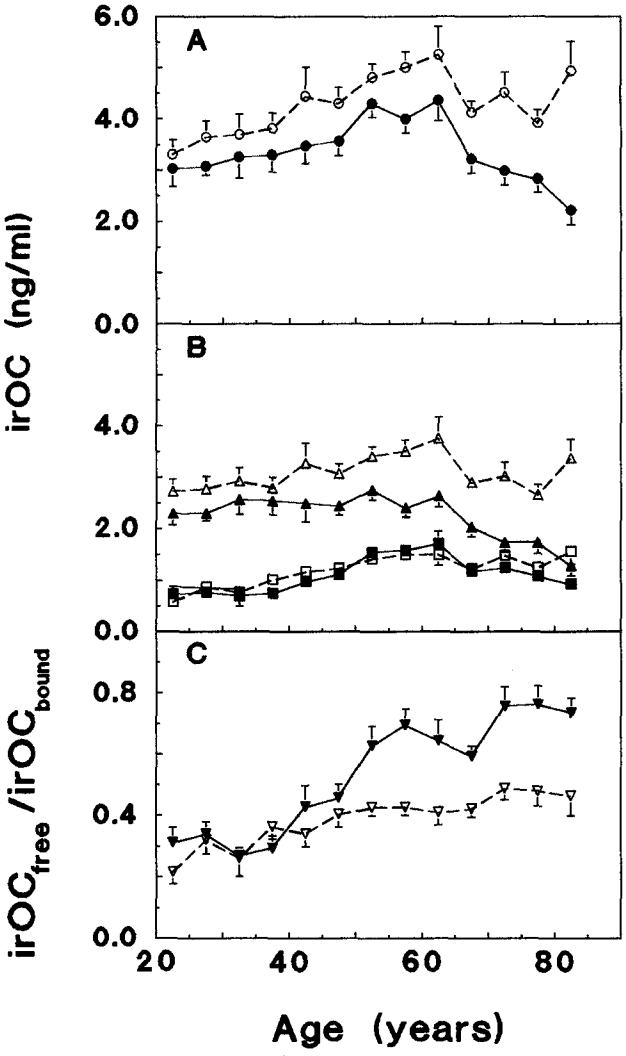

Fig. 1. Age-dependent changes in serum osteocalcin. Participants were divided into 13 age groups, each consisting of at least 10 women. **(A)** Total irOC and **(B)** $irOC_{free}$ (squares) and $irOC_{bound}$ (triangles) were measured before (closed symbols) and after (open symbols) 2 weeks of vitamin K administration. The ratio between $irOC_{free}/irOC_{bound}$ is given in **C**. All results are expressed as the means for each age group ± SE. The effect of vitamin K on total serum irOC **(A)** and on $irOC_{bound}$ **(B)** was significant ($P < 0.05$) in all groups older than 45 years; that on $irOC_{free}/irOC_{bound}$ **(C)** was significant in all groups older than 50 years.

Total irOC was subdivided into $irOC_{bound}$ and $irOC_{free}$ (Fig. 1B), which showed that the age-related increase of serum irOC was almost completely due to $irOC_{free}$, whereas the decrease after the age of 64 was caused by the substantial decrease of $irOC_{bound}$. Also, it appeared that the effect of vitamin K remained almost completely restricted to $irOC_{bound}$. Vitamin K–induced differences in the latter marker were significant in all groups of 45 years and older ($P < 0.01$ for all age groups). The mean effect of vitamin K in the women older than 50 years was an increase of the $irOC_{bound}$ from 2.25 (±0.08) to 3.23 (±0.09) ng/ml. Further details are summarized in Table 1.

In Figure 1C we have plotted the ratio between $irOC_{free}$ and $irOC_{bound}$ as a function of age. Before treatment with vitamin K this ratio amounted to 0.39 (±0.03) in women younger than 50 years, but it increased to 0.67 (±0.03) after

Table 1. Osteocalcin markers before and after vitamin K treatment in women younger than 50 years (n = 67) and older than 50 years (n = 78)[a]

Marker	Age group	Before treatment	P_{age}	P_{treat}	After treatment	P_{age}
$irOC_{total}$	<50	3.18 ± 0.15	<0.05	<0.005	3.87 ± 0.21	<0.005
	>50	3.62 ± 0.13		<0.005	4.63 ± 0.13	
$irOC_{free}$	<50	0.89 ± 0.07	<0.005		0.95 ± 0.15	<0.005
	>50	1.37 ± 0.05			1.40 ± 0.05	
$irOC_{bound}$	<50	2.30 ± 0.10		<0.005	2.92 ± 0.09	
	>50	2.25 ± 0.09		<0.005	3.23 ± 0.09	
$irOC_{free/bound}$	<50	0.39 ± 0.03	<0.005		0.33 ± 0.03	<0.005
	>50	0.67 ± 0.03		<0.005	0.44 ± 0.01	

[a] All results are given as the means ± SE. P_{age} stands for the significance of the difference of one marker between two age groups (two-sided unpaired Student's t-test); P_{treat} stands for the significance of the difference before and after vitamin K treatment in one age group (two-sided paired Student's t-test).

the age of 50. The difference between both age groups is significant at $P < 0.005$. After treatment of all women with vitamin K the $irOC_{free}/irOC_{bound}$ ratios of the younger women remained unchanged, but those of the elderly participants decreased to values which approached the premenopausal values.

To obtain more information about the nature of $irOC_{bound}$ and $irOC_{free}$ five serum samples from different subjects were dialyzed against buffered saline (pH 7.4) for 1 hour at 4°C. The molecular-weight cutoff value of the dialysis membrane was 3.5 kDa, which was found to be sufficient for preventing the loss of intact osteocalcin. Identical samples were incubated in plastic tubes at 4°C for the same period, and it was ascertained that both $irOC_{bound}$ and $irOC_{free}$ remained unchanged in these control samples. In the dialyzed samples at least 50% of the $irOC_{free}$ had disappeared within 1 hour, whereas the $irOC_{bound}$ had remained unchanged (data not shown). It seems, therefore, that in elderly women at least a major part of the circulating $irOC_{free}$ has a reduced size.

Correlation Between Serum Osteocalcin and Other Markers for Bone Metabolism

From the first trial it became apparent that the largest effects of vitamin K administration were found in the age group older than 50 years. To further identify subjects with a high response to vitamin K treatment, and to be able to correlate both forms of serum osteocalcin with other markers for calcium and bone metabolism, a second trial was started for which 834 apparently healthy women between the age of 50 and 80 years were recruited. Based on the calcium/creatinine ratios in their fasting urine, 70 fast losers of urinary calcium, as well as an age-matched group of normal losers, were selected. Both normal and fast losers were subdivided further into two similarly sized, age-matched groups: one received vitamin K treatment (1 mg/day for 3 months) and the other received a placebo. Blood and urine were collected at the outset, and at 1, 3, 4, and 6 months after starting the treatment. From the 140 participants thus selected 111 attended all examinations and completed the protocol: 58 in the fast-losers group (30 vitamin K-treated subjects and 28 placebo-treated ones) and 53 in the normal-losers group (28 vitamin K- and 25 placebo-treated subjects).

After 1 month of treatment the mean urinary calcium loss in the vitamin K–treated group of fast losers had decreased, whereas no effect was found in the normal losers group (Fig. 2). Also, in both placebo-treated groups the urinary calcium

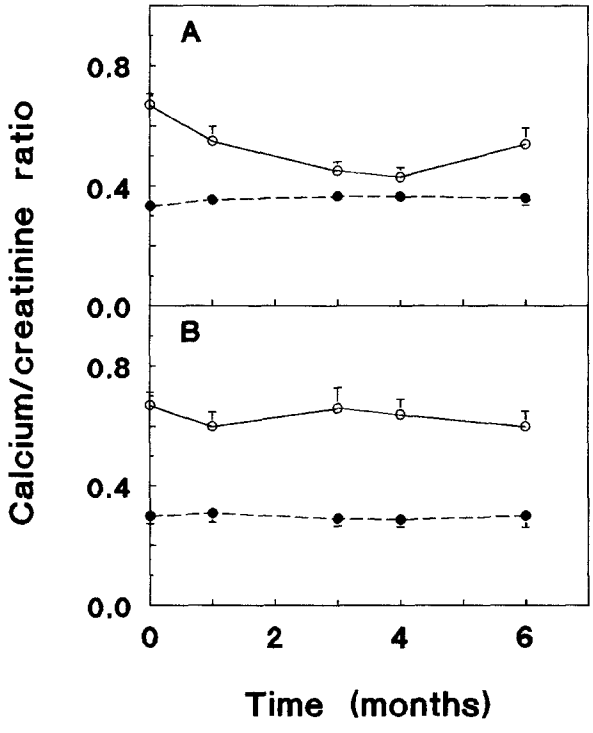

Fig. 2. Prolonged treatment of postmenopausal women (50–85 years) with vitamin K. Fast (open symbols) and normal (closed symbols) losers of urinary calcium were assembled and treated with either vitamin K (**A**) or placebo (**B**). $^*P < 0.05$, $^{**}P < 0.005$. All data are given ± SE.

loss remained constant during the trial. In the fast-losers groups the difference between vitamin K- and placebo-treated subjects was significant ($P < 0.01$) after 1 month, continued during the 2 subsequent months ($P < 0.005$, mean decrease: 30%), and gradually reversed after the treatment had stopped. In all groups the creatinine concentration in the samples had remained constant during the entire study period, and changes in the Ca/creatinine ratio were solely due to variations in the calcium excretion. These data demonstrate, therefore, that the effect of vitamin K administration on urinary calcium loss is most prominently seen among the fast losers of urinary calcium.

The effects of the treatment on serum irOC and bone-specific alkaline phosphatase (B-AP) are shown in Figure 3.

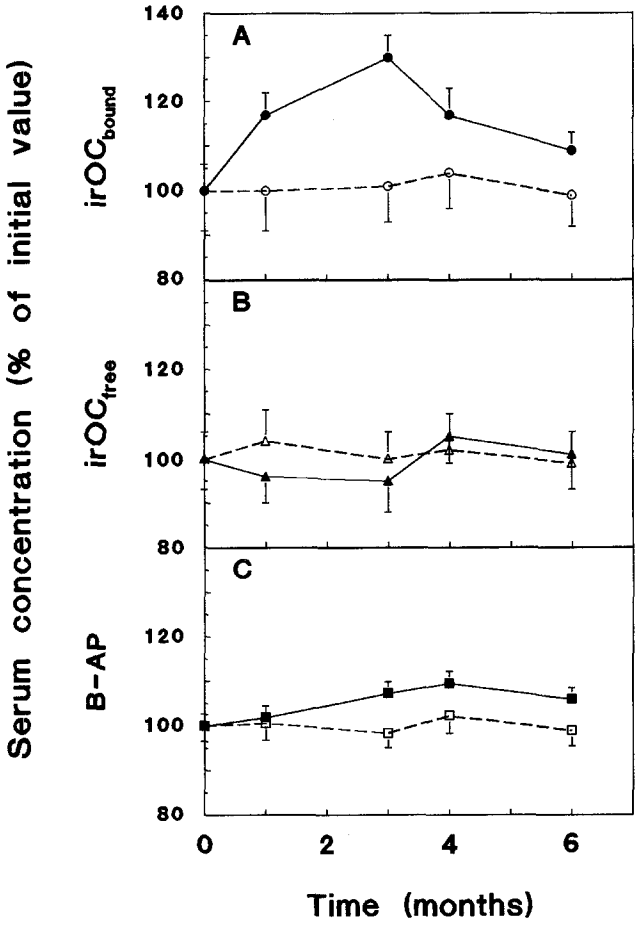

Fig. 3. Vitamin K–induced effects on various serum markers for bone metabolism. Markers tested were: irOC$_{bound}$ (**A**), irOC$_{free}$ (**B**), and B-AP (**C**). Closed symbols: vitamin K–treated groups; open symbols: placebo-treated groups. All data are expressed as a percentage of the starting values ± SE. Further details are as described in the legend to Fig. 2.

Also, in this trial the serum irOC$_{bound}$ significantly increased as a result of the vitamin K treatment ($P < 0.01$, Fig. 3A), whereas irOC$_{free}$ remained unaffected (Fig. 3B). A parallel increase of total alkaline phosphatase (data not shown) could be attributed completely to a rise in the B-AP levels ($P < 0.01$, Fig. 3C). These changes lasted for the entire treatment period and gradually reversed after the treatment was stopped. After 3 months of treatment the vitamin K–induced changes in irOC$_{bound}$ and B-AP were correlated; the r and P values were 0.541 and 0.002, respectively.

Subsequently we have tested whether serum irOC might correlate in some way to urinary calcium loss. For these calculations the pretreatment data of both the vitamin K and the placebo group were combined, and it turned out that the urinary calcium/creatinine ratio was positively correlated with irOC$_{free}$ ($r = 0.410$, $P = 0.0005$), as well as with the irOC$_{free}$/irOC$_{bound}$ ratio ($r = 0.398$, $P = 0.009$). No correlation was found between urinary calcium loss and either irOC$_{bound}$ or B-AP. After vitamin K treatment both the irOC$_{free}$/irOC$_{bound}$ as well as the calcium/creatinine ratio had shifted to lower values, and no correlation with calcium excretion was found at that time. On the basis of these data it is to be expected that irOC$_{free}$ in a population of fast losers of urinary calcium is higher than in a comparable group of normal losers. We have tested this assumption by comparing the data from all participants before the start of the second trial, and we found irOC$_{free}$ levels of 1.30 ± 0.07 and 1.78 ± 0.10 ng/ml for the normal- and the fast-losers group, respectively. This difference was significant at $P < 0.05$.

Discussion

In the literature there is no consensus about the relation between serum irOC and age. Whereas some authors report a continuous rise in irOC in women older than 40 [13, 14], others claimed this marker to be constant [15] or to decrease at increasing age [16]. In this paper we demonstrate that in adult women the serum level of total serum irOC gradually increases until the age of about 55 years, but it sharply decreases in the age groups older than 60. Further analysis showed that the curve for total irOC was composed of two apparently independent variables: irOC$_{free}$ and irOC$_{bound}$. Whereas irOC$_{free}$ exhibited an age-dependent increase which was most prominent between 50 and 60, the irOC$_{bound}$ remained nearly constant until age 60–65, and then sharply decreased at later ages. The ratio irOC$_{free}$/irOC$_{bound}$ remained low until the age of 45, but nearly doubled immediately afterward (Fig. 1C). Vitamin K administration only affected the serum levels of irOC$_{bound}$. The rate at which this effect took place (within 14 days) and the fact that the vitamin K–induced changes of irOC$_{bound}$ were strongly correlated to changes in the serum B-AP suggest that the circulating irOC$_{bound}$ originates from de novo synthesis by the osteoblasts. This has been proposed earlier for total serum irOC [17]. Moreover, the effect of vitamin K on B-AP suggests a direct effect of this vitamin on osteoblast activity. Similar effects of vitamin K have recently been reported for osteoblastic cells *in vitro*, which also responded to the vitamin with an increase of alkaline phosphatase production [18]. The physiological importance of these observations is unclear at this time.

It was striking that irOC$_{free}$ was insensitive for vitamin K. This poses serious questions to the earlier supposition that irOC$_{free}$ is a partly or noncarboxylated form of osteocalcin [10, 19]. An alternative explanation might be that irOC$_{free}$ has lost its Gla domain after limited proteolysis, either in serum or in bone. Preliminary experiments indicating a reduced mass for irOC$_{free}$ support the latter hypothesis. Moreover, the relative insensitivity of irOC$_{free}$ for vitamin K suggests that it does not originate from de novo synthesis, but from an already-existing pool. It seems probable, therefore, that at least part of the circulating irOC$_{free}$ is set free from bone tissue during osteoclastic resorption. This hypothesis implies that the proteolytic fragments are still detectable by radioimmunoassay, and the extent to which these fragments are recognized may be the major difference between the various commercial kits. More details about the nature and origin of both forms of osteocalcin may be obtained after two-site immunoassays based on well-defined monoclonal antibodies have become available.

It is well known that around the onset of menopause urinary calcium excretion and loss of bone mass may increase considerably [20]. It has been observed that around menopause irOC$_{free}$/irOC$_{bound}$ ratio increases, and that there is a correlation between irOC$_{free}$ and urinary calcium loss; both suggest that irOC$_{free}$ is somehow related to bone degradation. Here we want to put forward the hypothesis that rather than total irOC, the serum concentration of irOC$_{bound}$ is a marker for osteoblast activity, whereas serum irOC$_{free}$ levels reflect osteoclast activity. If so the ratio between irOC$_{free}$/irOC$_{bound}$ forms a marker for the metabolic state of

bone, and it may become an important diagnostic tool with which to identify subjects at risk for developing osteoporosis. Although in most women older than 50 years this ratio reacted strongly on vitamin K administration, only in fast losers of calcium did the vitamin have a significant effect on urinary calcium loss. This demonstrates that vitamin K status is not directly correlated with calcium excretion but that low nutritional intake or poor intestinal absorption of vitamin K may be a risk factor for increased urinary calcium loss.

As can be seen in Figures 2 and 3, the effect of vitamin K on $irOC_{bound}$, B-AP, and Ca/creatinine ratio was maximal after 3 months of treatment. It is also clear that for all three markers the effect was still detectable 3 months after the treatment had been stopped. Because the plasma half-life time of pharmacological doses of vitamin K is about 2 hours, we assume that at least part of the vitamin administered in our experiments had been stored in phospholipid membranes or adipose tissue and set free therefrom during the first few months following the treatment period.

Whether an insufficient vitamin K status may lead to an accelerated loss of bone mass cannot be concluded from our data. The first indications in favor of this suggestion may be found in the recent observations of Akiba et al., who showed that loss of bone mass could be retarded in hemodialysis patients with a low-turnover bone disease by vitamin K administration [21], and by papers from two independent groups showing that patients exposed to long-term treatment with vitamin K antagonists have a significantly lower bone mass than age- and sex-matched controls [22, 23]. As yet there are no indications for a regulation by Gla proteins of intestinal calcium absorption or glomerular filtration. A prospective vitamin K intervention study including yearly bone mass determinations (DEXA-scan) is in progress in our laboratory.

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