Effects of high vitamin K intake on bone and vascular health

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Your living is determined not so much by what life brings to you
as by the attitude you bring to life.
Not so much by what happens to you
as by the way your mind looks at what happens.

Circumstances and situations do color life
but you have been given the mind to choose what the color shall be.

John Homer Miller

Aan mijn ouders

Voor Cyril

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1 Introduction

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1.1 GENERAL INTRODUCTION

Discovery of vitamin K

Vitamin K was discovered in 1935 as a necessary factor for normal haemostasis [1]. Hendrik Dam described a bleeding disorder in chickens fed a cholesterol-depleted diet. The bleeding symptoms of these chickens were caused by the deficiency of a fat-soluble factor, which appeared to be essential for the maintenance of proper blood coagulation. After feeding the chickens with green plants such as clover and lettuce, the haemorrhages and spontaneous bleeding were stopped. A few years later, in 1939, two forms of vitamin K were isolated which were called vitamin K_1 (phylloquinone) and vitamin K_2 (menaquinone), respectively [2,3]. In the same years, a natural occurring antagonist of the vitamin was discovered. The vitamin K antagonist was isolated and characterized as 3,3'-methylene-bis-(4-hydroxycoumarin) and called dicumarol [4,5]. In the mid 1950's four clotting factors were discovered and it was shown that they require vitamin K for their biological activity. Later on, in 1974, eventually the biochemical function of vitamin K and coumarin antagonists was elucidated [6]. In the 50 years following its discovery, vitamin K was thought to be needed exclusively for the synthesis of blood clotting factors in the liver. In the last two decades the discovery of a diverse group of vitamin K-dependent proteins not related with blood coagulation has initiated renewed interest in vitamin K, notably the proteins involved in bone metabolism [7] and in vascular calcification [8] (Table 1).

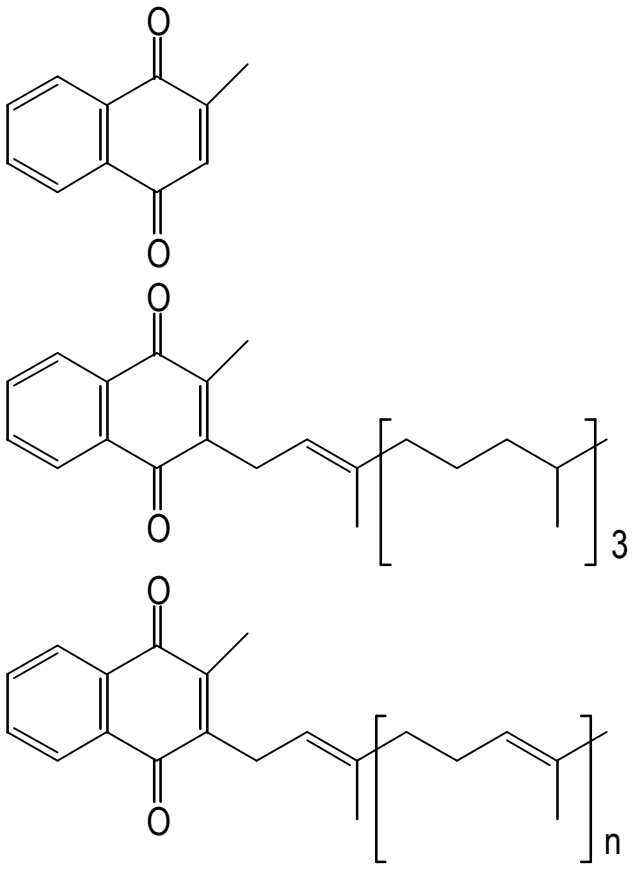
Table 1
Tissue distribution and roles of vitamin K-dependent (Gla) proteins.

Gla Protein	Tissue	Role
Prothrombin (FII), Factors VII, IX, and X	Liver (then plasma)	Procoagulants
Protein C, Z	Liver (then plasma)	Anticoagulants
Protein S	Liver (then plasma)	Anticoagulant role as cofactor for protein C
Osteocalcin (bone Gla-protein)	Bone, dentin	Regulator of orderly crystallization
Matrix Gla Protein (MGP)	Bone, cartilage, and most soft tissues	Inhibitor of ectopic mineralization
Gas-6 (growth arrest specific gene-6 protein)	Most soft tissues	Regulator of cell growth
Proline rich Gla-protein (PRGP)	Most soft tissues	Unknown
Transmembrane Gla-proteins (TMG)	Most soft tissues	Unknown

Structure and function of vitamin K

Vitamin K is a group name for several molecular forms that contain a common 2-methyl-1,4 naphthoquinone ring but which differ in the structures of the aliphatic side chains attached at the 3-position (Figure 1). Phylloquinone (vitamin K_1) is a single compound containing one

unsaturated and three saturated isoprenoid residues, and it is exclusively synthesized in green plants. Menaquinones (vitamin K_2) comprise a spectrum of related forms generally designated as menaquinone-*n* (MK-*n*), where *n* stands for the number of isoprenyl groups which are all unsaturated. A number of bacteria synthesize the higher menaquinones with MK-7 through MK-10 as the most common forms. MK-4 is mainly found in animal products (meat, dairy) and originates from conversion of both K_1 and K_3 (menadione). Vitamin K_3 is the synthetic water-soluble form of vitamin K, which is often added to animal food. K_1 and MK-4 are the two forms that are commercially available as synthetic products. It is generally accepted that the methylated naphtoquinone is the functional group, so the mechanism of action is similar for all K vitamins. Substantial differences may be expected, however, in intestinal absorption, transport, tissue distribution, and bioavailability. These differences are caused by the the various side chains and by the different food matrices in which they occur [9-13].



Menadione; Vitamin K_3

Phylloquinone; Vitamin K_1

Menaquinone; Vitamin K_2

Figure 1
Chemical structures of menadione (vitamin K_3), phylloquinone (vitamin K_1), and menaquinone (vitamin K_2). The (n) stands for number of isoprene residues.

The only known function of vitamin K in mammals is that it serves as a cofactor for the endoplasmic enzyme gammaglutamyl carboxylase, where it promotes the posttranslational conversion of selective protein-bound glutamate residues into gammacarboxy glutamate (Gla) [14-16]. The form of vitamin K required as the cofactor is not the stable quinone structure found in the diet, but the reduced quinol (or hydroquinone) structure (KH_2). The subsequent

oxidation of vitamin KH_2 to vitamin K 2,3-epoxide provides the energy to drive the carboxylation reaction yielding the final Gla product. The resulting Gla-residues are found in a limited number of proteins and they confer calcium-binding properties. During vitamin K deficiency, the carboxylation reaction cannot proceed, so the Gla proteins are released in an undercarboxylated form. In all cases in which the function of the Gla protein is known, Gla is essential to the biological activity of these proteins. An important point to mention with respect to the vitamin K cycle is that vitamin K-epoxide (KO) can be reconverted into the active KH2 by the action of the enzyme KO-reductase. Via this pathway each molecule of vitamin K may be recycled several thousand fold (see Figure 2), which explains its very low daily requirement as compared to other vitamins and cofactors. Well-known vitamin K-antagonists such as warfarin all block the KO-reductase, thus inducing an artificial vitamin K deficiency in all tissues.

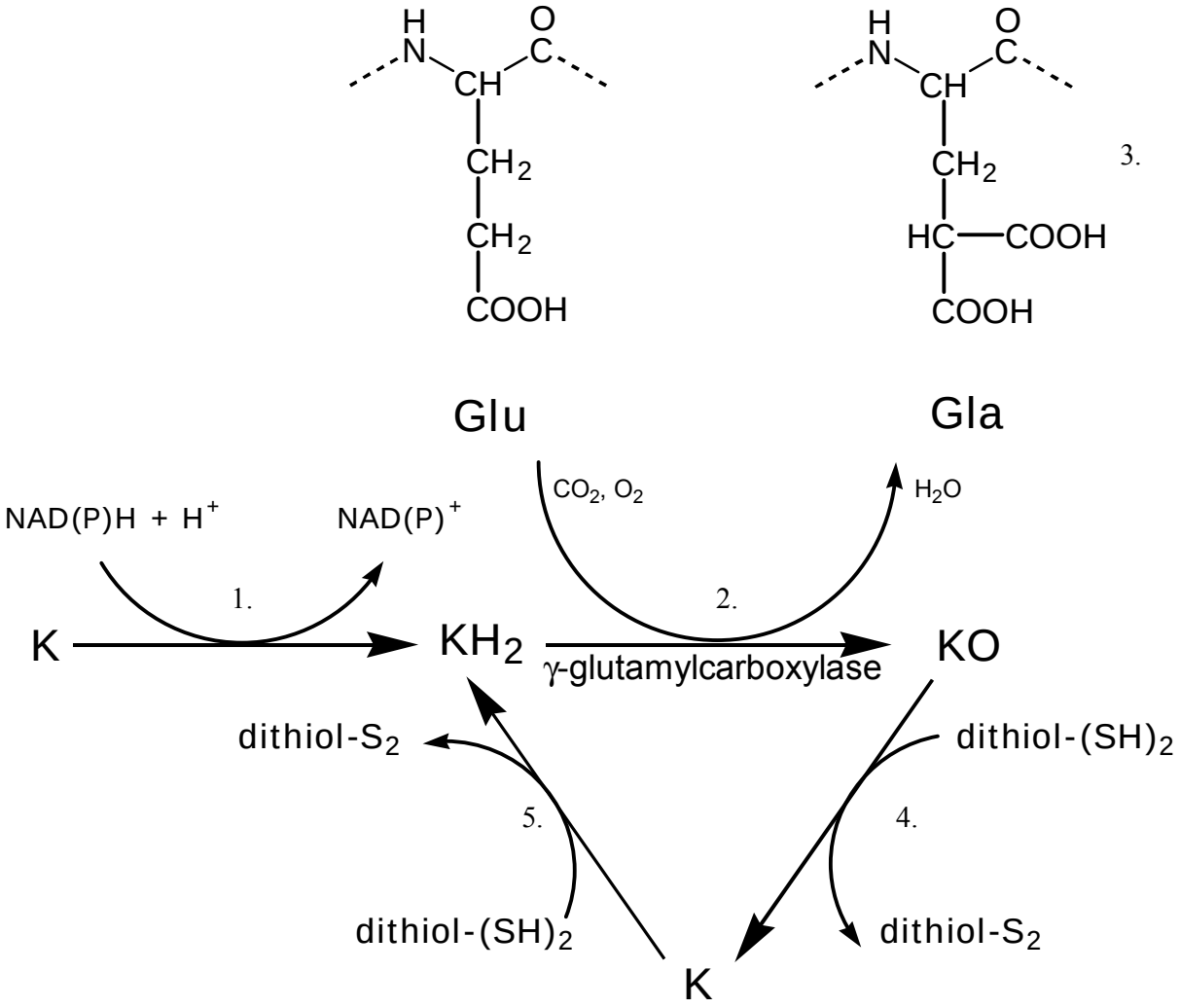


Figure 2

The vitamin K-cycle. Vitamin K-quinone (K) is first reduced to vitamin K-hydroquinone (KH_2), and then oxidized to vitamin K-epoxide (KO). During the oxidation step glutamic acid (Glu) is reduced to γ-carboxyglutamic acid (Gla). Finally KO is reduced to K. 1. = NAD (H)-dependent reductase, 2. = γ-glutamylcarboxylase enzyme, 3. extra introduced carboxy group, 4. = dithiol-dependent KO-reductase, 5. = dithiol-dependent K-reductase.

Occurrence and nutritional intake of K-vitamins

By far the most abundant form of dietary vitamin K is phylloquinone (vitamin K_1), which occurs in green vegetables such as kale, spinach, broccoli, and Brussels sprouts [17-19]. Except some plant oils [20], other foods have far lower phylloquinone contents. Menaquinones have a more restricted distribution in the diet than phylloquinone, with nutritionally significant amounts of MK-4 only occurring in animal meat and liver, and higher menaquinones (MK-6 through MK-9) in liver and some fermented products, such as cheese and curd cheese [19]. Only in Japan the MK-7 intake is high because of the abundance of this vitamer in the typical Japanese food product natto, which consists of fermented soybeans. Also the intestinal flora in the colon produces menaquinones, but the colonic absorption of these highly lipophilic compounds is probably very poor, and will not form a major contribution to the human vitamin K status. Several databases on vitamin K-content of food have been published at this time [17,18,21], but most of them focus on K_1. Recently the first detailed table for menaquinone content of various foods has been published [19]. In Table 2 part of the data obtained in our own group have been summarized. Using this table the vitamin K intake by the Dutch elderly population (> 55 years old) has been calculated which ranged between 124 μg/day (lowest quartile of intake) and 375 μg/day (highest quartile) for K_1 and between 10 and 45 μg/day for K_2 [21]. Whereas for K_2 intake only one population-based survey has been published, the obtained data for K_1 intake are comparable with those reported for the Framingham cohort (ranging from 59 to 262 μg/day) [22]. Other data have shown that K_1 intake of adolescents and young adults is substantially lower than that of adults, and may be below the RDA value in the majority of this age group [23]. When using these data it is important to realize that the efficacies of intestinal absorption of K_1 and K_2 are widely different. Whereas the poor extraction of K_1 from green leafy vegetables results in the absorption of 5-15% of the total vitamin K ingested [19,24], K_2 vitamins are taken up almost completely from their natural food matrix. It must be concluded, therefore, that although K_1 comprises about 90% of our total vitamin K intake, both K_1 and K_2 may contribute equally to the human vitamin K status.

Absorption and transport

In the small intestine vitamin K is extracted from the food and incorporated into micelles consisting of bile salts, free fatty acids and monoglycerides. These micelles are subsequently taken up by the intestinal mucosa, from which the vitamin – bound to chylomicrons – is set free in the lymphatic system and the circulation [25-27]. The intestinal absorption of dietary K-vitamins requires concomitant fat intake and normal bile secretion, which facilitate the solubilization of vitamin K into the mixed micelles. This explains the positive correlation between phylloquinone concentration in plasma and plasma triglycerides [28,29] and also why a striking impairment of vitamin K absorption is observed in patients with biliary obstruction (obstructive jaundice) and fat malabsorption problems [26]. In these patients intravenous or intramuscular injection of K_1 is indicated.

Chylomicrons are transported to the liver where they are taken up as chylomicron remnant particles. The genetically determined subtypes of apolipoprotein E within the chylomicrons, play a crucial role in the transport of chylomicrons and thus of vitamin K to the liver and other

target tissues [29,30]. The hepatic clearance is highest in the E4 subtype, followed by E3 and E2 [31]. A high chylomicron clearance corresponds with low plasma vitamin K levels [29,32].

Table 2
Vitamin K content of various foods (µg/100g).

Nutrient	K1	K2
Meat	0.5 - 3	1 - 8
Fish	0.1 - 1	0.1 - 1.6
Milk	0.5 - 1	0.2 - 2
Yoghurt	0.2 - 0.5	0.1 - 1
Cheese (all types)	2.5 - 15	0.5 - 80
Butter	9 - 20	10 - 20
Margarines (from plant oil)	80 - 110	0
Green vegetables	100 - 750	0
Natto (fermented soybeans)	20 - 30	800 - 1,000
Fruit	0.1 - 3	0
Bread	0.5 - 1	0

Tissue requirement

Whereas at low vitamin K intake most of the absorbed vitamin is transported to the liver, many different types of tissue accumulate K-vitamins at higher levels of intake [33]. Both in steady-state animal nutrition studies [33] as well as in single-dose experiments [34] it appeared that the ratio in which K_1 and K_2 accumulate is tissue-dependent, with relative high K_2 accumulation in the pancreas, the testis and the arterial vessel wall. In these studies it was also observed that in the latter three tissues K_2 was high even after long-term intake of exclusively K_1. The hypothesis that K_1 is converted into MK-4 by the intestinal flora appeared to be incorrect: also in germ-free rats high concentrations of MK-4 were found upon nutrition with K_1 [35,36], and it was concluded that tissues which accumulate high amounts of MK-4 have the remarkable capacity to convert up to 90% of the available K_1 into MK-4 in case MK-4 is lacking in their diet. Tissues characterized by preferential accumulation of K_1 (liver, heart) do not show the ability to convert K_1 into MK-4. Although the reason of the K_1-MK-4 conversion is presently unknown, it seems that in these tissues either MK-4 is preferentially used by the gammaglutamate carboxylase system, or MK-4 has a second, yet undiscovered function.

Present RDA values for vitamin K (1.5 µg/day/kg body weight) are solely based on the hepatic requirement for clotting factor synthesis. Various studies have demonstrated,

however, that the liver is extremely efficient in taking up vitamin K from the blood stream. Notably at low vitamin K intakes the liver was shown to be the principal target tissue for vitamin K, whereas at higher intakes other tissues (heart, pancreas, kidney, lung, aorta) accumulated vitamin K as well [33]. It is likely, therefore, that intakes well above the RDA value are required for adequate carboxylation of extrahepatic Gla-proteins because this process requires higher vitamin K intakes than that required for normal haemostasis. Obviously, the fact that in the recent past carboxylase was detected in many extrahepatic tissues [37] implies that our vitamin K intake may be adequate for covering the needs of the liver, but to a varying degree insufficient for other tissues. This phenomenon, described as the concept of tissue-dependent vitamin K-requirement, defines vitamin K-sufficiency as a state in which a certain tissue produces all its Gla-proteins in a fully carboxylated form. The required dietary vitamin K intake may depend on tissue-specific properties such as the blood flow and the apoE receptor density on the target cells. As a logical consequence, different RDA-values have to be defined for the maintenance of an optimal vitamin K-status in liver, bone, vessel wall, and other tissues. Another important point to realize is the large variety of K-vitamins present in our food. As was discussed above, K_1 is preferentially accumulated in the liver, whereas K_2 vitamins seem to be of more importance in extrahepatic tissues. One of the mechanisms underlying this phenomenon is probably the distribution of K-vitamins over the lipoprotein fractions: after oral ingestion most of K_1 is associated with the triglyceride fraction, whereas substantial amounts of K_2 are recovered in LDL and HDL [38]. Also, the hepatic clearance of higher menaquinones is much slower than that of K_1, so that they remain available much longer for extrahepatic uptake. This was observed, for instance for MK-9 from cheese and curd cheese, and for MK-7 from natto [38].

1.2 VITAMIN K AND BONE METABOLISM

Vitamin K-dependent proteins regulate cartilage calcification and new bone formation

The first indications for involvement of vitamin K-dependent proteins in bone metabolism were obtained in the mid-1970s, when serious bone malformations were observed in babies from women who had been treated with vitamin K-antagonists (oral anticoagulants) during the first trimester of pregnancy [39]. The defect is known as chondrodysplasia punctata or fetal warfarin syndrome, and is characterized by hypoplasia of the nasal bridge and distal phalanges and by punctate calcifications in the growth plates and rapidly growing bone, which appear as stippling on X-ray photographs. The excessive calcifications lead to irregular local termination of further bone development, which is the cause of the bone malformations. There are two Gla-proteins associated with bone formation: osteocalcin and matrix Gla-protein (MGP) [40]. Osteocalcin is a small protein (49 amino acid residues) containing 3 Gla-residues, and is exclusively synthesized by osteoblasts and odontoblasts in bone and dentin [41]. Osteocalcin is one of the most abundant proteins in the human body, but it is concentrated almost exclusively in the bone tissue. A small part of the *de novo* synthesized osteocalcin, however, is set free in the blood stream where it is available for

testing [42]. Circulating osteocalcin antigen is a well known marker for osteoblast activity and bone formation, but in the majority of the population it consists of both fully carboxylated as well as undercarboxylated species. In contrast to osteocalcin, MGP is synthesized in a wide variety of tissues; MGP mRNA has been found in various cells including chondrocytes [43], vascular smooth muscle cells [44] and epithelium [45]. MGP is a 10 kD protein containing 5 Gla-residues which is synthesized at low levels in most soft tissues. The function of both bone Gla-proteins became clear from transgenic mice in which either osteocalcin or MGP had been knocked out. Osteocalcin appeared to be a negative regulator of bone formation [46,47], whereas MGP is a strong inhibitor of soft tissue calcification [48]. Since bone is the only tissue to be calcified physiologically, one might ask whether mineralization is an active function of bone, or whether inhibition of mineralization is a function of all soft tissues [47]. The fact that MGP and other inhibitors of calcification such as fetuin [49] are constitutively expressed in a wide range of soft tissues is consistent with the view that prevention of calcification is an active function of soft tissues.

Comparable symptoms as seen in the human fetal warfarin syndrome could be induced in young experimental animals such as rats [50,51] and lambs [52]. The principle of the protocol was first described by Price et al. [50], and is based on the complete blockade of all KO-reductase by feeding the animals with excessive amounts of warfarin. To prevent their bleeding to death, the animals receive a balanced daily dose of vitamin K, which is used as an antidote for warfarin by the liver but not by extra hepatic tissues. In this way the clotting system remains operational, whereas severe vitamin K-deficiency is maintained in other tissues such as bone and vessel wall. As a result excessive tissue calcification is observed, notably of the epiphyses and other cartilage and arteries. These and other data have demonstrated that MGP is a strong inhibitor of calcification, chondrocyte mineralization, and ossification [43]. Since both vitamin K-dependent proteins identified in bone have a regulatory function in bone development, the question remains whether in humans long term low vitamin K intake or the use of vitamin K-antagonists may lead to impaired bone quality.

Effects of low vitamin K-status in humans

The first results providing evidence for the hypothesis that vitamin K is associated with bone metabolism came in 1984. It was shown that 16 osteoporotic patients with femur neck fractures had circulating vitamin K_1 levels which were less than 30% of those in 34 controls [53]. In subsequent studies, it was confirmed that in osteoporotic hip fractures and in spinal crush fractures the circulating vitamin K concentrations were less than 25% of those in matched controls [54,55]. It remained to be proven, however, that the low circulating vitamin K concentrations were indicative of poor vitamin K-status in the bone tissue. It was shown that high levels of undercarboxylated osteocalcin were detectable in serum from postmenopausal women, and that osteocalcin carboxylation quickly improved upon vitamin K administration [56]. The data, which strongly suggest that a mild vitamin K-deficiency is common in elderly women, were confirmed by others, and it turned out that circulating undercarboxylated osteocalcin is inversely correlated with bone mass [57,58] and that it is a strong risk factor for hip fracture [59,60]. Osteocalcin was isolated from the femoral heads removed during hip

replacement surgery in elderly subjects suffering from osteoporotic fractures or degenerative joint disease, and it turned out that there is a widespread under-carboxylation of osteocalcin in humans, a defect that may reflect inadequate levels of vitamin K in bone cells [61]. In a first population-based prospective study among over 72,000 elderly women it was demonstrated that subjects with nutritional vitamin K intake below 109 µg/day had a significantly higher age-adjusted relative risk of hip fracture than those with a higher intake [62]. A comparable study was carried out among both men and women of the Framingham Heart Study, and again an association was found between low vitamin K intakes and hip fracture risk [22]. Since the mean vitamin K intake in the American population is about 150 µg/day [63] we must conclude that the vitamin K status in a substantial fraction of the population is sub-optimal. Furthermore, in a recent meta-analysis Caraballo *et al.* concluded that regular intake of vitamin K-antagonists was associated with low bone mass [64]. In Japan postmenopausal osteoporosis is successfully treated with very high doses of MK-4 [65,66]. An answer to the question of what levels of vitamin K intake and which form of vitamin K is required for optimal bone maintenance will come from a number of clinical trials from our group and others, two of which are presented in this thesis.

1.3 VITAMIN K AND ARTERIAL CALCIFICATION

Vitamin K-dependent proteins regulate arterial calcification

Although the MGP null mutants were developed to study the effect of MGP-deficiency on bone metabolism, the most important and unexpected information from this transgenic model was the rapid calcification of a number of soft tissues including the large arteries, bronchi, and trachea. The profound impact of MGP-deficiency appeared from the fact that null mutants were all healthy at birth, but developed massive calcifications of the large vessels within the first weeks of life leading to death by rupture of the thoracic and abdominal aorta within 8 weeks after birth [48,67]. Re-evaluation of the model based on coumarin / vitamin K treatment showed similar calcifications, typically starting at the elastic lamellae of the arterial media, with subsequent proliferation extension of calcifications at longer periods of treatment [68,69]. In humans, vascular calcification may occur in two distinct forms. Calcification of the tunica media (also known as Mönckeberg's sclerosis) often occurs in the absence of overt vascular disease and is frequently seen in patients with diabetes mellitus and in those with dialysis-dependent renal failure. Intima calcification, on the other hand, is widely associated with atherosclerosis. For many years it has been thought that vascular calcification is a passive process in which high local calcium concentrations were the result of dying cells within necrotic vascular lesions. Recent studies have shown, however, that the calcifying vessel wall shares many similarities with bone [8,70]. On one hand vascular smooth muscle cells seem to be able to differentiate to cells with osteogenetic / osteoblastic protein expression type [71], on the other hand many proteins generally regarded as characteristic for bone and cartilage (osteocalcin, MGP, osteopontin, osteonectin, bone morphogenetic proteins, bone sialoprotein) have been identified in the calcifying vessel wall [72,73]. It seems

therefore, that calcification of the vessel wall is a process regulated by similar proteins and processes as those known from bone metabolism. Among the proteins identified, the two vitamin K-dependent ones (osteocalcin and MGP) are known as potent mineralization inhibitors. Hence local vitamin K-deficiency in the vessel wall might lead to undercarboxylated (i.e. non-functional) forms of osteocalcin and MGP and thus to increased calcium deposition. In a population-based study Jie and colleagues observed an inverse correlation between dietary intake of vitamin K_1 and mineralization of the abdominal aorta [74]. Recently, a much stronger inverse correlation was found between vitamin K_2 intake and artery calcification, with a 50% risk reduction for myocardial infarction and total cardiac death in the highest tertile of K_2 intake [75]. These data are consistent with a recent publication from Kawashima et al., who used hypercholesterolemic rabbits to demonstrate that high menaquinone intake suppresses the progression of atherosclerotic plaques, intimal thickening and pulmonary atherosclerosis, and that it significantly suppressed circulating cholesterol levels, cholesterol-ester deposition in the aorta, and plasma lipid peroxidation [76]. *In situ* hybridisation studies demonstrated that the vitamin K-dependent MGP is particularly expressed around atherosclerotic lesions, and in vascular smooth muscle cells located in the tunica media [8]. From subsequent immunohistochemical data with antibodies directed against human MGP it appeared that MGP accumulates in vascular areas prone to calcification such as the tissue surrounding the growing plaque and the elastin fibres in the tunica media [77]. The current hypothesis is that this strong increase of MGP expression is a defence mechanism triggered by the increased local calcium concentration, but that precipitation of calcium salts is not inhibited because the vascular vitamin K status is insufficient to support the carboxylation of all MGP. Unless vitamin K-containing supplements are taken, most of the MGP will be synthesized in an undercarboxylated (i.e. inactive) form. Hence patients and subjects at risk for cardiovascular disease are not optimally protected against arterial calcification because of a dietary inadequacy of vitamin K.

Recently, it was shown that high local calcium concentrations or precipitated calcium salts strongly induce MGP synthesis, probably in an attempt to minimize calcification and tissue damage [78]. However, since the local vitamin K supply is insufficient, merely undercarboxylated and hence inactive MGP is produced. An answer to the question whether vitamin K supplements may contribute to optimal vessel wall condition comes from a clinical trial from our group, which is described in this thesis.

1.4 THE OBJECTIVE OF THIS THESIS

The main objective of the work presented in this thesis was to study the effects of high vitamin K intake on bone and vascular health. In part I, two intervention studies on the effects of vitamin K_1 supplementation on bone mineral density are described. Although many observational studies have shown an association between vitamin K-status and bone mineral density, no randomised intervention study has been published until now. Postmenopausal women form a high risk group for bone loss, due to the reduction in oestrogen production right at the onset of the menopause. The same is true for female athletes who often suffer from amenorrhoea due to extreme training intensity. In both high risk groups for bone loss, the effects of vitamin K supplementation were studied. In 181 postmenopausal women the effect of 1 mg vitamin K_1 per day, co-administered with minerals and vitamin D, was investigated on bone mineral density during a follow-up period of three years (chapter 2). In 115 female athletes with high training intensity, the rate of bone loss was quantified as a function of menstrual status and the potential effects of vitamin K_1 (10 mg/per day) supplementation was investigated during a follow-up period of two years (chapter 3).
In part II of this thesis, investigations are described on different aspects of the association between vitamin K and vascular health. To investigate the association between vitamin K and intermediary markers of cardiovascular disease, the data of the well-known epidemiological study, the Framingham offspring cohort, were analysed (chapter 4). In a follow-up study the process of ageing (chapter 5) and the effects of vitamins D + K on vessel wall characteristics were investigated (chapter 6). The results of these studies contribute to a better understanding in the mechanisms underlying the association between vitamin K and vascular biology. Finally, in chapter 7, an assay for measuring the vitamin K-dependent protein matrix-gla protein (MGP) in serum is described. With this assay it is possible to study the potential utility of MGP as a serum marker for cardiovascular disease.

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2 Vitamin K_1 supplementation retards bone loss in postmenopausal women between 50 and 60 years of age

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ABSTRACT

Although several observational studies have demonstrated an association between vitamin K status and bone mineral density (BMD) in postmenopausal women, no placebo-controlled intervention trials on the effect of vitamin K_1 supplementation on bone loss have been reported thus far. In the trial presented here we have investigated the potential complementary effect of vitamin K_1 and a mineral + vitamin D supplement on postmenopausal bone loss. In total 181 healthy postmenopausal women between 50 and 60 years old were recruited for this randomised double-blind placebo-controlled intervention study; 155 of whom completed the study. During the 3-year treatment period participants received a daily supplement containing either placebo, or calcium, magnesium, zinc and vitamin D, or the same formulation with additional vitamin K_1. The main outcome measure was the change in BMD of the femoral neck and lumbar spine after three years, as measured by DEXA. The group receiving the supplement containing additional vitamin K_1 showed reduced bone loss of the femoral neck; the differences in decrease of BMD (relative to baseline) with both the placebo (1.7%) (95% CI: 0.35 to 3.44) and the mineral + D group (1.3%) (95% CI: 0.10 to 3.41) were statistically significant. No significant differences were observed between the three groups with respect to change of BMD at the site of the lumbar spine. If co-administered with minerals and vitamin D, vitamin K_1 may substantially contribute to reducing postmenopausal bone loss at the site of the femoral neck.

INTRODUCTION

The role of calcium and vitamin D in the maintenance of skeletal health has long been recognized. Supplements containing vitamin D and calcium were reported to be effective in the prevention of hip fractures in women with a low dietary vitamin D and calcium intake [1,2]. However, vitamin D supplements did not decrease hip fracture rate in women with normal calcium intakes [3].

Accumulating evidence suggests that poor vitamin K status may be an independent risk factor for postmenopausal bone loss. The two most important forms of vitamin K are vitamin K_1 (phylloquinone) and the group of K_2 vitamins (menaquinones). Green vegetables such as broccoli and spinach, as well as some plant oils are the major sources of phylloquinone in the human diet [4-7]. Menaquinones are primarily found in meat (menaquinone-4) and fermented foods like cheese and curds (menaquinone-6-9) [7]. The mean dietary vitamin K intake by the Dutch elderly population (> 55 years) ranges between 124 µg/day (lowest quartile of intake) and 375 µg/day (highest quartile) for phylloquinone and between 10 and 45 µg/day for the combined menaquinones. Vitamin K functions as a cofactor in the posttranslational carboxylation of several bone proteins, the most abundant one of which is osteocalcin. Vitamin K-deficiency of bone tissue results in the synthesis of undercarboxylated osteocalcin (ucOC), and the concentration of circulating ucOC is generally accepted as a sensitive marker for vitamin K status [8-10]. Several authors have demonstrated that low serum concentrations of either vitamin K_1 [11,12] or ucOC [13-15] are associated with low bone mineral density (BMD) and an increased risk for osteoporotic hip fractures. Also, a number of clinical studies have demonstrated that the use of vitamin K antagonists (oral anticoagulants) is associated with low BMD and increased hip fracture risk [16-18]. Such association was not found in all studies, however [19-21]. The first intervention study with vitamin K showed that supplementation with 1 mg vitamin K_1 per day for 2 weeks induced a decrease of urine markers for bone resorption and an increase of serum markers for bone formation [10]. So far, there has been no intervention trial on the effects of vitamin K_1 on BMD. A number of Japanese studies, however, have shown significant reduction of osteoporotic bone loss after supplementing patients with high doses (45 mg/day) of vitamin K_2 (menatetrenone) [22].

In the present study, we have investigated the effect of minerals + vitamin D supplementation on postmenopausal bone loss, and the potential additive effect of vitamin K_1 on this treatment. It is the first intervention trial in which the potential prevention of bone loss by vitamin K_1 supplementation has been monitored.

METHODS

Protocol

Participants were recruited by local newspaper calls. Inclusion criteria were: apparently healthy women, Caucasian race, between 50 and 60 years old and at least two years postmenopausal. Exclusion criteria were: bone fractures during the preceding year, metabolic bone disease, use or recent use (≤ one year) of oral anticoagulants, hormone replacement therapy, bisphosphonates, vitamin concentrates or food supplements, drugs known to interfere with calcium metabolism (such as corticosteroids), and alcohol consumption of > 2 glasses/day. In total 188 women met the criteria for participation, and were randomized into our study. The enrollment of participants took place between November 1997 and March 1998. Follow-up measurements were performed until March 2001. The primary outcome measures of the study were the BMD of the femoral neck and lumbar spine both measured with a dual-energy x-ray absorptiometer (DEXA; Lunar, Madison, WI). Scans of the lumbar spine (L2-L4) and femoral neck were performed at baseline and after one, two and three years of treatment. To measure the effect of seasonal influences on BMD we performed also a measurement after 1.5 years; these data are beyond the scope of this paper and will be published elsewhere. Measurements were performed by the same two experienced in-hospital technicians throughout the whole study-period. The mean coefficient of variation of repeated measurements was 1.4% for the femoral neck and 1.0% for the lumbar spine. Together with BMD, height and weight were measured each year with standardized equipment. Fasting blood and 2-h morning urine samples were collected at t = 0, 3, 12, and 36 months to measure biochemical markers for bone metabolism. Participants were asked to refrain from taking the supplement on the day preceding blood and urine sampling. Markers tested in serum were: undercarboxylated osteocalcin (ucOC, Takara, Japan) and total osteocalcin (Cis Bio International, France) for compliance and bone vitamin K status, 25OH-vitamin D_3 (Incstar, USA) for vitamin D status, and bone alkaline phosphatase (Hybritech, Belgium) for bone formation. In urine we measured deoxypyridinoline (Metra Biosystems, USA) for bone resorption, as well as calcium, measured by an atomic absorption spectrophotometer. For both urinary markers, a correction was made for creatinine excretion. Between measurement points the same investigator visited the participants at home to check their compliance at regular intervals. All participants gave written informed consent and the trial was approved by the University Hospital medical ethics committee.

Assignment

Participants were randomized into three groups, the first one (n=60) receiving a placebo (maltodextrine), the second one (n=58) receiving a supplement containing 500 mg calcium (natural calcium complex derived from milk), 10 mg zinc, 150 mg magnesium and 8 μg vitamin D_3 (minerals + vitamin D = MD-group), whereas subjects in the third group (n=63) received a supplement containing the same constituents as the MD-group but with additional

1 mg vitamin K_1 (minerals + vitamins D+K = MDK-group). The participants were allowed to choose between a supplement in the form of either a tasteless powder (to be mixed with water before intake) or chocolate-coated tablets with a crunchy malt core. The percentage of subjects using the powder or tablets was equally distributed across the three groups. Participants were instructed to take one sachet with powder or three tablets per day during evening hours, preferably after the meal. Also, they were advised to maintain their usual diets and to avoid taking supplements containing either calcium, vitamin D, or vitamin K throughout the study. After randomization, the women received their first batch of supplements and were supplied with subsequent portions every six months. Novartis Consumer Health SA (Nyon, Switzerland) prepared and provided all supplements.

Masking

Randomization was performed according to a computer-generated randomization list and the randomization codes were kept apart from the study site during the trial. The participant randomization codes were allocated sequentially in the order in which the participants were enrolled. One investigator who supervised the whole study was responsible for the enrollment and assignment of participants to the respective groups. Because the three different types of supplements were similar in appearance and taste, neither participants nor investigators were aware of group assignment.

Statistical Analysis

The sample size was calculated on the assumption that the desired minimal detectable effect was a 15% decrease of bone loss in one of the treatment groups compared to placebo with a 90% power and a 0.05 level of significance. With the assumption of a dropout rate of 10% per year we calculated that 180 subjects had to be included. Statistical analysis was performed using the Statistical Package SPSS (SPSS Corp, Chicago, IL). Only the results on women who had more than one measurement were included in the analysis. Results are presented as means $\pm$ SD, unless indicated otherwise. For each subject separately, all follow-up measurements were related to the corresponding baseline-value by expressing changes as % from baseline. Values of bone loss for each participant were adjusted for the exact time she had been in the trial. At all time-points changes in BMD from baseline were compared between treatment groups and placebo and between both treatment groups using linear regression analysis. In these analyses, the change in BMD from baseline was used as the dependent variable and the initial BMD value, the treatment groups, and other covariates were used as explanatory variables. Age, BMI, and years since menopause were chosen as covariates, because their influence on the rate of bone loss or response to supplementation could not be excluded. Data were analysed according to the intention-to-treat principle.

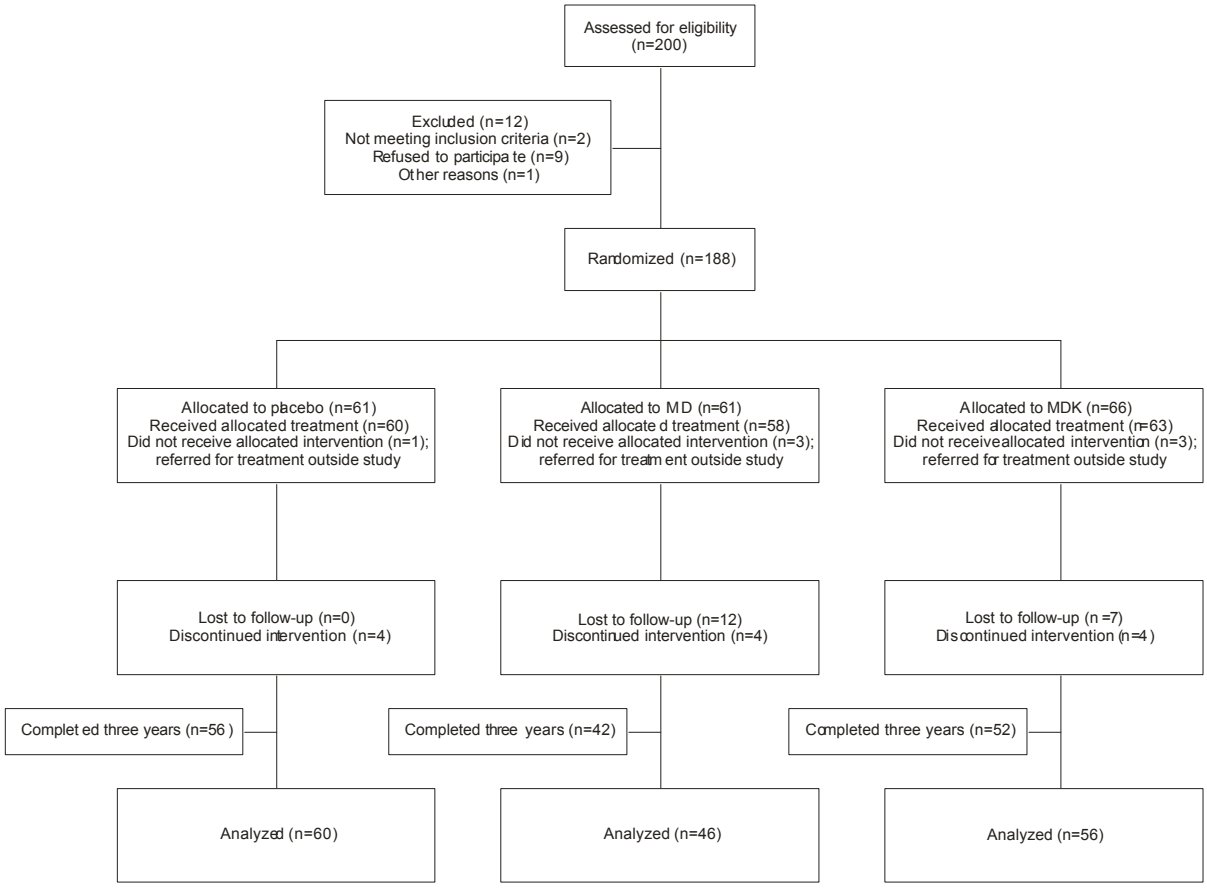

Figure 1
Flow diagram of participants through the different stages of the study.

RESULTS

Participant flow and follow-up

Figure 1 shows the flow of participants through each stage for the separate groups. After the first bone densitometry, seven subjects (1 in the placebo group, 3 in the MD-group, 3 in the MDK-group) were removed from the study and referred for treatment because their BMD was > 2.5 SD below the reference population. From the remaining 181 subjects who entered the study, 31 subjects discontinued their participation, reasons given for discontinuation were: personal reasons (n=13: 2 in the placebo group, 8 in the MD-group, 3 in the MDK-group), illness (n=6: 2 in the MD-group, 4 in the MDK-group), glucocorticosteroid (1 in the placebo group) or estrogen therapy (n=3: 1 in the placebo group, 2 in the MD-group) or multivitamin supplements (1 in the MDK-group), whereas seven participants withdrew because of complaints of mild constipation (4 in the MD-group, 3 in the MDK-group). No other adverse events were reported during the study. The majority of the subjects who discontinued treatment did so during the first year (n=19), so no follow-up data could be collected on these subjects. Drop-out rates in both treatment groups were higher than in the placebo. Given the specific reasons for drop-out as mentioned in the results it is unlikely that the treatment

assignment is responsible for the higher drop-out rate. The subjects who discontinued treatment during the second and third year (n=12) were included in the analysis. There were no significant differences between the three groups in baseline characteristics and the initial mean values for BMD at both sites measured were similar in the three groups (Table 1). Omission of the drop-outs from the analyses did not change these baseline data.

Table 1
Baseline characteristics (mean ± standard deviation) in the three treatment groups.

Baseline-characteristics	Placebo (n=60)	Mineral + vitamin D (n=46)	Mineral + vitamin D+K (n=56)
Age (yr)	54.6 ± 2.8	55.7 ± 2.9	55.3 ± 2.8
Weight (kg)	69.5 ± 11.4	69.4 ± 10.3	67.4 ± 11.4
Height (m)	1.63 ± 0.05	1.65 ± 0.07	1.64 ± 0.06
BMI (kg/m^2)	26.1 ± 4.3	25.5 ± 3.9	25.1 ± 25.1
Postmenopausal age (yr)	5.0 ± 3.5	7.0 ± 5.2	6.0 ± 5.2
Non-smokers (%)	75.0	73.9	85.0
L2-L4 BMD (g/cm^2)	1.16 ± 0.15	1.10 ± 0.14	1.13 ± 0.16
Neck BMD (g/cm^2)	0.95 ± 0.14	0.93 ± 0.10	0.96 ± 0.14

Abbreviations used: body mass index (BMI) and bone mineral density (BMD).

Bone mineral density

The changes in femoral neck BMD during the study are shown in Figure 2. In the placebo group the BMD decreased regularly, as was to be expected. During the first year of the study the BMD of the femoral neck had remained constant in both treatment groups. At two and three years after the start, the femoral neck BMD had also declined significantly in both treatment groups, although the rate of bone loss was lower in the MDK-group than in either the placebo or the MD-group. The differences in % BMD loss from baseline between the MDK-group and placebo (1.7%) (95% CI: 0.35 to 3.44) and between the MDK- and the MD-group (1.3%) (95% CI: 0.10 to 3.41) were statistically significant after three years ($p<0.05$) after adjustment for baseline BMD, age, BMI, and years since menopause. After three years, there was no significant difference in the % change from baseline in femoral neck BMD between the MD-group and placebo (mean difference: 0.4%, 95% CI: -1.51 to 1.79).

During the first year of the study we found no significant decrease in the lumbar spine BMD of the MD-group, whereas in both other groups the BMD decreased significantly. The difference in % bone loss from baseline after three years between the MD-group and placebo was 1.3% (95% CI: -0.28 to 2.69) and between the MD- and MDK-group the difference was 0.9% (95% CI: -0.70 to 2.23) after adjustment for baseline BMD, age, BMI, and years since menopause. Although the bone loss in the MD-group seems to be less than in both other

groups, the differences in % bone loss between the three groups were not statistically significant. Hence a positive effect of the mineral + D supplement at the level of lumbar spine cannot be concluded. Neither a beneficial effect of vitamin K could be demonstrated at this site (difference with placebo: 0.5%, 95% CI: -0.95 to 1.82).

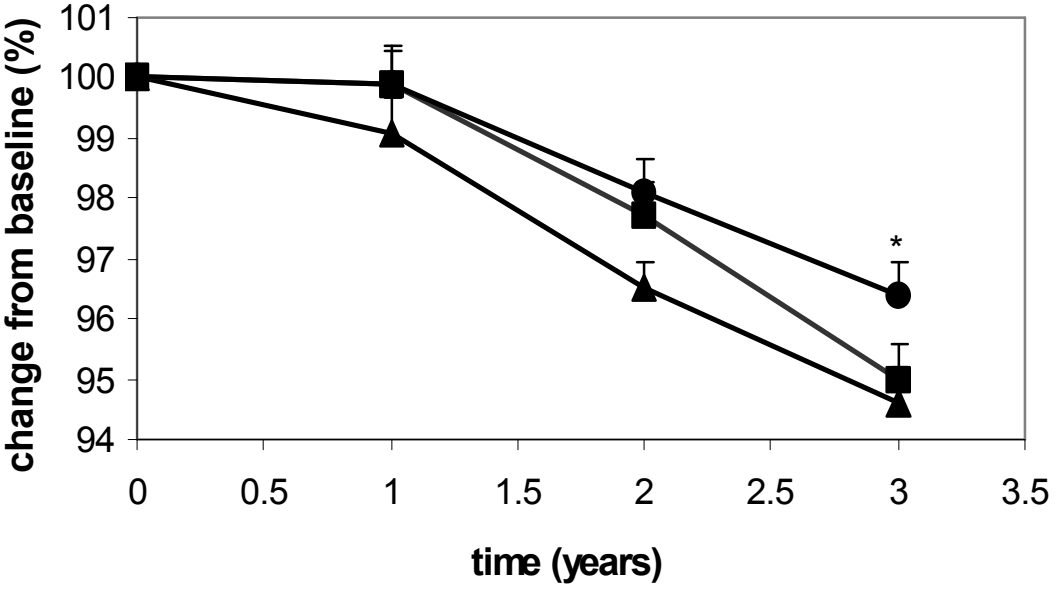

Figure 2

Mean percent change from baseline (± standard error of the mean) in femoral neck bone mineral density. Explanation of symbols: placebo (n=60) (▲), group MD (n=46) (■), group MDK (n=56) (●). *: $p < 0.05$, significantly different from placebo and MD-group.

Biochemical markers for bone metabolism

The data on biochemical markers are summarized in Table 2. As was to be expected, ucOC strongly declined in the MDK group, but not in both other ones, thus showing the compliance to treatment as well as the increase in vitamin K status in this group. In both treatment groups serum vitamin D increased during the first year, thus confirming compliance to treatment and the increase was statistically significant both as compared to baseline as well as to placebo. The elevated vitamin D levels were maintained throughout the study, although at T=36 months significance was lost in the MDK-group. During the first year all markers for bone metabolism showed a trend for decrease in both treatment groups, but not in all cases this trend did reach the level of statistical significance. In the MDK-group the decline in bone metabolism seems to be (at least partly) maintained at this time, although the effects are weak and may not be clinically relevant.

Table 2
Bone markers (mean ± standard deviation) in the three treatment groups at all timepoints.

Marker	Group	T=0	T=3 months	T=12 months	T=36 months
ucOC (ng/mL)	Placebo	2.77 ± 0.90	n.m.	2.77 ± 1.12	2.84 ± 1.07
	MD-group	2.58 ± 0.78	n.m.	2.18 ± 1.15	2.49 ± 0.93
	MDK-group	3.01 ± 0.95	n.m.	0.78 ± 0.48*#	0.54 ± 0.27 *#
25OH-vit D3 (ng/mL)	Placebo	20.4 ± 5.6	19.4 ± 5.5	22.3 ± 5.1*	19.8 ± 4.9
	MD-group	22.5 ± 5.8	24.8 ± 5.2*#	25 ± 4.6*	24.5 ± 5.4*#
	MDK-group	22.9 ± 7.4	27.5 ± 4.5*#	25 ± 5.9*	23.7 ± 5.8
OC (ng/mL)	Placebo	23.8 ± 6.1	24.2 ± 6.5	21 ± 6.1*	23.3 ± 7.6
	MD-group	24.6 ± 7.3	23.7 ± 7.1	21.2 ± 7.6*	23.3 ± 7.9
	MDK-group	25.5 ± 6.4	23 ± 4.9*#	19.1 ± 4.7*#	23.8 ± 8.6
BAP (ng/mL)	Placebo	14.5 ± 5.8	14.3 ± 4.7	13.9 ± 4.6	15.8 ± 4.5
	MD-group	14.8 ± 4.7	14 ± 5.5*	12.1 ± 3.8*	15 ± 5.0
	MDK-group	15 ± 5.8	14.5 ± 6.0*	13.3 ± 5.3*	14.9 ± 5.6
DPD/creat (µmol/mol)	Placebo	7.4 ± 3.2	7.3 ± 2.6	6.7 ± 2.5	7.2 ± 3.2
	MD-group	7.6 ± 4.6	6.5 ± 1.8	6.4 ± 1.6	7.4 ± 3.7
	MDK-group	7.1 ± 3.4	7.5 ± 2.8	6.5 ± 2.1	6.9 ± 3.1
Ca/creat (mol/mol)	Placebo	0.3 ± 0.17	0.27 ± 0.13	0.28 ± 0.14	0.3 ± 0.16
	MD-group	0.36 ± 0.22	0.3 ± 0.19*#	0.35 ± 0.19	0.36 ± 0.25
	MDK-group	0.32 ± 0.18	0.33 ± 0.16	0.32 ± 0.15	0.36 ± 0.20

*significant difference compared to baseline ($p<0.05$)
#significant difference compared to placebo ($p<0.05$)
Abbreviations used: not measured (n.m.), undercarboxylated osteocalcin (ucOC), osteocalcin (OC), bone alkaline phosphatase (BAP), deoxypyridinoline (DPD), creatinine (creat) and calcium (Ca).

DISCUSSION

The efficacy of calcium and vitamin D in retarding postmenopausal bone loss is presently not clear. A general tendency in many (although not in all) studies published thus far, is that most clear effects were observed in cohorts with a low baseline calcium and vitamin D intake [1,2]. Also, short-term (1-2 years) studies often show a positive effect of treatment [23], whereas potential benefits are less obvious during longer treatment periods [24,25]. From our study it appeared that also a more complex mineral supplement containing calcium, magnesium, and zinc as well as vitamin D_3 resulted in a transient, but no long-term retardation of bone loss at the site of the femoral neck. Addition of vitamin K_1 to this supplement, however, showed complete protection during the first year and a partial but persisting protective effect thereafter, leading to a 35% reduction of bone loss (as compared to placebo) after three years. From the fact that - after a transient delay - bone loss also in the MDK group continued (though at a lower pace), we conclude that the bone balance had remained

negative. This phenomenon has also been described for other inhibitors of postmenopausal bone loss [26]. The bone densitometry data are consistent with the biochemical markers for bone metabolism, showing a trend towards a decreased bone turnover for both treatment groups during the first year of the study, which returned to baseline values after 3 years. However, the differences with baseline are small and not in all cases statistically significant. Osteocalcin has a vitamin D-responsive element in its promotor gene, and the fact that the vitamin D-containing supplement did not increase circulating osteocalcin levels suggests that all subjects were adequate in vitamin D at baseline. The circulating ucOC, and its sharp decrease after vitamin K treatment demonstrates that the participants were sub-optimal in vitamin K. Some Gla-proteins (such as rat prothrombin) exhibit a strongly decreased cellular secretion in their undercarboxylated form. From the fact that the improved osteocalcin carboxylation did not result in an increase in circulating osteocalcin total antigen, we conclude that both carboxylated and undercarboxylated species of human osteocalcin are secreted by the osteoblast comparably well.

Our study is the first intervention trial showing a retardation of postmenopausal bone loss by increased vitamin K_1 intake, and is consistent with a number of observational studies in which an association between vitamin K status and either femoral neck BMD or fractures were demonstrated [11-15,27]. Comparable intervention trials among the Japanese population indicated that vitamin K_2 has a more complete effect on bone loss both at the site of the lumbar spine and the femoral neck. The doses used in these studies were extremely high, however [22]. No clear functional differences between vitamin K_1 and K_2 are known, although several recent observations suggest that the liver is the main target tissue for vitamin K_1, whereas vitamin K_2 is accumulated and used preferentially in extra-hepatic tissues [28]. This might explain why vitamin K_2 could have a more profound effect on bone than vitamin K_1.

In our study we did not find an effect of treatment on the rate of bone loss at the site of the lumbar spine. Although it seems remarkable that an effect of vitamin K_1 was only seen on the femoral neck and not the lumbar spine, it must be kept in mind that several factors interfere with DEXA measurement of the vertebrae. Other calcifications, such as osteoarthritis with occurrence of osteophytes, or extensive aortic calcification may hamper the correct reading of true vertebral BMD. Therefore, these data have to be interpreted with caution. The mechanism underlying the protective effect of vitamin K_1 on bone loss of the femoral neck is probably related with the higher carboxylation degree of vitamin K-dependent proteins in bone. This needs not remain restricted to osteocalcin (which was only used as a marker protein in this study), since several other Gla-proteins have been identified in bone [29,30]. The data presented here do not warrant any speculation regarding the molecular mechanism underlying the observed effects.

In conclusion, we have demonstrated that a supplement containing minerals and vitamins D and K has a long-term preventive effect on bone loss in healthy postmenopausal women, although a complete halt of bone loss was not achieved. If the observed effect sustains during longer periods of intake, it may result in postponement of osteoporosis to later ages. The nutritional supplement does not replace classical anti-osteoporotic therapy, and this study does not allow to draw any conclusions concerning fractures.

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3 Factors affecting bone loss in female endurance athletes: a two-year follow-up study

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ABSTRACT

Low bone mass leading to stress fractures is a well known and as yet unsolved problem in female elite athletes. In a prospective study among healthy athletes we have quantified the rate of bone loss, and we have investigated the potential effects of oestrogen supplementation, vitamin K supplementation, as well as other factors such as dietary calcium intake. In total 115 female endurance athletes (between 15 and 50 years old) were selected and classified into amenorrheic, eumenorrheic, or oestrogen-supplemented women and randomised to receive either placebo or vitamin K_1 (10 mg/day) for two years. Primary outcome measure was the change in bone mineral density (BMD) of the femoral neck and lumbar spine after two years, as measured by DEXA. The results showed that the femoral neck BMD had decreased significantly after two years in all three subgroups. The decrease was higher in amenorrheic (-6.5 ± 4.0%) than in eumenorrheic (-3.2 ± 4.1%, p< 0.01) and oestrogen-supplemented athletes (-3.9 ± 3.1%, p=0.07). In all three subgroups the lumbar spine BMD remained constant. Amenorrhea was the only significant predictor found for bone loss in this study (ß-coefficient: 3.2%, 95% CI: -5.7 to –0.612). Supplementation with vitamin K did not affect the rate of bone loss. In conclusion, the rate of bone loss in female endurance athletes was unexpectedly high. Oestrogen supplementation may retard, but not prevent the bone loss. High training intensity maintained over several years must be regarded as a significant risk factor for osteoporosis, and protocols for optimal treatment should be developed.

INTRODUCTION

Normal physical activity positively contributes to peak bone mass and may decrease the rate of bone loss in postmenopausal women [1,2,3]. Also, a less active lifestyle has been associated with an increased risk for osteoporotic fractures [4]. Hence moderate exercise is generally recommended to decrease the rate of bone loss after menopause [5]. On the other hand, high training loads may have a detrimental effect on bone both in men and women. It was reported, for instance, that lumbar spine bone mineral density (BMD) was significantly lower in young male runners than in an age-matched group of non-runners [6]. It was hypothesised that the high weekly mileage of runners may result in low testosterone and high cortisol levels, resulting in a catabolic effect on bone. Thus, a hormonal mechanism may be responsible for reduced BMD in endurance athletes engaged in a high training load [7,8]. The different effects of normal and high exercise intensity on BMD may be explained by assuming that until a threshold level of exercise bone formation is stimulated, but that at higher training intensity the beneficial effect is lost and – in extreme cases – may even result in actual bone loss [9]. Bone loss in early life is especially of concern for women, since their peak bone mass is lower than that of men, and because they experience higher bone loss after menopause. Female endurance athletes possess a number of risk factors for bone loss that are intimately related, such as: large training volumes with a high intensity affecting the oestrogen status, high incidence of amenorrhea, low body mass index (BMI), low body fat content, and relatively low energy and calcium intake [8,10-14]. In these women there is a high incidence of skeletal problems related to osteopenia such as stress fractures [15], and little is known about their bone development at later ages. It is assumed that the combination of oestrogen deficiency with any of the above factors increases the risk of bone loss [16,17]. Thus far, the problem of bone loss in female elite athletes has mainly been addressed in cross-sectional studies, whereas longitudinal studies – especially in relation to treatment – have been rare. Oestrogen supplementation is a common treatment for amenorrheic athletes, but whether this prevents further bone loss is not completely clear.

During recent years it has been shown that vitamin K is involved in bone metabolism. Vitamin K functions as a cofactor in the synthesis of a group of calcium-binding proteins generally known as the 'Gla-proteins', where Gla stands for the unusual amino acid γ-carboxy glutamic acid [18]. The bone Gla-protein osteocalcin is involved in the regulation of calcium deposition in bone and biochemical vitamin K deficiency of bone tissue is common in large parts of the population [19,20]. Osteoporotic hip and vertebral fractures where shown to be associated with depressed levels of circulating vitamin K [21-23], high concentrations of Gla-deficient osteocalcin [24,25] and low dietary intakes of vitamin K [26]. A number of Japanese studies [27,28] as well as a placebo-controlled clinical trial from our group (Braam et al., unpublished data) have shown beneficial effects of vitamin K supplementation on BMD to postmenopausal women and renal dialysis patients. Moreover, data from a small pilot study suggest that high vitamin K intake may lead to an improved bone balance in female athletes [29].

In this paper we report a 2-year follow-up study in which we have monitored the BMD change in female athletes as a function of their menstrual status and oestrogen supplementation. To find additional ways for protection against further bone loss, the potential effects of vitamin K supplementation were also investigated.

METHODS

Subjects

Female endurance athletes from the Netherlands, Belgium and Germany between 15 and 50 years of age were selected in collaboration with sports physicians of the Royal Dutch and Belgian Athletics Union and the German Athletics Union. All participants completed a questionnaire providing information on their training schedules, menstrual status, dietary habits and use of medication and food supplements. Inclusion criteria were: apparently healthy, performance on an international/national top or medium level (training intensity of at least 7 hours per week). Exclusion criteria were: recent (< 1 year ago) bone fractures, use of oral anticoagulants, use of medication known to influence calcium metabolism. In total 115 women were enrolled in the study between October 1998 and September 1999. The study protocol was approved by the University Hospital Medical Ethics Committee, and written informed consent was obtained from all participants.

Study Design

On basis of the information obtained from the questionnaires the study cohort was subdivided into the following three groups: amenorrheic/oligomenorrheic athletes (in this paper further described as amenorrheic athletes): those who experienced absence of menstruation or who had fewer than 6 menstrual cycles in the last 12 months, in all athletes the amenorrhea was secondary (n=29); eumenorrheic athletes: those who had 10-13 menstrual cycles in the previous year and not using oral contraceptives (n=49); oestrogen-supplemented athletes: those who were using oral contraception, the duration of supplementation ranging from 1-17 year (n=37). Subjects in each of these subgroups were randomised for treatment with either vitamin K_1 (10 mg/day, n=59) or placebo (corn starch gran, n=56). Randomisation was performed using a computer-generated randomisation list and the randomisation codes, which were kept apart from the study site during the trial, were allocated sequentially in the order in which the participants were enrolled. One investigator who supervised the whole study was responsible for the enrollment and assignment of participants to the respective groups. Capsules with vitamin K and placebo were kindly supplied by Roche Vitamins Ltd (Basel, Switzerland). Because the two different types of supplements were similar in appearance and taste, participants and investigators were not aware of group assignment. The subjects were instructed to take one capsule per day during evening hours, preferably after the meal. After randomisation and baseline measurements, the women received supplements for the first year, and after one year they were supplied with a new batch for the second year.

Measurements

The BMD of the lumbar spine (L2-L4) and femoral neck were measured using a dual energy X-ray absorptiometer (DEXA, Lunar Co, Wisconsin, USA). Scans of the lumbar spine (L2-L4) and femoral neck were performed at baseline and after two years of treatment. The same two experienced in-hospital technicians performed the measurements throughout the whole study-period. The mean coefficient of variation of repeated measurements was 1.4% for the femoral neck and 1.0% for the lumbar spine. Blood samples were drawn at baseline and after two years. The participants were not allowed to perform any kind of exercise in the morning before blood sampling. Serum was prepared by leaving the samples for 2h at room temperature, followed by centrifugation for 15 min at 2000 x g. Urine was collected during the last two hours of a 16-hour fasting period to exclude dietary influences. All samples were immediately frozen and kept at –80 ºC until use. In serum, total osteocalcin (Cis Bio International, France) and bone alkaline phosphatase (Hybritech, Belgium) were measured as markers of bone formation by radio immunoassay. In urine, total calcium (atomic absorptiometry) and deoxypyridinoline (competitive immunoassay from Metra Biosystems, USA) were used as markers for bone resorption; correction for creatinine excretion was not justified in this case because of the association between creatinine excretion and training intensity. The habitual dietary calcium intake of our subjects was measured with a validated food frequency questionnaire (FFQ), as described elsewhere [30]. Height and weight were measured using standardized equipment, body mass index (BMI) was calculated as weight (kg) / height^2 (m^2). In the two years between baseline and end-point measurement, the responsible investigator contacted the participants every 6 months to check their motivation and compliance.

Statistical Analysis

The sample size was calculated on basis of the assumption that the desired minimal detectable effect of vitamin K treatment would be a 30% decrease of bone loss in the treatment group compared to the placebo group with a 90% power and a 0.05 level of significance. With the assumption of a dropout rate of 15% per year we calculated that 115 subjects had to be included. Statistical analysis was performed using the Statistical Package SPSS (SPSS Corp, Chicago, IL). Results are presented as means ± SD, unless indicated otherwise. The baseline characteristics and changes in bone mineral density and bone markers after two years between the three subgroups were compared by analysis of variance (ANOVA). The follow-up measurement of each subject was related to the corresponding baseline-value by expressing change as % from baseline. The total cohort was analysed to study the effect of treatment. Changes in BMD from baseline between the vitamin K group and placebo were compared using a student T-test. Linear regression analysis was used to study the effects of treatment after adjustments for potential confounders and to assess significant predictors of bone loss. Change of BMD after two years was used as the dependent variable and age, BMI, calcium-intake, stress fractures (yes/no), age of menarche and of start training, amenorrhea (yes/no), use of oral

contraceptives (yes/no) and vitamin K treatment (yes/no) were used as independent variables.

Table 1
Baseline characteristics in three groups of female athletes classified according to menstrual status.

Variables	Amenorrhea	Eumenorrhea	Oestrogen users
	Mean ± SD (n=17)	Mean ± SD (n=36)	Mean ± SD (n=26)
age (yr)	22.2 ± 8.4	31.8 ± 10.9*	28.7 ± 9.8
BMI (kg/m^2)	19.3 ± 1.1	20.5 ± 2.0	20.4 ± 1.8
age menarche (yr)	14.5 ± 1.5	13.6 ± 1.2	14.2 ± 1.6
age of start training (yr)	14.8 ± 9.3	19.2 ± 10.3	18.1 ± 8.8
training intensity (hours/week)	9 ± 2	10 ± 2.5	9 ± 1.5
calcium intake (mg/day)	781 ± 363	753 ± 453	864 ± 579
lumbar spine BMD (g/cm^2)	1.2 ± 0.1	1.3 ± 0.2	1.2 ± 0.1
femur neck BMD (g/cm^2)	1.1 ± 0.1	1.1 ± 0.2	1.1 ± 0.2
serum BAP (ng/ml)[1]	17.6 ± 8.2	15.2 ± 10.3	11.3 ± 4.1*
serum osteocalcin (ng/ml)[2]	30 ± 12.5	23 ± 11.8	23 ± 8.4
urinary DPD (mmol/2h)[3]	5.7 ± 3.4	5.4 ± 2.3	6.1 ± 2.6
urinary calcium (mmol/2h)	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
% vitamin K treatment	58%	40%	50%

* significant difference with group A ($p<0.05$)
Abbreviations used: body mass index (BMI), bone alkaline phosphatase (BAP), deoxypyridinoline (DPD) and bone mineral density (BMD).
[1] mean value in premenopausal females: 8.7 ± 2.9 (ng/ml) (Hybritech, Belgium)
[2] mean value in premenopausal females: 17.1± 3.5 (ng/ml) (Cis Bio International, France)
[3] mean value in premenopausal females: 4.5 ± 1.4 (mmol/2h) (Metra Biosystems, USA)

RESULTS

After inclusion, 36 of the 115 participants discontinued the study prematurely; reasons for drop out were: lack of motivation, sudden ending of athletics career, and non-specified personal reasons. The number of dropouts was equally distributed across the three subgroups and both treatment groups. In Table 1 the baseline characteristics of the 79 participants who completed the study are shown. There were no significant differences in baseline values between the 79 participants who completed the study and the group of 36 subjects who discontinued treatment. The main form of training of our participants consisted of endurance running for about 9 hours per week (i.e. except the regular competitions). The three subgroups were comparable in all variables measured except age, which was 22 years in the amenorrheic group, and 29 and 32 years in the oestrogen-users and eumenorrheic group, respectively. Also, bone alkaline phosphatase in the oestrogen-users was slightly lower than in the amenorrheic group, but other markers for bone metabolism were similar in all three groups. In all three groups the bone markers were well above the normal range for this age group, indicating a high bone turnover (see Table 1).

Table 2 shows the relative change of the variables monitored after two years. It appeared that the BMD decreased in all three groups. At the site of the lumber spine the decrease was not statistically significant, but a very high rate of bone loss was found in the femoral neck. At this site the difference from baseline was statistically significant in all three subgroups ($p<0.000$), whereas the decrease in the amenorrheic group was substantially higher than in both other groups. There was a tendency for all bone markers to increase during the study period, although this increase reached the level of significance only for bone alkaline phosphatase in the oestrogen-supplemented group and for urinary calcium in the eumenorrheic athletes.

We have also analysed the total cohort for the effect of vitamin K treatment. Effects of vitamin K treatment did not differ by menstrual status (data not shown); hence, the results of vitamin K treatment within each group of menstrual status were pooled. There were no significant differences in baseline characteristics between the vitamin K and the placebo group (data not shown), and we did not found an effect of vitamin K on the rate of bone loss (Table 3). Also in this analysis we observed a general tendency for bone markers to increase, with an exception for serum osteocalcin which decreased as a result of vitamin K treatment.

In a subsequent multiple regression analysis we have tested the independent contribution of a number of variables on the observed bone loss including vitamin K supplementation to study also the effect of treatment after adjustment for potential confounders (Table 4). It turned out that the variable amenorrhea was the only significant predictor of bone loss in our study population. Other variables such as BMI, calcium-intake, and age at menarche showed to be no significant predictors of bone loss in our study.

Table 2
Relative change in BMD and bone markers after two years of follow-up in three groups of female athletes classified according to menstrual status.

% change	Amenorrhea Mean ± SD (n=17)	Eumenorrhea Mean ± SD (n=36)	Oestrogen users Mean ± SD (n=26)
lumbar spine BMD	-0.6 ± 4.4	-0.5 ± 2.8	-0.2 ± 2.6
femur neck BMD	-6.5 ± 4.0#	-3.2 ± 4.1*#	-3.9 ± 3.1#
BMI	+2.4 ± 6.0	+1.0 ± 5.2	+1.3 ± 4.1
serum BAP	+23.2 ± 25.2	+16.0 ± 26.5	+30.8 ± 36.8#
serum osteocalcin	+3.0 ± 39.9	+7.7 ± 32.6	+5.0 ± 38.6
urinary DPD	+27.2 ± 72.6	+8.6 ± 50.2	+17.0 ± 72.5
urinary calcium	+40.7 ± 101.1	+52.9 ± 96.8 #	+51.5 ± 117.0

*significant difference with group A ($p<0.05$)
#significant change from baseline ($p<0.05$)
Abbreviations used: body mass index (BMI), bone alkaline phosphatase (BAP), deoxypyridinoline (DPD) and bone mineral density (BMD).

Table 3
Relative change in BMD and bone markers after two years follow-up in two groups of female athletes classified according to vitamin K intake.

% change	Placebo Group Mean ± SD (n=42)	Vitamin K Group Mean ± SD (n=37)
lumbar spine BMD	-0.37 ± 3.6	-0.49 ± 2.5
femur neck BMD	-3.5 ± 4.6#	-4.8 ± 2.8#
BMI	+1.3 ± 6.1	+1.5 ± 3.5
serum BAP	+16.4 ± 32.8	+29.9 ± 25.5#
serum osteocalcin	+14.9 ± 39.9	-5.6 ± 27.1*#
urinary DPD	+29.6 ± 68.2	-2.0 ± 50.9*
urinary calcium	+49.3 ± 104.1	+49.2 ± 97.8
% amenorrheic	17%	27%
% eumenorrheic	52%	38%
% oestrogen-users	31%	35%

* significant difference with placebo group ($p<0.05$)
# significant change from baseline ($p<0.05$)
Abbreviations used: body mass index (BMI), bone alkaline phosphatase (BAP), deoxypyridinoline (DPD) and bone mineral density (BMD).

Table 4
Regression coefficients (95% confidence intervals) for the effect of possible predictors of femoral neck bone loss in the total study population.

Independent variables	ß -coefficient	p-value	95% CI
age (yr)	-0.040 ± 0.074	0.587	-0.189 to 0.108
BMI (kg/m^2)	-0.170 ± 0.261	0.517	-0.692 to 0.351
calcium-intake (mg/day)	0.002 ± 0.001	0.891	-0.002 to 0.003
age of start training (yr)	0.070 ± 0.08	0.501	-0.001 to 0.002
age menarche (yr)	0.001 ± 0.003	0.406	-0.092 to 0.224
amenorrhea (yes/no)	-3.180 ± 1.300	0.010	-5.740 to -0.612
use oral contraceptives (yes/no)	-0.776 ± 1.050	0.463	-2.870 to 1.320
vitamin K treatment (yes/no)	-1.200 ± 0.889	0.182	-2.970 to 0.573
previous stress fractures (yes/no)	0.929 ± 0.976	0.345	-1.018 to 2.880

Abbreviations used: body mass index (BMI). The level of significance (p-value) was used to test which independent variables are significant predictors of femoral neck bone loss and to test the hypothesis that vitamin K treatment significantly influences bone loss after adjustments for potential confounders.

DISCUSSION

To our knowledge this is the largest prospective study in which bone loss among female elite athletes is monitored. A first observation is that there were no significant differences in baseline lumbar spine and femoral neck BMD between the three subgroups stratified for menstrual status. These findings are in contrast to results of previous cross-sectional studies in which a significantly decreased lumbar spine BMD was found in amenorrheic as compared to eumenorrheic athletes [7,8,11,31]. Only a few studies showed also reduced BMD at the site of the femoral neck [32-34]. A possible explanation for the discrepancy between these findings and our study is the significantly younger age of the amenorrheic group: the total period of high training intensity (and thus the period of potential bone loss) was 3.2 – 5.2 years shorter than in the other groups. At the observed rate of bone loss extrapolation to the ages in both other groups would lead to substantial lower bone mass in the amenorrheic athletes. Although we did not observe baseline differences, the rate of bone loss at the site of the femoral neck was significantly higher in the amenorrheic athletes than in the eumenorrheic athletes. The lumbar spine BMD remained constant in all three subgroups. Whereas there are many cross-sectional studies in female athletes, not many follow-up studies are available for comparison of our findings. Taaffe [35] also found a greater decrease in femoral neck BMD (-1.2%) than lumbar spine BMD (-0.2%) in female runners after a follow-up period of 8 months. In a 12-month longitudinal study of Bennel [36], small increases in BMD were found in endurance athletes. For a long time, it has been thought that only trabecular and not cortical bone would

be affected by loss of menses, because it would be more susceptible to hormonal stimuli that lead to demineralization. These assumptions were based on studies showing no significant differences in radius BMD between different groups of menstrual status. However, the radius is not a weight-bearing bone and in long distance runners only the weight-bearing bones are subjected to repeated mechanical stresses. An explanation for the difference between the two skeletal sites found in our study could be a relative higher pressure on the lower extremities rather than on the spine in runners. However, more follow-up studies in female athletes are needed to unravel the mechanism underlying the differential effect of physical activity and oestrogen on cortical and trabecular bone.

With 3.3% per year the rate of femoral bone loss was unexpectedly high in the group of amenorrheic athletes, this value being about twice that found in the first years after menopause of non-sporting women. Remarkably, also in both the eumenorrheic athletes and the oestrogen-supplemented group we observed considerable bone loss, amounting 1.6 and 2.0% per year, respectively. These rates of bone loss in amenorrheic and eumenorrheic athletes are higher than those found by others [35-37]. Earlier studies have shown that especially calcium intake may influence bone changes in association with exercise [31,38]. In an earlier study [7] it was concluded that the calcium requirement for amenorrheic women should approximate that described for postmenopausal women, namely 1500 mg per day. In this perspective, the mean intake (792 mg) of our athletes must be regarded as well below the recommended levels. Furthermore, our reported intakes are also below the observed calcium-intakes (1100 mg/day) in a large population-based study in the Netherlands among 20921 non-athletic men and women [39]. The fact that calcium intake was not a significant predictor of the observed bone loss, is maybe partly due to the low number of subjects in our study.

It should be pointed out that in the group of oestrogen-users most athletes received oestrogen because they had been amenorrheic previously. Thus, although supplementation of amenorrheic athletes with oestrogen may decrease the rate of bone loss, additional treatment will have to be found in order to completely solve the problem. Several other studies have addressed the question whether oestrogen therapy has beneficial effects on bone mineral density. It was shown that the bone mineral density in female athletes increased after therapy, but not to levels found in normally menstruating healthy young women. Until now, no consensus has been reached on the potential benefits of oestrogen therapy for athletes [40].

Another observation in our study is that amenorrheic athletes had started their training at much younger age than eumenorrheic ones. This means that girls starting endurance athletics at young age not only may have a longer athletics career, but that they are also at risk for higher bone loss than those starting at later ages. Accordingly, an early start of extensive endurance training at high intensity is questionable and measures to limit these effects should be defined. The high bone loss was consistent with the high bone turnover (a well-known risk factor for bone loss) at baseline. High bone turnover reflected by high bone formation and resorption markers has been demonstrated before in male runners [41]. However, other studies have shown a reduced bone turnover among female athletes [42].

In the same cohort we have analysed also the effect of vitamin K administration. Since effects of vitamin K treatment did not differ by menstrual status, the results of vitamin K treatment within each group of menstrual status were pooled. Vitamin K treatment had no effect on the rate of bone loss. This seems to be in contrast with an earlier study from our group, in which we found a 35% decrease of bone loss in post-menopausal women during a 3-year supplementation period (Braam et al., unpublished data). In the latter study, however, calcium, magnesium, zinc, and vitamin D were co-administered with vitamin K. One possible reason for the different outcome of both studies is, therefore, a synergistic effect of minerals, and vitamins D and K with a much lower effect of each of the components separately. Another reason for the different outcomes of both trials may be the very different characteristics of the respective study populations. The female athletes described in this study differed from postmenopausal women in that they had a low BMI and low calcium intake, in combination with an extreme training intensity. These factors have been identified as risk factors for developing early osteoporosis in female athletes [34,40]. Together with their relatively low oestrogen production, these conditions probably all contribute to the high rate of bone loss observed, and – like oestrogen supplementation – also increased vitamin K intake may not be sufficient to overcome these negative effects. The poor effect of vitamin K on bone maintenance was reflected in the markers for bone turnover, which were high at baseline and which (except osteocalcin) had a tendency to even increase. Only osteocalcin decreased as a result of vitamin K treatment, but this may be related to more complete gammacarboxylation, resulting in an increased affinity for the hydroxyapatite matrix in bone rather than to decreased bone turnover. One limitation of this study is a high drop-out rate of our participants. As a consequence a considerable amount of follow-up data was missing which could potentially introduce bias. The reasons for drop-out were not related to the menstrual status or treatment assignment of our participants and the number of drop-outs was equally distributed across the subgroups and treatment groups. Furthermore, there were no significant differences in baseline values between the drop-outs and the subjects who completed the study. Therefore we expect the potential bias to have minimal effect on the results.

By multiple regression analysis it was shown that the variable amenorrhea was the only significant predictor of bone loss in our study population. Oestrogen supplementation to amenorrheic athletes should be encouraged, therefore. This does not explain, however, why also in the eumenorrheic and oestrogen-supplemented groups considerable bone loss took place. In other studies it was demonstrated that besides amenorrhea, also body weight and age of menarche were important predictors of bone loss in athletes [33,34]. Although in our study we have not made a direct comparison with non-sporting women, the mean age at menarche of the athletes was relatively high. This is consistent with comparative studies by others [31,32,43]. Delayed puberty could be associated with a lower rate of bone mineral accretion during adolescence leading to a lower peak bone mass. Endurance athletes are known to continue their high training intensity longer than in many other sports, sometimes for 20 years or more. Extrapolation of our data would mean that at the end of their sport career a substantial part of these athletes will be osteopenic. Since it is not known whether

bone will quickly recover from many years of bone loss, it seems that these subjects are at disadvantage when entering the menopause. It is regrettable, therefore, that the mineral intake by athletes from all three participating countries was low. A low calcium intake has also been demonstrated in the USA where inadequate calcium intakes below 900 mg/day were found in sporting girls between 12 and 19 years [44]. It seems likely that dietary recommendations are primarily focussed on optimal performance. Our study demonstrates that medical supervision, dietary recommendations and hormonal substitution should consider optimal skeletal development with high priority. High training intensity maintained over several years must be regarded as a significant risk factor for osteoporosis, and protocols for optimal treatment should be developed.

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4 Vitamin K dietary intakes and cardiovascular risk factors in the Framingham Offspring Cohort

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ABSTRACT

The objective of this study was to determine associations between reported dietary intakes of two forms of vitamin K (phylloquinone and menaquinones) and intermediary markers of CVD risk in a population-based cohort of men and women. Dietary phylloquinone- and menaquinone-intakes were assessed by food frequency questionnaire in 1338 men and 1603 women. All subjects were free-living participants in the Framingham Offspring Study. Lifestyle characteristics and lipid profiles, including total-, LDL- and HDL-cholesterol and triacylglycerol concentrations, were measured. Cross-sectional associations were estimated across increasing quintiles of reported phylloquinone and menaquinone intakes. Participants in the highest quintile of reported phylloquinone intake were older (P<0.001), had lower BMI (P=0.04), smoked less (P<0.001), and consumed more fruit, vegetables and dietary supplements (P<0.001) and less meat and total fat (P<0.001) compared to participants in the lowest quintile. Consistent with this overall heart healthy lifestyle, higher phylloquinone intakes were associated with lower triacylglycerol concentrations and higher HDL-cholesterol concentrations. Participants in the highest quintile of reported menaquinone intake were younger (P<0.001), had higher BMI (P<0.001), and consumed less fruit and fiber (P<0.001) and more meat and total fat (P<0.001), yet were associated with lower LDL-cholesterol concentrations (P=0.005) and higher HDL-cholesterol concentrations (P=0.03). In this study it was demonstrated that high phylloquinone intakes were associated with an overall heart healthy lifestyle, whereas high menaquinone intakes were not. However, reported dietary intakes of both forms of vitamin K were associated with a more favorable lipid profile.

INTRODUCTION

The vitamin K-dependent step in the synthesis of vitamin K-dependent proteins is the posttranslational carboxylation reaction in which specific glutamate residues are converted into gamma-carboxyglutamate (Gla) residues [1]. The Gla-residues form the calcium-binding groups in vitamin K-dependent proteins, and are thought to be required for functionality. Vitamin K-dependent carboxylase, the endoplasmic enzyme involved in the synthesis of Gla residues, has been identified in various organs, including liver and vessel wall [2].

Vitamin K-dependent proteins produced by the vessel wall include protein S, growth-arrest-specific gene 6 protein (Gas6) and matrix Gla protein (MGP). Protein S is associated with local inhibition of thrombosis [3], whereas Gas6 is thought to stimulate normal cell growth and to prevent apoptosis in growth-arrested cells [4]. MGP is a potent inhibitor of calcium precipitation, as was demonstrated in MGP-null mice, which died by eight weeks of life from massive arterial calcification and rupture of the thoracic or abdominal aorta [5]. Aorta mineralization was also observed in rats after 4 to 6 weeks of treatment with warfarin, a vitamin K antagonist, suggesting that vitamin K is essential for the function of MGP [6]. Collectively these observations are suggestive of a role for vitamin K in atherosclerosis and cardiovascular disease (CVD).

Two natural forms of vitamin K are found in the human diet: phylloquinone (vitamin K_1), which occurs in leafy green vegetables and in some plant oils [7], and menaquinones (K_2 vitamins), which occur in meats (menaquinone-4; MK-4) and fermented foods like cheese and curds (menaquinone-6-9; MK-6 to MK-9) [8-10]. To date, only dietary phylloquinone intakes have been assessed in different populations [7]. An inverse relationship between reported dietary phylloquinone intakes and risk for age-related chronic diseases, such as atherosclerotic calcification [11] and hip fracture risk [12,13] has been reported in population-based studies. These findings should be interpreted with some degree of caution because dietary phylloquinone may be a marker for an overall healthy lifestyle, given that it is concentrated in green, leafy vegetables [14,15]. In contrast, it is less probable that any association between reported menaquinone intakes and risk for age-related chronic diseases is confounded by diets that reflect an overall healthy lifestyle because menaquinones are concentrated in animal products, such as meats and cheeses.

In this study, we examined the associations among reported dietary phylloquinone and menaquinone intakes and lifestyle characteristics, including dietary patterns that are indicative of an overall heart healthy lifestyle, in men and women participating in the Framingham Offspring Study. Furthermore, we examined the associations among reported dietary intakes of these two forms of vitamin K and plasma lipid and lipoprotein profiles, which are related to CVD risk.

METHODS

Subjects

The Framingham Offspring Study is a longitudinal community-based study of cardiovascular disease among the children, and their spouses, of the original participants in the Framingham Heart Study Cohort [16]. In 1971, 5135 participants were enrolled into the Offspring Study and have returned every 3-4 years for an extensive physical examination, comprehensive questionnaires, anthropometric measurements, blood chemistries and assessment of cardiovascular and other risk factors by trained clinical personnel [17]. Between 1991 and 1995, there were 3799 participants in the 5th examination cycle of the Framingham Offspring Study. Valid food frequency questionnaire (FFQ) data were available for 3418 participants. Of these, participants were excluded from the analysis of the current study if they were using medication to control blood glucose (insulin n=36; oral hypoglycemic n=90), or if they were taking cholesterol-lowering medication (n=214). Participants with missing covariate information were also removed, reducing the final sample to 2941 (1338 men and 1603 women). The Institutional Review Board for Human Research at Boston University and the Human Investigation Research Committee of New England Medical Center approved the protocol.

Dietary Assessment

Usual dietary intakes for the previous 12 months were assessed at the 5th examination using a 126-item semiquantitative FFQ, as described elsewhere [18]. This FFQ has been validated for numerous nutrients [18], including phylloquinone [13], but not for menaquinones. Questionnaires were mailed to the subjects prior to the exam and once completed, were returned to the examination site. Questionnaires with reported energy intakes below 2.51 MJ/d and above 16.74 MJ/d (600 and 4000 kcal, respectively), or with more than 12 food items left blank were considered invalid and excluded from further analysis. Vitamin and mineral supplement use and specific type of breakfast cereal most commonly consumed were used in the estimate of total micronutrient intakes. Daily phylloquinone intake was calculated by multiplying the phylloquinone content per serving of each food [19,20] by the reported frequency of consumption, and summing over all foods. Phylloquinone intakes as reported here do not include intake from multivitamin and mineral preparations, or other nutrient supplements. The menaquinone intake was a compilation of MK-4 and MK-5-10 using the menaquinone contents of different food items [8,10]. All values found in the literature were converted to µg per 100 g of food. When multiple published values were reported for the same food item, the median value was used for the menaquinone content of that food. Daily menaquinone intakes were calculated by multiplying the menaquinone contents per serving of each food by the reported frequency of consumption, and summing over all foods, similar to that of phylloquinone. Initially, MK-4 and MK-5-10 intakes were analyzed separately, in addition to analysis of total menaquinone intake. However, as the results were consistent across intakes of individual menaquinones, only the total menaquinone intakes are reported here.

Serum Lipids

Fasting blood samples were drawn after an overnight fast for measuring lipid concentrations. Serum lipid measurements included enzymatic measurement of total cholesterol and triacylglycerol concentrations [21]. The HDL fraction (HDL-C) was measured after precipitation of LDL and VLDL with dextran sulfate magnesium [22]. LDL-cholesterol concentrations (LDL-C) were calculated using Friedewald equation [23] for only those individuals with triacylglycerol concentrations less then 400 mg/dl (n=2879).

Covariate Information

Height and weight were measured with the subject standing. Body mass index (BMI) was calculated as the weight in kilograms divided by the square of the height in meters (kg/m^2). Subjects were classified as hypertensive if both of the two measurements for diastolic blood pressure and systolic blood pressure was > 90 mm Hg or > 140 mm Hg, respectively, or if use of antihypertensive medications was reported (Joint National Committee on Detection) [24]. Current smokers were defined as those who reported smoking at least one cigarette per day during the previous year. Physical activity was assessed as a weighted sum of the proportion of a typical day spent sleeping and performing sedentary, slight, moderate, or heavy physical activities [25]. Estrogen replacement therapy among postmenopausal women was classified as current or no use at the time of the physical examination. Alcohol consumption was estimated in grams per day from the FFQ. Multivitamin use was classified as current or no use at the time of the physical examination.

Statistical Analysis

SAS statistical software (release 8.0, SAS institute, Cary NC) was used for all statistical analysis. Dependent variables were total-C, LDL-C, the ratio of LDL-C to HDL-C and the natural logarithms of HDL-C, triacylglycerol concentrations and the ratio of total-C to HDL-C. To express transformed variables to their natural scale, geometric means were computed by exponentiation of adjusted least squares means. There were no systematic interactions by sex between vitamin K (phylloquinone and menaquinone) intakes and lipid concentrations.

All analyses between the lipid concentrations and phylloquinone or menaquinone intake were adjusted for the following variables: sex, age (years), body mass index (BMI), energy intake (kcal/d), multivitamin supplementation use (Y/N), alcohol intake (g/d), blood pressure medication (Y/N), current cigarette smoking (Y/N), physical activity score (continuous), current estrogen replacement therapy among women (Y/N) and saturated fatty acid (SFA) intake (percentage of total energy intake, continuous). Dietary fiber intake was adjusted for associations with phylloquinone intakes because the predominant sources of phylloquinone are green leafy vegetables, which are fiber-rich foods. Statistical significance of trends across quintiles of phylloquinone or menaquinones intake was assessed with linear regression models (for continuous outcome variables) or Mantel-Haenszel test of trend (for categorical variables). Associations were considered to be statistically significant at $P<0.05$. All P-values were 2-sided. The data were presented as means ± SD, unless stated otherwise.

RESULTS

The mean ± SD reported daily intakes of phylloquinone and total menaquinones were 162 ± 114 µg and 16 ± 11 µg, respectively. Of the menaquinones, there was a mean ± SD intake of 7.5 ± 3.8 µg/d of MK-4, and 7.3 ± 9.5 µg/d of MK-5-10. There were no significant differences in reported phylloquinone or menaquinone intakes between men and women after adjustment for energy intake. Consistent with other studies using this FFQ [12,13] reported phylloquinone intakes were higher than the current adequate intake of 90-120 µg/d of phylloquinone (Institute of Medicine) [26]. The food items on the FFQ that contributed the most to phylloquinone were broccoli (17%), iceberg lettuce (17%), spinach (14%), romaine lettuce (7%), and cabbage/coleslaw (5%). The highest contributors to the total dietary MK-4 were chicken (38.1%), red meat (26.5%) and eggs (11.5%); for the higher menaquinones, dietary intake was almost exclusively derived from cheese (97.2% of total MK-5-10 intake).

The age, sex and energy-adjusted descriptive characteristics of the study population across quintiles of phylloquinone- and menaquinone-intake are shown in Tables 1 and 2, respectively. When compared to the lowest quintile of intake (median: 63 µg/d), participants in the highest quintile of phylloquinone intake (median: 282 µg/d) were older and had lifestyle characteristics and dietary patterns consistent with an overall heart healthy lifestyle, such as lower BMI, less smoking, greater estrogen use among women, higher intakes of fruit, vegetable, fish, folate, vitamin E and dietary fiber and lower intakes of meat. In contrast, participants in the highest quintile of menaquinone intake (median: 28 µg/d) were younger, had a higher BMI, a lower intake of carbohydrate, dietary fiber, folate and fruit, and a higher intake of monounsaturated and saturated fatty acids, cholesterol and animal products when compared to those in the lowest quintile of menaquinone intake (median: 7 µg/d). There was no significant association between reported phylloquinone and menaquinone intakes in this study group. These contrasting dietary and lifestyle patterns between high phylloquinone and high menaquinone intakes are not unexpected given the unique dietary sources of each form of vitamin K.

The lipid concentrations compared across quintiles of reported phylloquinone intake are shown in Table 3. When compared from the lowest to the highest quintile of phylloquinone intake, HDL-C was significantly higher ($P=0.003$), triacylglycerol was significantly lower ($P<0.001$), and the ratios of LDL-C to HDL-C and total cholesterol to HDL-C were significantly lower (more favorable). No trends were observed for LDL-C or total cholesterol concentrations. The lipid concentrations compared across quintiles of reported menaquinone intakes are shown in Table 4. When compared from the lowest to the highest quintile of reported menaquinone intake, HDL-C was significantly higher ($P=0.031$), whereas LDL-C, the ratio of LDL-C to HDL-C and total cholesterol to HDL-C were all significantly lower ($P<0.01$). There was a non-significant trend across quintiles of menaquinone intake for total cholesterol, but not for triacylglycerol concentrations.

Table 1
Characteristics of Framingham Offspring participants (n=2941; 1,338 men and 1,603 women) by quintiles (Q1-Q5) of phylloquinone intake in examination 5 (1991-1995)[1].

Phylloquinone intake	Q1	Q2	Q3	Q4	Q5	P for trend[2]
µg/d	63	103	139	185	282	
N	588	588	589	588	588	
Age (years)	52.5	54.1	53.7	54.9	55.1	<0.0001
BMI (kg/m^2)	27.1	26.9	27.0	26.8	26.5	0.04
Current smoker (%)	27.9	21.8	16.6	14.3	16.2	<0.0001
Estrogen use[3] (%)	6.3	6.1	7.5	12.1	13.8	<0.0001
Multivitamin user (%)	24.7	23.1	29.4	35.0	35.5	<0.0001
Hypertension (%)	18.2	19.6	20.0	17.9	18.9	0.94
PA score	34.8	34.5	34.9	35.2	35.2	0.10
Carbohydrate (% of E)	47.4	47.1	47.6	47.6	49.1	<0.0001
Protein (% of E)	15.6	16.4	16.9	17.4	17.9	<0.0001
Total fat (% of E):	28.4	28.6	27.8	27.1	25.4	<0.001
SFA	11.6	11.1	10.7	10.3	9.3	<0.0001
MUFA	11.8	11.8	11.4	11.0	10.2	<0.0001
PUFA	5.0	5.7	5.7	5.8	5.9	<0.0001
Cholesterol (mg/day)	204	207	211	210	202	0.47
Dietary fiber (g/day)	12.8	15.3	17.6	18.9	22.4	<0.0001
Alcohol (g/day)	4.5	4.9	5.2	5.8	6.1	<0.0001
Vitamin E (mg/d)	4.5	5.5	5.9	6.2	7.0	<0.0001
Vitamin E, incl. supplements (mg/d)	46.2	44.2	44.4	54.3	63.9	0.01
Folate (µg/d)	217	258	285	306	366	<0.0001
Fruit (servings/wk)	5.8	6.9	8.6	9.6	12.7	<0.0001
Vegetables (servings/wk)	8.1	12.1	16.0	20.5	32.9	<0.0001
Meat (servings/wk)	6.2	6.1	5.7	5.1	4.0	<0.0001
Fish (servings/wk)	1.6	1.9	2.2	2.5	3.1	<0.0001

[1] All analyses were adjusted for sex, age and total energy
[2] P for linear trend
[3] Estrogen use among postmenopausal women
Abbreviations used: body mass index (BMI), physical activity (PA), total energy (E), saturated fat (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA).

Table 2
Characteristics of Framingham Offspring participants (n=2941; 1,338 men and 1,603 women) by quintiles (Q1-Q5) of total menaquinone intake[1] in examination 5 (1991-1995)[2].

Menaquinone intake	Q1	Q2	Q3	Q4	Q5	P for trend[3]
μg/d	7	10	15	19	28	
N	588	588	589	588	588	
Age (years)	55.7	54.3	53.8	53.6	52.8	<0.0001
BMI (kg/m^2)	26.3	26.4	27.1	27.0	27.6	<0.0001
Current smoker (%)	20.8	17.2	19.9	19.7	19.2	0.94
Estrogen use[4] (%)	11.6	9.0	8.2	8.7	8.3	0.11
Multivitamin user (%)	26.9	34.4	27.0	28.2	31.3	0.55
Hypertension (%)	22.8	16.7	20.5	16.8	17.7	0.08
PA score	35.1	35.0	34.9	35.1	34.6	0.23
Carbohydrate (% of E)	53.1	49.8	47.4	45.8	42.6	<0.0001
Protein (% of E)	15.0	16.4	16.8	17.4	18.5	<0.0001
Total Fat (% of E):	23.8	26.2	27.5	29.0	31.1	<0.0001
SFA	8.5	10.0	10.6	11.4	12.8	<0.0001
MUFA	9.9	10.8	11.3	11.8	12.5	<0.0001
PUFA	5.4	5.4	5.6	5.8	5.8	<0.0001
Cholesterol (mg/day)	149	195	213	237	259	<.0001
Dietary fiber (g/day)	17.5	17.8	17.1	16.9	16.0	<.0001
Alcohol (g/day)	4.9	4.8	5.9	5.4	5.2	0.40
Vitamin E (mg/d)	5.6	5.7	5.8	5.9	5.8	0.14
Vitamin E incl.supplements (mg/d)	48.7	54.0	51.3	42.8	55.5	0.75
Folate (ug/d)	279	292	284	283	269	0.009
Fruit (servings/wk)	9.7	9.3	8.5	8.5	7.5	<.0001
Vegetables (servings/wk)	17.7	17.8	17.8	17.3	18.0	0.85
Meat (servings/wk)	4.2	4.8	5.2	6.0	7	<.0001
Fish (servings/wk)	2.2	2.2	2.3	2.3	2.3	0.48
Phylloquinone (μg/d)	165	160	162	154	159	0.43

[1] Total menaquinone intake is the sum of MK-4 and MK-5-10 intakes
[2] All analyses were adjusted for sex, age and total energy
[3] P for linear trend
[4] Estrogen use among postmenopausal women
Abbreviations used: body mass index (BMI), physical activity (PA), total energy (E), saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA).

Table 3
Lipid concentrations of Framingham Offspring participants (n=2941; 1,338 men and 1,603 women) by quintiles of phylloquinone intake in examination 5 (1991-1995)[1].

Phylloquinone intake	Q1	Q2	Q3	Q4	Q5	P for trend[2]
μg/d	63	103	139	185	282	
N[3]	588	588	589	588	588	
HDL-C (mmol/l)	1.22	1.22	1.24	1.24	1.27	0.003
Triacylglycerol (mmol/l)	1.73	1.60	1.58	1.56	1.50	<.0001
Total cholesterol (mmol/l)	5.25	5.22	5.28	5.20	5.20	0.33
LDL-C (mmol/l)	3.13	3.13	3.15	3.08	3.10	0.49
LDL : HDL-C	2.7	2.7	2.7	2.6	2.6	0.04
Total : HDL-C	4.3	4.3	4.2	4.1	4.1	<.0001

[1] All analyses were adjusted for sex, age, total energy, saturated fat (% of total energy), BMI, physical activity, alcohol intake (g/d), smoking, estrogen use, treatment for blood pressure and multivitamin use
[2] P for linear trend
[3] There were 576 participants per quintile for LDL-C and the ratio of LDL to HDL-C.

Table 4
Lipid concentrations of Framingham Offspring participants (n=2941; 1,338 men and 1,603 women) by quintiles of total menaquinone intake[1] in examination 5 (1991-1995)[2].

Menaquinone intake	Q1	Q2	Q3	Q4	Q5	P for trend[3]
μg/d	7	10	15	19	28	
N[4]	588	588	589	588	588	
HDL-C (mmol/l)	1.22	1.20	1.25	1.25	1.25	0.03
Triacylglycerol (mmol/l)	1.62	1.61	1.57	1.56	1.59	0.47
Total cholesterol (mmol/l)	5.29	5.22	5.21	5.21	5.19	0.15
LDL-C (mmol/l)	3.20	3.17	3.09	3.09	3.05	0.005
LDL : HDL-C	2.8	2.8	2.6	2.6	2.6	0.005
Total : HDL-C	4.3	4.3	4.1	4.1	4.1	0.009

[1] Total menaquinone intake is the sum of MK-4 and MK-5-10 intakes
[2] All analyses were adjusted for sex, age, total energy, saturated fat (% of total energy), BMI, physical activity, alcohol intake (g/d), smoking, estrogen use, treatment for blood pressure and multivitamin use
[3] P for linear trend
[4] There were 576 participants per quintile for LDL-C and the ratio of LDL to HDL-C.

DISCUSSION

In the present study, we examined associations among reported dietary intakes of vitamin K, lifestyle characteristics and serum lipid concentrations in a population-based cohort. Of the two forms of vitamin K analyzed, participants with a reported high phylloquinone intake appeared to have characteristics and dietary patterns consistent with an overall heart healthy lifestyle. The mean reported phylloquinone intake of 162 µg/d is similar to those reported in the Framingham Study original cohort (mean age: 72 y) [12] and the Nurses' Health Study [13] using the same FFQ. While this FFQ overestimates phylloquinone intakes when compared to diet records [13], it is suitable for the purpose of ranking individuals according to reported intake. Green, leafy vegetables were the predominant sources of phylloquinone, which is consistent with other studies [12,13]. Furthermore, higher reported phylloquinone intakes were associated with a lipoprotein profile indicative of decreased risk of developing cardiovascular disease (higher HDL-C, lower LDL-C, and lower ratio of total cholesterol to HDL-C).

Participants with a higher phylloquinone intake tended to smoke less, have a higher intake of fruits, vegetables and fish, had lower meat intakes, and were more likely to take multivitamin supplements. There was also more estrogen use among the post-menopausal women with higher phylloquinone intakes. Most of these characteristics have also been associated with reduced CVD risk [27-36]. The strong overlap among chronic disease risk factors complicates the interpretation of studies with inverse associations between reported dietary phylloquinone intakes and chronic disease risk. However, phylloquinone intakes may be a suitable surrogate marker of a heart healthy lifestyle in a population-based cohort where more detailed data are impractical to collect or are unavailable.

In contrast to phylloquinone, reported dietary menaquinone intakes were associated with an overall less heart healthy lifestyle pattern, including higher BMI, higher intakes of total fat, SFA, cholesterol and meat, and lower intakes of dietary fruit and fiber. There were no significant associations between menaquinone intakes and multivitamin or vitamin E supplement use. This was an expected finding given that dietary menaquinones are primarily found in meats and cheeses in the U.S. diet, but may not be a global phenomenon because there are populations with a high consumption of fermented foods such as natto in Japan [37].

There are several major caveats in the interpretation of the reported menaquinone intakes in the current study. In the absence of menaquinone food composition data for the U.S. food supply, we used data from Europe [10] and Asia [8]. The current food composition data for menaquinones are very limited, and geographical variation in content has not yet been examined. Secondly, the low endogenous concentrations of menaquinones in circulation preclude validation of this FFQ with a biological marker. Finally, there is a tendency to underreport meats and dairy products using this FFQ, especially among men [38]. However, this underreporting may attenuate our ability to detect associations between reported dietary menaquinone intakes and lifestyle patterns. It is plausible that use of additional food composition data and other methods of dietary assessment may result in a stronger association than reported here.

Dietary phylloquinone intakes were inversely associated with triacylglycerol concentrations in the current study. Unfortunately, the absence of plasma phylloquinone concentrations in the current study precludes comparison with those that have a reported positive association between plasma phylloquinone and triacylglycerol concentrations [39,40]. Although plasma phylloquinone concentrations are associated with recent dietary intakes [7], few data exist on the vitamin K diet-plasma association relative to lipid and lipoprotein concentrations in a population-based cohort. Surprisingly, reported menaquinone intakes in the current study were also associated with more favorable lipid concentrations, despite an overall less heart healthy lifestyle. Pharmacologic doses of menaquinone-4 have been reported to lower total cholesterol concentrations in patients on peritoneal dialysis [41]. While suggestive, these effects were associated with menaquinones doses that were approximately 2,000 times greater that the dietary intakes reported in the current study. Until a common biological mechanism is identified to explain these associations, the existing data are difficult to interpret. In conclusion, reported dietary phylloquinone intakes are positively associated with heart healthy lifestyle characteristics in a population-based cohort. Assessment of phylloquinone intakes may have potential as a marker for CVD risk on a population-wide basis.

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5 Effects of ageing on vessel wall characteristics in postmenopausal women: a longitudinal study

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ABSTRACT

Many cross-sectional studies have shown in healthy subjects that the elastic properties of major arteries decrease with increasing age, but prospective studies on this have been rare. In the present paper we describe the effects of ageing on vascular characteristics of the common carotid artery in a group of 60 postmenopausal women who were followed for three years. At baseline and after three years, compliance, distensibility, and intima-media thickness were measured with an ultrasound technique, and the changes in these variables over three years were evaluated. It was shown that the distensibility coefficient and compliance coefficient decreased with 10% (mean decrease: 1.8 Mpa^{-1}, 95% CI: 0.73 to 2.9) and 6% (mean decrease: 0.05 mm^2/kPa, 95% CI: 0.02-0.09), respectively, and the intima-media thickness (IMT) increased with 9% (mean increase: 0.05 mm, 95% CI: 0.02 to 0.07). In postmenopausal women ageing causes a decrease in the elastic properties and an increase in the wall thickness of the common carotid artery. These results are in line with previously reported cross-sectional studies.

INTRODUCTION

Changes in the elastic properties of the main arteries have major implications for the development of vascular disease. Arteries, especially the larger elastic arteries such as the common carotid artery, become stiffer during ageing [1]. Arterial stiffness was shown to be associated with cardiovascular disease and this association is corroborated by an increased pulse pressure following increased arterial stiffness and arteriosclerosis [2]. In cross-sectional studies it has been shown that the distensibility and compliance of the elastic common carotid artery decreased linearly with age, but that the reduction in compliance is less pronounced than the reduction in distensibility [3,4].

Women tend to catch up with men after menopause with respect to the risk of cardiovascular disease [5]. The disappearance of the risk difference between men and women has been attributed to the decrease of female sex hormones, notably estrogen, after menopause [6-8]. Therefore, also postmenopausal women are regarded nowadays as a high-risk group for developing cardiovascular disease. The decrease in arterial stiffness with increasing age is suggested to be more marked in women between 45 and 60 years than in men of the same age, and changes in vessel wall properties may probably be detected faster in these women [4].

An increasing amount of research is focused on the evaluation of arterial properties, facilitated by the recent availability of accurate non-invasive methods to measure arterial stiffness [9,10]. However, until now there has been no extensive prospective study to investigate the effect of ageing on compliance and distensibility. It was the aim of the present investigation to monitor changes of the distensibility coefficient (DC) and the compliance coefficient (CC) in a group of healthy postmenopausal women, during a 3-year study period. With the same technique, also the intima-media thickness (IMT) of the artery wall can be measured [11]. IMT is assumed to represent the endothelial adaptive response to physiological and pathophysiological processes [12-14] and IMT has been used as an indicator of arteriosclerosis in various studies [15,16]. Several population-based studies have investigated changes in IMT over time [17], however this has never been investigated in coherence with DC and CC. The Young's Modulus (E) expresses the elasticity of tissue independent of its volume [18]**.** The present study also included the effects of ageing on changes in IMT and E.

METHODS

Subjects

The study was performed in 60 healthy female volunteers between 50 and 60 years who were at least 2 years postmenopausal. Information on cardiovascular risk factors, current health status, medical history, drug use and smoking behavior was obtained before the start of the study. No history of important systemic disease was reported and none of the volunteers were using or had recently used hormonal replacement therapy. Baseline measurements were performed between November 1997 and March 1998, whereas the

follow-up examination took place between December 2000 and March 2001. All participants gave written informed consent and the trial was approved by the University Hospital Medical Ethics Committee.

Study Design

The participants were enrolled in a 3-year double-blind placebo-controlled clinical trial in which the effects of certain supplements were investigated on bone mineral density and vessel wall characteristics. The participants were selected based on strict in- and exclusion criteria, which were defined for the specific purposes of this clinical trial. Because participants were enrolled in a three-year controlled study, they were monitored frequently and therefore regular check-ups on health status and medication use took place. The vascular examination took place at baseline and at the end of the study after 3 years. Here we report the change of vessel wall characteristics in the placebo group of the study, thus elucidating the effects of ageing on the vessel wall characteristics.

Measurements

The ultrasonic vessel wall tracking system (WTS) to determine arterial wall properties has been described in detail before [9,19]. This ultrasound system provides estimates of the arterial end-diastolic adventitia-adventitia diameter (d) and the change in diameter from diastole to systole (Δd) normalized for the end-diastolic diameter (Δd/d) for each captured heartbeat. Simultaneously with measuring diameter changes, arterial blood pressure measurements were obtained at the level of the brachial artery by means of a semiautomated oscillometric device (DINAMAP). Pulse pressure (PP), defined as systolic minus diastolic blood pressure, was determined by averaging the three measurements nearest to the distension measurements. From d, Δd and Δp (PP), vessel wall properties were calculated according to the following equations:

$$DC = (2d\Delta d + \Delta d^2)/(d^2\Delta p)$$

$$CC = \pi(2d\Delta d + \Delta d^2)/4\Delta p$$

The intima-media thickness was measured simultaneously at the same location (2-3 cm proximal to the bifurcation) where the end-diastolic diameter and diameter changes were measured. Only the IMT of the posterior wall was assessed, because here the reflections from the blood-intima and media-adventitia transition are distinctly visible, whereas, at the anterior wall, the trailing edge of the adventitia reflections may obscure the media and intima signal. At the end of the session, recorded IMT-files were processed employing the wall thickness program. The threshold for the derivative was maintained at 0.025 [11]. Each heart beat within a recording resulted in an estimate of wall thickness; the median of the estimates per recording was used for further evaluation. The end-diastolic diameter, DC and IMT were also used to calculate the ratio of the end-diastolic diameter to IMT (D/IMT) and E ((D/IMT)/DC).

To save time we have investigated only the right common carotid artery. To our knowledge no different ageing effects have been reported in the wall properties of the right and the left common carotid artery. The same investigator performed all examinations at the start and the end of the study and for each participant several repeated measurements (5-7) were made during one session. Reproducibility was evaluated for the assessed common carotid artery distension and diameter. Before the vascular examination, height and weight were measured with standardized equipment to estimate the body mass index (weight/height^2).

Statistical Analysis

Statistical analysis was performed using the Statistical Package SPSS (SPSS Corp, Chicago, IL). For every participant, the percentage change from baseline in all parameters was calculated and results were presented as means ± standard deviation (SD), unless indicated otherwise. Only participants who had completed the study were included in the analysis. Furthermore, participants who during the study had started to use medication, known to have a direct effect on the vessel wall, were excluded from analysis. Also, participants with atherosclerotic plaques in the common carotid artery and a high variability in their results (arterial translation of > 2 mm and beat-to-beat variation in distension of > 20%) were excluded. A paired t-test was used to evaluate the change in the vessel wall characteristics over the three years within the group. We considered a level of $p<0.05$ to be statistically significant.

Table 1
Baseline characteristics study population (mean ± SD).

Baseline-characteristics	Mean ± SD (n=40)
Age (yr)	54.1 ± 2.97
Weight (kg)	69.5 ± 11.9
Height (m)	1.65 ± 0.05
BMI (kg/m^2)	25.6 ± 4.27
Years postmenopausal	4.6 ± 3.7
% non-smokers	97.5%

Abbreviations used: body mass index (BMI)

RESULTS

Subjects

Baseline characteristics of the participants are presented in Table 1. From the 60 control subjects who entered the study, 4 subjects discontinued their participation and were not available for the follow-up measurement. Of the 56 subjects who completed the study, we excluded 16 subjects from the analysis: 2 subjects started to use medication, known to have

direct effects on the vessel wall, in 3 subjects we found atherosclerotic plaques in the common carotid artery and 11 subjects had arterial translations of more than 2 mm or beat-to-beat variations in distension of more than 20%. Analysis was performed on 40 participants who completed the study and who did not have any of the above mentioned interfering factors.

Table 2
Change in vessel wall characteristics (mean ± SD) in study population after 3 years.

N=40	T= 0	T= 3 yr	Difference (T=3)-(T=0) (paired t-test)
Diameter (μm)	7162 ± 562	7358 ± 594	196 (p<0.001)
Distension (μm)	372 ± 118	351 ± 107	-21 (p<0.05)
Pulse Pressure (mmHg)	51.9 ± 11	54.6 ± 13	2.7 (p=0.093)
Distensibility (MPa^{-1})	15.8 ± 5.24	14.0 ± 4.8	-1.8 (p<0.01)
Compliance (mm^2/kPa)	0.64 ± 0.23	0.59 ± 0.19	-0.05 (p<0.01)
Intima-media thickness (mm)	0.62 ± 0.11	0.67 ± 0.10	0.05 (p<0.001)
Heart rate (beats/min)	60.8 ± 9.2	63.9 ± 10.1	3.04 (p<0.01)
Diameter / IMT	11.7 ± 1.9	11.1 ± 1.6	-0.6 (p<0.05)
E (kPa)	811 ± 296	885 ± 333	+74 (p=0.115)

Follow-up results

Figures 1 and 2 represent scatter plots of the individual values of DC and CC at baseline and after three years. These figures allow comparison of the individual changes of the participants. Table 2 summarizes the mean values and paired-levels of significance of all vascular parameters at the start and the end of the study. As compared to baseline, the DC had decreased by 10% (mean decrease: 1.8 Mpa^{-1}, 95% CI: 0.73 to 2.9, p<0.01) and the CC by 6% (mean decrease: 0.05 mm^2/kPa, 95% CI: 0.02-0.09, p<0.01). On the other hand, the arterial diameter had increased by 2% (mean increase: 196 μm, 95% CI: 102 to 291, p<0.001) and also the heart rate (beats/minute) had increased with 5% (mean increase: 3.0, 95% CI: 0.8 to 5.3, p<0.01). The pulse pressure increased by 5%, but this increase was not significant (mean increase: 2.7 mmHg, 95% CI: -0.48 to 5.9, p<0.10). Figure 3 shows the individual values of IMT at baseline and after three years of follow-up in the participants. The intima-media thickness (IMT) had increased by 9% relative to baseline (mean increase: 0.05 mm, 95% CI: 0.02 to 0.07, p<0.001), the D/IMT ratio decreased by 3.8% (mean decrease: 0.56, 95% CI: 0.06 to 1.06) and the E also increased by 13% relative to baseline (mean increase: 74 kPa, 95% CI: -19 to 167).

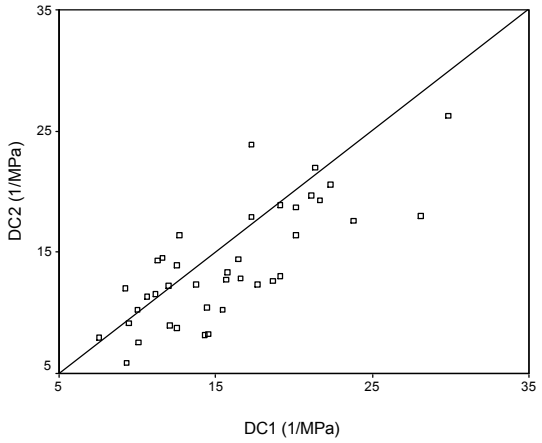

Figure 1
Scatter plot of individual values of distensibility coefficient at baseline (DC1 at x-axis) and after three years (DC2 at y-axis) in a group of healthy postmenopausal women (n=40).

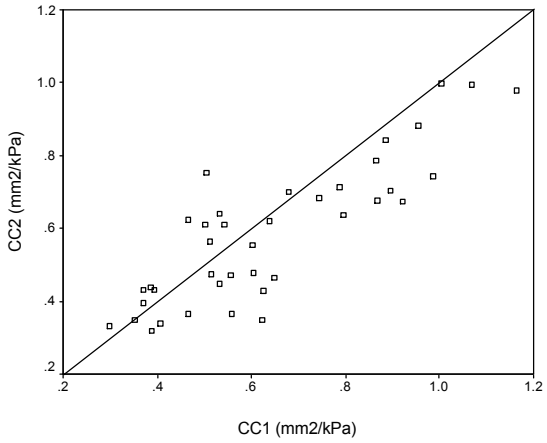

Figure 2
Scatter plot of individual values of compliance coefficient at baseline (CC1 at x-axis) and after three years (CC2 at y-axis) in a group of healthy postmenopausal women (n=40).

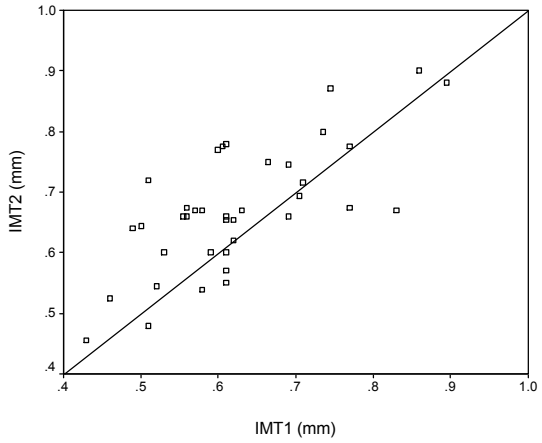

Figure 3
Scatter plot of individual values of intima-media thickness at baseline (IMT1 at x-axis) and after three years (IMT2 at y-axis) in a group of healthy postmenopausal women (n=40).

DISCUSSION

In this prospective study on effects of ageing on vascular characteristics in healthy volunteers, we observed a significant decrease in the distensibility and compliance of the common carotid artery after a 3-year follow-up period in postmenopausal women. The decrease in compliance was smaller than the decrease in distensibility. In parallel with the decrease in DC and CC we observed a significant increase in the arterial diameter and the intima-media thickness; the increase in E did not reach the level of significance. The less pronounced decrease in compliance can be explained by the concomitant increase in arterial diameter of the common carotid artery. Our results show that ageing causes a decrease in the elastic properties and an increase in the wall thickness of the common carotid artery (and possibly in other arteries as well), which may result in an increased risk of development of vascular diseases.

Although several cross-sectional studies have already shown the effects of ageing on the elastic properties of major arteries and a few prospective studies have shown the effects of ageing on IMT, to our knowledge no prospective studies have been reported showing the concomitant effects of ageing on DC, CC and IMT in the same group of healthy subjects. In a cross-sectional study by Reneman et al. [3] it was found that in healthy subjects between 50 and 60 years the DC decreased with 2.2% / year, which is slightly lower than our findings (-3.3% / year) and the CC was reported to decrease with 1.3% / year, which is also slightly lower than the 2% / year decrease found in our study. In a comparable study [4] in which the pressure strain elastic modulus (Ep) was used as an inverse estimate of distensibility, the distensibility decreased with 2.7% / year in women between their 45th and 60th year of age. The figures from these cross-sectional studies are fairly consistent with the data obtained using the follow-up design in our trial. In a recent longitudinal study [20], Barenbrock et al. evaluated the vessel wall properties in a group of 24 normotensive and 24 hypertensive renal transplant patients. Within two years the DC decreased by 6.3% (3.2 % / year). Although the results from the latter study are similar with our data, it must be kept in mind that the renal transplant patients form a specific and inhomogeneous population, which is not comparable with our study population. In the mentioned cross-sectional studies, linear extrapolation is used to estimate changes in the vessel wall properties over years while in longitudinal studies changes are detected in the same group of subjects. To find out if there might be a faster non-linear decrease in DC and CC in women in the early postmenopausal period, as is suggested by the study of Hansen [4], a longitudinal study with more subjects and longer duration is needed. Changes in IMT over time have been investigated in a few prospective studies; a two-year follow-up study in 100 Finnish men [12] showed a mean increase of 0.03 mm / year in subjects without atherosclerotic lesions, which is comparable to the increase in IMT in our study. Furthermore, our results are also in line with the results of the highest age-group (56-60 years) in a 6-year follow-up study of 472 men and women showing a mean increase in IMT of 0.03 mm / year [21]. Our results on E are comparable with the results of an earlier study [10], but differ from that of a recent cross-sectional study [22] in which a 20% lower

E was found, mainly due to a higher IMT and distension. This discrepancy might be explained by differences in recording techniques.
Reliable assessment of distensibility and compliance requires the determination of pulse pressure at the site of the measurement of the end-diastolic diameter and distension. The brachial pulse pressure tends to deviate from the pulse pressure in the common carotid artery [23]. One should realize, however, that errors due to differences in location of blood pressure measurement and artery wall property assessment will be methodological in nature and of the same order of magnitude at the various moments of determination, because intra-subject variation was determined in time. Therefore, the absolute error made in the determination of pulse pressure has not significantly influenced the outcome of our study.
In summary, in this study we have found that during three years in healthy postmenopausal women the elastic properties of the common carotid artery significantly decreased, whereas the intima-media thickness significantly increased.

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6 Beneficial effects of vitamin K on the elastic properties of the vessel wall in postmenopausal women: a follow-up study

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Submitted for publication

ABSTRACT

Matrix-Gla Protein (MGP) is a strong inhibitor of vascular calcification, the expression of which is vitamin D dependent. MGP contains five g-carboxyglutamic acid (Gla)-residues which are formed in a vitamin K-dependent carboxylation step and which are essential for its function. Hence vascular vitamin K-deficiency will result in undercarboxylated, inactive MGP which is a potential risk factor for calcification. In the present study we describe the effects of vitamin K_1 and D supplementation on vascular properties in postmenopausal women. In a randomized placebo-controlled intervention study 181 postmenopausal women were given either a placebo or a supplement containing minerals and vitamin D (MD-group), or the same supplement with vitamin K_1 (MDK-group). At baseline and after three years, vessel wall characteristics including compliance coefficient (CC), distensibility coefficient (DC), intima-media thickness (IMT) and the Young's Modulus (E) were measured to assess the effect of the supplements on the change of these parameters. The results showed that the elastic properties of the common carotid artery in the MDK-group remained unchanged over the three-year period, but decreased in the MD- and placebo-group. Comparing the MDK- and placebo-group, there were significant differences in decrease of DC (8.8%; $p<0.05$), CC (8.6%; $p<0.05$), and in increase of PP (6.3%; $p<0.05$) and E (13.2%, $p<0.01$). There were no significant differences between the MD-group and placebo. No significant differences were observed in the change of IMT between the three groups. It is concluded that a supplement containing vitamin K_1 and D has a beneficial effect on the elastic properties of the arterial vessel wall.

INTRODUCTION

Vitamin K is a cofactor in the post-translational carboxylation of selective protein-bound glutamate residues which are converted into gamma-carboxy glutamate (Gla) [1]. Presently only a dozen mammalian Gla-containing proteins (shortly: Gla-proteins) have been identified. Vitamin K deficiency results in the synthesis of under-carboxylated, biologically inactive Gla-proteins. Examples of Gla-proteins are several blood coagulation factors (all synthesized in the liver), osteocalcin (synthesised in bone) and matrix Gla-protein (MGP), which is synthesized in a number of non-hepatic tissues, notably cartilage and the arterial vessel wall. Today MGP is regarded as a major inhibitor of soft tissue calcification [2].

Vascular calcification is an important factor that contributes to considerable morbidity and mortality, notably in diabetics and in hemodialysis and atherosclerotic patients [3]. Calcification may occur either in the tunica intima in association with atherosclerosis or in the tunica media where it is known as Mönckeberg's sclerosis [4]. Medial vascular smooth muscle cells (VSMCs) synthesize most of the MGP in the vessel wall [5]. It was found that the overall arterial expression of MGP was decreased in Mönckeberg's sclerosis suggesting that low levels of MGP may predispose to calcification [6]. However, in VSMCs adjacent to the calcium salt deposits, MGP mRNA expression was substantially elevated which may represent a response to the locally increased calcium concentration in order to enhance calcium clearance [5]. Using monoclonal antibodies against MGP, Schurgers et al [7] demonstrated that MGP also accumulates around the calcified areas.

Although the precise sequence of events in the association between MGP-expression and calcification still needs to be elucidated, a number of animal studies have established unequivocally that MGP is a potent inhibitor of calcification. The vascular phenotype of the transgenic MGP null mouse showed massive calcification of the large arteries within 4 weeks after birth [8]. The fact that comparable results were obtained in normal rats after treatment with the vitamin K-antagonist warfarin demonstrated that Gla-residues are essential to the calcification inhibitor function of MGP [2].

Whereas vitamin K is involved in the posttranslational processing of MGP, vitamin D has a role in the regulation of MGP gene expression. Fraser et al have shown that the MGP promotor contains a vitamin D response element that is responsible for a 2-3 fold enhancement of MGP expression after vitamin D binding [9]. To our knowledge, a direct association between vitamin D deficiency and arterial media calcification has not yet been demonstrated, however. With respect to vitamin K, it was shown in postmenopausal women that low vitamin K_1 intake is a risk factor for aortic calcification [10]. In an independent population-based study among 4500 elderly subjects an inverse correlation was demonstrated between vitamin K_2 intake and aortic calcification, myocardial infarction and sudden cardiovascular death [11]. Based on these findings, it is suggested that in a substantial part of the population the vitamin K status of the arterial vessel wall is inadequate to support full MGP carboxylation [7]. We wish to put forward the hypothesis that local vitamin K deficiency forms a risk factor for vascular hardening, increasing stiffness, and loss of elastic properties.

The present study forms part of a large intervention trial, in which we investigated the effects of minerals (calcium, magnesium, and zinc) and vitamins D and K on bone mineral density and vascular properties. Here we report the effects of minerals + vitamin D, and minerals + vitamins D and K supplementation on the vascular properties during a three-year follow-up period. Measures of functional vascular properties are distensibility and compliance as they characterize the arterial stiffness of the vessel wall, which may be modified by medial calcification. The intima-media thickness (IMT) is assumed to represent an endothelial adaptive response to physiological and pathophysiological processes [12-14] and is regarded as an indicator of atherosclerosis [15,16]. The ratio of diameter to IMT corrects the functional parameter DC for the tissue volume involved, resulting in a parameter for the structural elasticity, known as the Young's Modulus (E) [17]. The present study also included the effects of vitamin K_1 and D on changes in IMT and E. We selected a group of healthy postmenopausal women to assess the effects of vitamin K_1 and D supplementation on changes in vessel wall characteristics, because it has been suggested that changes in vessel wall properties occur faster in postmenopausal women between 45 and 60 years than in men of the same age [18].

METHODS

Subjects

The participants were enrolled in a 3-year double-blind placebo-controlled clinical trial in which the effects of minerals, vitamins D and K were investigated on bone mineral density and vessel wall characteristics. The participants of this trial were recruited by newspaper advertisements. Inclusion criteria were: apparently healthy women, Caucasian race, between 50 and 60 years old and at least 2 years postmenopausal. Exclusion criteria were: use or recent use (< 1 year) of oral anticoagulants, corticosteroids, hormone replacement therapy, vitamin concentrates or food supplements, and alcohol consumption of > 2 glasses/day. In total 188 women met the criteria for participation, and were randomized into our study. Information on cardiovascular risk factors, current health status, medical history, drug use and smoking behavior was collected before the start of the study. No medical history of important systemic diseases was reported. Within this trial participants were seen every 3 months to check for compliance and physical health; on a number of these occasions bone densitometry (DXA) and blood sampling were performed. The vascular examination only took place at baseline and at the end of the study after 3 years. Baseline measurements were performed between November 1997 and March 1998, and the follow-up examination took place between December 2000 and March 2001. All participants gave written informed consent and the trial was approved by the University Hospital medical ethics committee.

Study Design

The subjects were randomized into three groups. In the first group (n=60) participants received a placebo (maltodextrine, i.e. placebo group), in the second group (n=58)

participants received a supplement containing 500 mg calcium (natural calcium complex derived from milk), 10 mg zinc, 150 mg magnesium and 8 µg vitamin D_3 (minerals + vitamin D = MD-group), and in the third group (n=63) participants received a supplement containing the same constituents as the MD-group but with additional 1 mg vitamin K_1 (minerals + vitamins D+K = MDK-group). In the present study, we compared the changes of vessel wall characteristics between treatment groups and placebo to elucidate the effects of vitamins K and D on the vessel wall characteristics.

The randomization of the participants to the three groups was performed according to a computer-generated randomization list and the randomization codes were kept apart from the study site during the trial. The participant randomization codes were allocated sequentially in the order in which the participants were enrolled. One investigator who supervised the whole study was responsible for the enrollment and assignment of participants to the respective groups. Because the three different types of supplements were similar in appearance and taste, participants and investigators were not aware of group assignment. Participants were allowed to choose between a supplement in the form of a tasteless powder (to be mixed with water before intake) or in the form of chocolate-coated tablets with a crunchy malt core. The percentage of subjects who used the powder or tablets was equally distributed across the three groups. Participants were instructed to take one sachet with powder or three tablets per day during evening hours, preferably after the meal. Also, they were advised to maintain their usual diets and to avoid taking supplements containing either calcium, vitamin D, or K two months before and throughout the study. Novartis Consumer Health SA (Nyon, Switzerland) prepared and provided all supplements. After randomization, the women received the first batch of supplements and were supplied with a new batch of supplements every six months.

Measurements

The primary outcome measures for the purpose of this study were the vessel wall characteristics of the common carotid artery measured with ultrasound (7.5 MHZ, ATL Mark V). The ultrasonic vessel wall tracking system (WTS) to determine arterial wall properties has been described in detail previously [19,20]. It provides estimates of the arterial end-diastolic adventitia-adventitia diameter (d) and the change in diameter (distension) from diastole to systole (Δd) normalized for the end-diastolic diameter (Δd/d) for each captured heart beat. In parallel with diameter change measurements, arterial blood pressure was recorded at the level of the brachial artery by means of a semiautomated oscillometric device (DINAMAP). Pulse pressure (PP), defined as systolic minus diastolic blood pressure, was determined by averaging the three measurements nearest to the distension measurements. From d, Δd and Δp (PP), vascular distensibility coefficient (DC) and compliance coefficient (CC) were calculated according to the following equations:

$$DC = (2d\Delta d + \Delta d^2)/(d^2\Delta p)$$

$$CC = \pi(2d\Delta d + \Delta d^2)/4\Delta p$$

The intima-media thickness (IMT) was measured simultaneously at the same location (2-3 cm proximal to the bifurcation) of the common carotid artery where the diameter and diameter changes were measured. Only the IMT of the posterior wall was assessed, because here the reflections from the blood-intima and media-adventitia transition are distinctly visible, whereas, at the anterior wall, the trailing edge of the adventitial reflections may obscure the medial and intimal signal. At the end of the session, recorded IMT-files were processed employing the wall thickness program. The threshold for the derivative was maintained at 0.025 [21]. Each heart beat within a recording resulted in an estimate of wall thickness; the median of the estimates per recording was used for further evaluation. The E follows from the ratio of the end-diastolic diameter to IMT (d/IMT), normalized with respect to the DC (d/IMT)/DC).

To save time we have investigated only the right common carotid artery. To our knowledge no significant differences between the wall properties of the right and the left common carotid artery have ever been reported. The same investigator performed all examinations at the start and the end of the study and for each participant several repeated measurements (5-7) were made during one session. Reproducibility was evaluated for the assessed common carotid artery distension and diameter. Before the vascular examination, height and weight of each participant were measured with standardized equipment to estimate the body mass index (weight/height^2).

Statistical Analysis

The sample size was calculated on the assumption that the desired minimal detectable effect was a 15% reduced decrease in distensibility of the MDK-group compared to the placebo group with a 90% power and a 0.05 level of significance. With the assumption of a dropout rate of 10% per year we calculated that 180 subjects had to be included. Statistical analysis was performed using the Statistical Package SPSS (SPSS Corp, Chicago, IL). Results are presented as means ± standard deviation (SD), unless indicated otherwise. Only participants who had completed the study were included in the primary outcome analysis. Furthermore, participants who during the study had started to use medication, that directly affects the vessel wall, were excluded from analysis. Also, participants with atherosclerotic plaques in the common carotid artery and a high variability in their results (arterial translation of > 2 mm and beat-to-beat variation in distension of > 20%) were excluded. The primary outcome analyses were repeated to exclude the possibility of bias by drop-outs and excluded subjects. In these repeat analyses, the missing values of drop-outs and excluded subjects were replaced by mean values of outcome variables of the total population of 108 participants. A paired t-test was used to evaluate the change in the vessel wall characteristics over the three years within each group. We considered a level of $p<0.05$ to be statistically significant. For every participant, the percentage change from baseline in all parameters was calculated and the mean change from baseline was calculated per group. Primary outcome analysis consisted of comparison of the change in DC, CC, PP, IMT, d/IMT and E between the MD-group and placebo and between the MDK-group and placebo using linear regression analysis. In this analysis, the change in vascular parameters relative to

baseline was used as dependent variable and the treatment groups and several covariates were used as explanatory variables. Baseline values of age, weight, smoking (yes or no), heart rate and mean arterial pressure were chosen as covariates, because their influence on the change in vascular properties or response to the supplementation could not be excluded.

Table 1
Baseline characteristics (mean ± standard deviation) in the three treatment groups.

Baseline-characteristics	Placebo (n=40)	MD-group (n=30)	MDK-group (n=38)
Age (yr)	54.1 ± 3	55.9 ± 2.8*	55.4 ± 2.8
Weight (kg)	69.5 ± 11.9	70.6 ± 11.1	66.3 ± 9.5
Height (m)	1.65 ± 0.05	1.65 ± 0.07	1.63 ± 0.06
BMI (kg/m^2)	25.6 ± 4.3	26 ± 4.4	25.1 ± 3.1
Postmenopausal age (yr)	4.6 ± 3.7	7.6 ± 5.1**	5.1 ± 4.3
Non-smokers (%)	75	73.9	85
Diameter (µm)	7162 ± 562	7314 ± 582	7173 ± 411
Distension (µm)	372 ± 118	353 ± 83	332 ± 83
Pulse Pressure (mmHg)	51.9 ± 11.1	52.9 ± 10.1	53.7 ± 14.3
Diastolic Blood Pressure (mmHg)	73.5 ± 7	73.9 ± 8.7	73.3 ± 8.6
Heart Rate (beats/min)	60.8 ± 9.2	63.1 ± 8.9	60.6 ± 6.6
CC (mm^2/kPa)	0.64 ± 0.23	0.61 ± 0.2	0.56 ± 0.17
DC (MPa^{-1})	15.8 ± 5.2	14.5 ± 4	14 ± 4
IMT (mm)	0.63 ± 0.11	0.64 ± 0.1	0.61 ± 0.08
Diameter / IMT	11.7 ± 1.9	11.6 ± 1.8	12 ± 1.7
E (kPa)	822 ± 300	852 ± 266	941 ± 298

* significant different from placebo ($p<0.05$)
** significant different from placebo and MDK-group ($p<0.05$)
Abbreviations used: body mass index (BMI), compliance coefficient (CC), distensibility coefficient (DC) and intima-media thickness (IMT).

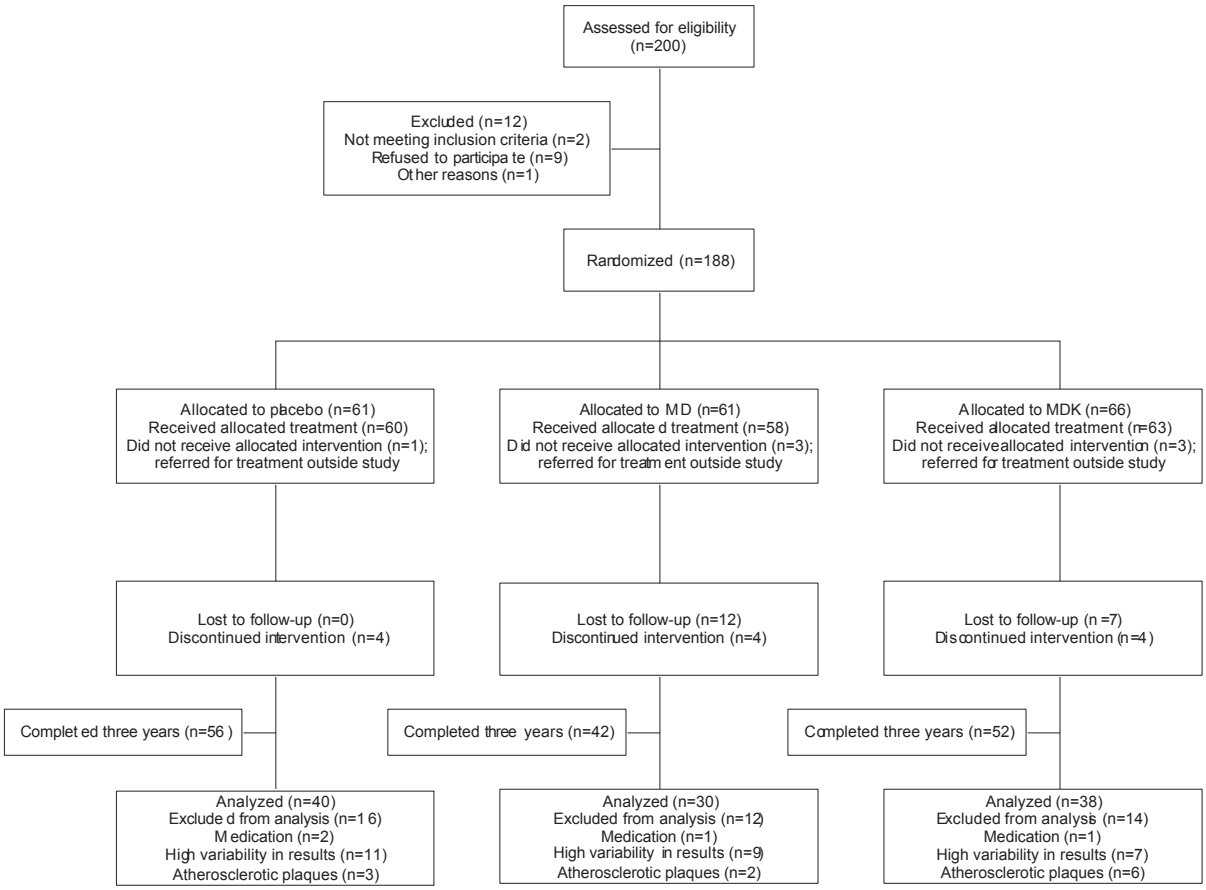

Figure 1
Flow diagram of participants through the different stages of the study.

RESULTS

Baseline characteristics

Baseline characteristics of the selected participants are presented in Table 1. The MD group differed slightly but significantly from the placebo and the MDK group with respect to age and the number of years since menopause. No significant differences were observed in the baseline values of the vessel wall characteristics. Figure 1 shows for the separate groups the flow of participants through each stage. From the 181 subjects who entered the study, 31 discontinued their participation during the course of the study, and were not available for the follow-up measurement. Of the remaining 150 participants who completed the study, we excluded 42 subjects from the analysis: 4 women started to use medication, known to have direct effects on the vessel wall (ACE-inhibitors, β-blockers or Calcium-antagonists), in 11 subjects atherosclerotic plaques in the common carotid artery were clearly visible and they were referred for treatment outside the study, and 27 subjects had a high variability in their repeated measurements (arterial translation of > 2 mm and beat-to-beat variation in distension of > 20%). Analysis was performed on 108 participants who completed the study and who did not have any of the above mentioned interfering factors. There were no significant differences in baseline values of the vessel wall characteristics between the 108

selected participants and those who discontinued treatment or were exluded (n=73). The only side-effect of the allocated intervention reported to the investigator were complaints of mild constipation in a few participants of the MD-group (n=4) and the MDK-group (n=3). No further adverse events occurred during the study.

Vascular parameters of elasticity

Table 2 summarizes per group the differences between the mean values at baseline and the end of the study for all vascular parameters with their paired-levels of significance. The DC and CC in the placebo group decreased significantly with 10% and 6%, respectively. The PP, on the other hand increased by 7%, but the increase did not reach the level of significance. In the MD-group, DC decreased significantly with 7% and CC decreased with 4%, while the PP increased with 6%, however these latter two changes did not reach the level of significance. In the MDK-group, the DC, CC and PP remained constant over the three years period, with even a tendency for the CC to increase (+3%). Figure 2 illustrates the change in DC and CC of the three groups. After adjustment for baseline heart rate, mean arterial pressure, age, weight and smoking, there were significant differences between the MDK-group and the placebo with respect to DC (8.8%; 95% CI: 1.9 to 21.4), CC (8.6%; 95% CI: 1.8 to 20.3), and PP (-6.3%; 95% CI: -17.1 to –0.7). In the same analysis no differences were found between the MD- and placebo-group with respect to DC (2.5%; 95% CI: -6.3 to 14.8), CC (2.2%; 95% CI: -6.3 to 13.8), and PP (-0.11%; 95% CI: -12.1 to 5.6). The repeat analyses in which mean values were allocated to drop-outs and excluded subjects, showed the same trends as those described above; as to be expected the differences between the MDK-group and placebo in change of DC, CC and PP were smaller, but they remained statistically significant.

Table 2
Change in vessel wall characteristics (mean ± SD) in study population after 3 years.

Change between T=0 and T=3 years (paired t-test)	Placebo (n=40)		MD-group (n=30)		MDK-group (n=38)	
Diameter (μm)	196 ± 295	(p<0.01)	154 ± 179	(p<0.01)	131 ± 226	(p<0.01)
Distension (μm)	-21 ± 61	(p<0.05)	-12.6 ± 47	(p=0.15)	-3.9 ± 49	(p=0.63)
Pulse Pressure (mmHg)	2.7 ± 9.9	(p=0.09)	2.8 ± 10.1	(p=0.14)	-0.2 ± 7.6	(p=0.89)*
DC (MPa^{-1})	-1.8 ± 3.4	(p<0.01)	-1.4 ± 3.0	(p<0.05)	-0.4 ± 3.0	(p=0.43)*
CC (mm^2/kPa)	-0.05 ± 0.1	(p<0.01)	-0.04 ± 0.1	(p=0.10)	0.01 ± 0.11	(p=0.75)*
IMT (mm)	0.05 ± 0.08	(p<0.01)	0.02 ± 0.09	(p=0.32)	0.06 ± 0.06	(p<0.01)
Diameter / IMT	-0.56 ± 1.5	(p<0.05)	-0.14 ± 1.5	(p=0.63)	-0.85 ± 1.1	(p<0.01)
E (kPa)	74 ± 279	(p=0.12)	68 ± 254	(p=0.17)	-27 ± 236	(p=0.50)*

* significant different from placebo (p<0.05)
Abbreviations used: compliance coefficient (CC), distensibility coefficient (DC) and isntima-media thickness (IMT).

Intima-media thickness

Changes of the intima-media thickness relative to baseline are also given in Table 2. In all three groups the IMT increased: 9% in the placebo ($p<0.01$), 10% in the MDK-group ($p<0.01$), but only 4% in the MD-group ($p=0.32$). The d/IMT ratio decreased significantly in the placebo and MDK-group with 3.8% and 6.5% respectively, while in the MD-group the ratio remained constant. The Young's Modulus (E) increased in the placebo and MD-group by 13.2% and 13.7% respectively, however these changes did not reach the level of significance. In the MDK-group E remained constant. Figure 2 illustrates the change in IMT and E of the three groups. In the multivariate analysis with adjustments for baseline heart rate, mean arterial pressure, age, weight, and smoking, the difference in increase of IMT between the MDK-group and placebo was 1.3% (95% CI: -3.2 to 9.2) and between the MD-group and placebo the difference was -4.5% (95% CI: -8.9 to 4.2). Hence neither for the MD- nor for the MDK-supplement a significant beneficial effect on the age-related increase of the IMT could be demonstrated. However, there was a significant difference in the change of E between the MDK-group and placebo (-13.2%; 95% CI: -35.8 to -5.3), while no significant difference was observed between the MD-group and placebo (0.46%; 95% CI: -23.2 to 9.4) and neither significant differences were observed between the groups in change of the d/IMT ratio.

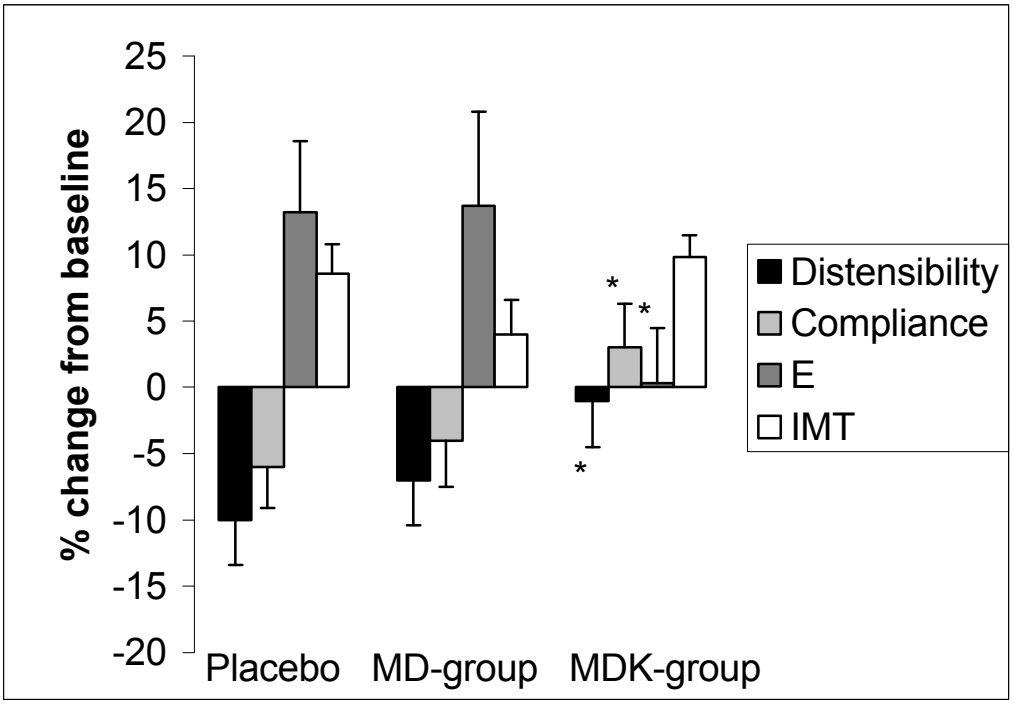

Figure 2

Mean percent change (± standard error of the mean) from baseline (0%) in distensibility coefficient, compliance coefficient, intima-media thickness and the Young's Modulus (E) of the common carotid artery in the placebo (n=40), group MD (n=30) and group MDK (n=38) after three years of treatment. *: $p < 0.05$, significantly different from placebo.

DISCUSSION

In the present investigation, we have demonstrated a long-term beneficial effect of a supplement containing vitamin K_1 and D on the elastic properties of the carotid artery. In contrast, no effect was found on the IMT. Vitamin D alone did not influence these variables. To our knowledge, this is the first study which shows a longitudinal beneficial effect of vitamins K_1 + D supplementation on vascular properties.

During recent years the classical role of vitamin K in blood coagulation has been extended to widely different functions in extra-hepatic tissues, such as bone and the vessel wall. Since the first observation of the association of poor vitamin K_1 status and age-related osteoporosis [22], population-based studies on correlations between low dietary vitamin K_1-intakes and undercarboxylated osteocalcin on one hand and fracture risk and low bone mineral density on the other hand have been accumulating [23-26]. The occurrence of vitamin K-dependent proteins in the arterial vessel wall was discovered more recently, and only limited data are available on a possible relation between vitamin K intake and vessel wall properties. In animals it was found that pharmacological doses of vitamin K_2 prevent the progression of atherosclerosis by supression of plaque formation, intima-thickening and pulmonary atherosclerosis [27]. One of the few studies in humans showed an inverse correlation between dietary vitamin K_1 intake and aortic calcification in postmenopausal women [10]. In a recent population-based study an inverse correlation was found between vitamin K_2 intake and aortic calcification, myocardial infarction and sudden cardiovascular death [11]. The latter study suggests that vitamin K_2 may be a more powerful inhibitor of arterial calcification than vitamin K_1. Furthermore, vitamin K_2 was shown to decrease the total circulating cholesterol concentration [28]. Most of the studies published thus far have focused on effects of vitamin K_2 and not of vitamin K_1.

When hypothesizing about the mechanism underlying our observations, we would like to focus on the vitamin K-dependent protein MGP. It is synthesized by the vascular smooth muscle cells [5], and accumulates in or around the elastic fibres in the tunica media [7]. In this respect it is noteworthy to mention that transgenic MGP-deficient mice developed arterial calcifications starting in the media, but even at later stages there was no neo-intima formation or atherosclerosis. Rather, the type of calcification was similar to that found in Mönckeberg's sclerosis of the media such as is often seen in diabetics and hemodialysis patients. In these patients calcification starts from the elastic lamellae of the media and occurs without inflammation. DC and CC are related to the functions of elastin and collagen in the vascular media as they represent the elastic properties of the vessel wall. IMT, on the other hand, is regarded as endothelial response to pathophysiological processes as in atherosclerosis. While DC and CC represent functional characteristics of the vessel wall, the Young's Modulus (E) expresses the structural characteristics of the tissue. In this study it was shown that supplementation with vitamin D+K exerted beneficial effects on both structural and functional characteristics of the vessel wall. There was no age-related change in structural characteristics (Young's Modulus) in contrast to the age-related change in the functional characteristics as shown in the placebo group.

In many papers it has been suggested that the vitamin K requirement of extra-hepatic tissues is substantially higher than that of the liver. Although these conclusions are mainly based on the bone Gla-protein osteocalcin, which was found to be undercarboxylated in the majority of the population (notably elderly) [29], also for MGP substantial undercarboxylation has been reported [30]. Assuming that at baseline also in our study population part of the MGP was synthesized in an undercarboxylated (i.e.: inactive) form, this must be expected to form a risk factor for media sclerosis and vascular stiffening, but not for atherosclerosis. In this view it is conceivable that increased vitamin K intake will increase the vascular vitamin K status and hence the production of active MGP, thus contributing to the inhibition of calcification and protection against arterial stiffening. Although this is precisely the effect observed in our study, it cannot be excluded that other vitamin K-dependent proteins are involved in maintaining vascular elasticity.

Since the MGP promotor contains a vitamin D-responsive element, it is theoretically possible that a low vitamin D status also affects the level of MGP expression. To make certain that the participants were sufficient in vitamin D, the treatment included supplementation with vitamin D (1 x RDA) together with the vitamin K_1 (8 x RDA). In a third arm of the study it was demonstrated that vitamin D alone had no effect on any of the vessel wall characteristics measured. No significant differences were observed in longitudinal changes between the placebo and the vitamin D group.

To which extent vessel wall characteristics of the carotid artery represent a measure for risk on cardiovascular disease remains to be investigated. In various studies increased IMT of the carotid artery was shown to be a risk factor for atherosclerosis, especially if the IMT is > 1 mm. Since in our study population the IMT in all three groups remained between 0.6 – 0.7 mm, it cannot be excluded that under different conditions (e.g. in older age groups, or after prolonged treatment) also an effect of vitamin K on IMT will be seen. This may especially occur at later stages of atherosclerosis in which calcification of the lesions forms an end stage process. Less information is available on the correlation of distensibility and compliance with cardiovascular disease, although in previous studies also arterial stiffness was shown to be associated with myocardial infarction and coronary artery disease [31,32]. In a recent study of 110 end-stage renal disease patients, a strong correlation was found between calcification score and arterial stiffness, especially with E, but also with DC, CC and IMT [33]. It has been suggested in this study that measurement of arterial parameters, exploring both structural and functional properties, could be helpful in the assessment of risk on cardiovascular disease and in the evaluation of risk reduction by treatment. However, extrapolation of these results to other groups may not be justified because of the particular characteristics of these patients. The fact that our data suggest a more pronounced effect of vitamin K_1 supplements on (medial) calcification than on IMT thickening warrants further studies in other populations, for instance diabetics or patients with severe atherosclerosis.

One limitation of our study is the fact that we observed a higher drop-out rate in the treatment groups than in the placebo group. Given the specific reasons for drop-out as mentioned in the results, it is unlikely that the treatment assignment was responsible for the higher drop-out rate. However, it cannot be excluded that the higher drop-out rate was due to minor

differences in taste of the different supplements. Another limitation of this study was the exclusion of part of our randomized population. Extensive analysis of the collected ultrasound data lead to necessary exclusions because of technical difficulties in measuring diameter changes within regular heart cycles, as exhibited by example arterial translations of > 2 mm and beat-to-beat variations in distension of > 20%. Additional analyses were performed to exclude the possibility that the high drop-out rate and exclusion of subjects had seriously biased our results. This turned out not to be the case. The study presented in this paper is a first step to corroborate the role of vitamin K in vascular tissue. Clinical trials measuring other endpoints and progression of calcification are needed to provide further evidence that vitamin K contributes to the prevention of cardiovascular disease.

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7 Assay for human matrix Gla-protein in serum: potential applications in the cardiovascular field

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ABSTRACT

Matrix Gla-protein (MGP) is synthesized in a vitamin K-dependent way in smooth muscle cells of the healthy vessel wall and its mRNA transcription is substantially upregulated in atherosclerotic lesions. Here we report the preparation of a monoclonal antibody against human MGP and its use in an enzyme-linked immuno sorbent assay. The intra- and inter-assay variations in serum samples were 5.4 and 12.6%, respectively, and the lower detection limit was 8.5% of the normal serum value. Individual within-day variations were below 11%, and did not show a distinct circadian pattern. Day-to-day variations in fasting morning samples were below 8%. In a first explorative survey, serum MGP concentrations were found to be significantly increased in patients with severe atherosclerosis, whereas they were normal in those with low bone mass and osteoporosis. This is consistent with the high MGP mRNA-expression observed in atherosclerotic vessels and plaques. Furthermore, in this study it was shown that the serum MGP levels were significantly correlated to one of the MGP-polymorphisms. More elaborate studies are required to assess the potential clinical utility of the newly developed assay.

INTRODUCTION

Vitamin K is a cofactor in the posttranslational conversion of glutamate residues into γ-carboxyglutamate (Gla). At this time 10 mammalian Gla-containing proteins have been described in detail, and the number of Gla-residues per molecule varies from 3 (osteocalcin) to 13 (protein Z). In all cases in which their function was known, the activity of the various Gla-proteins was strictly dependent on the presence of the Gla-residues [1]. One of the Gla-proteins is Matrix Gla-Protein (MGP); it is synthesized by chondrocytes and vascular smooth muscle cells [2,3]. Small amounts of MGP mRNA have also been detected in various other tissues [4], but this may reflect - at least in part - synthesis in small vessels and capillaries. Although its mode of action on a molecular level has remained obscure until now, recent data in rodents strongly suggest that MGP plays a key role in the inhibition of tissue calcification. MGP-deficient mice were generated by Luo et al. [2], who observed excessive cartilage and growth plate mineralization resulting impaired growth of the long bones. An even more prominent phenomenon, however, was that all animals showed massive calcification of the main arteries and died within eight weeks after birth due to rupture of the thoracic or abdominal aorta. The importance of Gla-residues for MGP to exert its mineralization-inhibitory function was demonstrated in rats in which extrahepatic protein carboxylation had been blocked by treatment with warfarin [5]. After 3-4 weeks of treatment these animals developed arterial calcifications starting around the elastic lamellae of the media in a similar way as was reported for MGP-deficient mice. Whether this effect was due to poor cellular excretion of undercarboxylated MGP or because of its lack of functionality has remained unclear in this experiment. It is generally assumed, however, that - like in other Gla-proteins - also in MGP the Gla-residues are important for its function. Taken together, the available data in rodents demonstrate that MGP is a potent inhibitor of tissue calcification, and that its posttranslational carboxylation is essential for exerting this activity in vivo. A recent publication from Munroe et al. suggested that vascular calcification in humans may be more complex than in rodents. These authors reported data for three unrelated patients with Keutel syndrome (KS), which is an autosomal recessive disorder characterized by abnormal cartilage calcification [6]. These authors showed that the three KS patients had (different) mutations in their MGP gene predicting frame shift or premature frame shift in the mature protein. KS patients may be regarded therefore as a human model for MGP-deficiency, but remarkably arterial calcification is not a common feature in KS. It should be noticed, however, that neither histological nor pathological examination of arteries had not been performed in the patients described, so that the effect of MGP-deficiency on human vascular biology remains to be investigated.

Cardiovascular disease is one of the major life-threatening diseases in the Western society, but biomarkers to monitor the severity or the progression of the disease are presently not available. Also, the number of biochemically detectable risk factors (e.g. serum cholesterol, triglycerides, ApoE genotype) is surprisingly low. Based on the - limited - data available, serum MGP is a good candidate to become a biomarker associated with arterial calcification. In this chapter we report the production of a monoclonal antibody (mAb^{3-15}) against human

MGP and the development of a microtiter plate-based assay with which circulating MGP was demonstrated and quantified in human serum. The assay may be used to explore the potential value of circulating MGP as a marker in the field of cardiovascular disease. Furthermore, the assay was also used to investigate if certain promoter polymorphisms in the MGP-gene are associated with fluctuations in MGP serum concentrations. The hypothesis is that polymorphisms may be present in the promoter region of MGP that could result in interindividual variation in transcription and tissue expression of MGP. The assay may help us to understand the role of MGP in human vascular biology.

METHODS

Materials

Synthetic peptides homologous to the sequences 3-15 and 63-75 of human MGP and the sequences 1-16 and 29-43 of human osteocalcin were synthesized and purified by Pepscan Systems (Lelystad, The Netherlands) and will be designated below as MGP^{3-15}, MGP^{63-75}, OC^{1-16}, and OC^{29-43}, respectively. All chemicals used were of analytical or high-performance liquid chromatography.

Preparation of recombinant MGP

mRNA's coding for MGP and osteocalcin (OC) were isolated from cultured human osteoblasts and used for preparing the corresponding cDNA's. Both cDNA's were inserted in the pQE-40 vector (Qiagen, Hilden, Germany) and expressed in E.coli M15 as chimeric proteins with murine dihydrofolate reductase (DHFR) equipped with an N-terminal 6-His tag for rapid purification (H.M.H. Spronk, unpublished data). Following expression, bacteria were lysed in buffer A (8 mol/L urea, 0.3 mol/L NaCl, and 0.01 mol/L Tris-HCl, pH 8.0). After centrifugation for 30 min at 10,000 x g the supernatant was passed over a Ni^{2+}-nitrilotriacetic acid agarose column (Qiagen, Hilden, Germany) in buffer A, and the 6-His tagged protein was eluted with buffer B (8 mol/L urea, 0.5 mol/L imidazole, pH 8.0). Unfortunately, the preparation thus obtained was insoluble under physiological conditions, which hampered its use as a reference material in the MGP assay.

Preparation of antibodies.

Balb/C mice were immunized intraperitoneally with the peptide MGP^{3-15}, which was coupled to keyhole limpet hemocyanin (Pierce Chemical Co). Twenty µg of antigen in Freund's complete adjuvant were used for the first immunisation, followed by three boosts (20 µg each) in Freund's incomplete adjuvant with 2-week intervals. Post-immune sera were screened for their affinity towards purified recombinant MGP, which was used as a chimeric construct with murine dihydrofolate reductase (DHFR, se below). At one week after the last boost, splenocytes of the best responder mouse were fused with an American Type Culture Collection (Manassas, Va) mouse myeloma cell line (Sp 2/01-Ag, CRL 8006) according to standard procedures, and growing hybridomas were screened by an enzyme-linked

immunosorbent assay (ELISA) in which recombinant proteins were coated to the microtiter plate. Positive clones were selected on the basis of specific recombinant DHFR-MGP recognition, whereas recombinant DHFR-osteocalcin served as a negative control. A clone with a strong and specific reaction with rMGP was selected for the large scale preparation of monoclonal antibodies (mAb^{3-15}). In a final step the IgG was isolated from the culture medium by protein G affinity chromatography.

MGP assay

Urea-solubilized rMGP (1 g/L) was diluted 50-fold with coating buffer (0.1 mol/L Na-carbonate, pH 9.6) and used for the coating of microtiter plates (50 µL/well). After incubation for 1 h at 37 °C remaining protein binding sites were blocked with 100 µL/well of blocking buffer (Hoffmann-La Roche, Basel, Switzerland, cat. no: 1 112 589) and incubated for another 1 h at 37 °C. After repeated washing (with washing buffer: 0.3% (w/v) tween-20 in PBS (0.15 mol/L NaCl, 10 mmol/L Na-phosphate, pH 7.4)) the plates were ready for use. The serum samples were diluted (as indicated) with PBS and 125 µL of sample were supplemented with 25 µL of mAb^{3-15} (6 mg/L in PBS containing 2% (w/v) non-fat dry milk protein (Nutricia, Zoetermeer, The Netherlands)) and incubated for 5 min at room temperature. Subsequently, 50 µL of sample were transferred to the microtiter plate and incubated for 1 h at 37 °C. After three washing cycles with PBS-tween-20 washing buffer (see above), the mAb^{3-15} bound to the plate was quantified using a second antibody (rabbit anti-mouse total IgG conjugated with horse radish peroxidase (Dako, 1 mg/L in PBS-tween)) and stained with 3,3',5,5'-tetramethylbenzidine (TMB from Hoffmann-La Roche). After 10 min the staining was stopped by adding 200 µL 1 M H_2SO_4, and the plate was read at 450 nm. The MGP content of pooled reference serum from 30 healthy individuals was arbitrarily defined to be 100 Units/L.

Subjects

Unless stated otherwise, fasting blood samples were taken. Serum was left at room temperature for 2 h before centrifugation (15 min, 2,000 x g) and storage at -80 Cº until use. Genomic DNA was extracted from leukocytes**.** For assessment of the normal range and reference groups, apparently healthy subjects were recruited among the Maastricht population. The day-to-day and within-day variations were determined in a group of 12 healthy men (20-35 years old), from whom blood was taken by venipuncture at nine time points on one day, and on 4 different days at 09.00 a.m. with one week intervals. Samples were also obtained from 200 healthy subjects (55-65 years old) in whom the intima/media thickness of the carotid artery was measured by ultrasound as described by Hoeks et al. [7]. In a collaborative study with the University of Cambridge, these samples were also used to determine the presence of certain promoter polymorphisms in the MGP gene. Blood sampling, MGP analysis and DNA extraction were performed at Maastricht, genomic analysis were accomplished at Cambridge [8].

Patient samples were obtained via the University Hospital Maastricht. The study was approved by the local Medical Ethics Committee, and informed consent was obtained from all participants according to the institutional guidelines.

Statistical analysis

The Student t-test (for groups n = 30), and the Mann Whitney U-test (for groups n < 30) were performed to assess whether observed differences between patient groups were statistically significant ($p < 0.05$). ANOVA and the Kruskal-Wallis test were used to detect differences in MGP-levels between the different types of several MGP-polymorphisms.

RESULTS

A. Calibration curve and test characteristics

Calibration curves were made on 12 different days using six different dilutions of pooled reference serum. Each dilution was measured in duplicate, and the mean optical densities (OD) at 450 nm (± SD) were expressed as a function of the serum concentration (Figure 1A). At increasing dilutions of the serum sample, more anti-MGP was bound to the plate, with the buffer value as a theoretical maximum. The lower detection limit was defined as the mean OD + three times the standard deviation of the buffer value, and amounted 2.096 – 3 × 0.089 = 1.829, corresponding with a MGP concentration of 8.5 U/L. The intra- and inter-assay variation of the test were determined using a four-fold dilution of the reference serum. The intra-assay variation was calculated by expressing the standard deviation as a percentage of the mean obtained from 21 replicates, repeated on three different days and amounted 5.4%. For assessment of the inter-assay variation, duplicate measurements were made on 14 consecutive days after which the standard deviation was expressed as a percentage of the means to give a value of 12.6%.

The validation of the assay was performed in a number of control experiments, which are partly summarized in Figure 1B. To eliminate the possibility of a false positive signal because of cross reaction of the second antibody with microtiter-bound proteins, the assay was performed in the absence of $mAb^{3\text{-}15}$. No response was obtained under these conditions. To eliminate the possibility that human serum contains auto-antibodies against MGP which might interfere with the assay, serum was transferred in seven subsequent steps across microtiter plate wells coated with rMGP before it was used in the MGP assay. Dilution curves of sera with and without this pre-treatment were identical, thus denying the occurrence of pre-existing anti-MGP. Next, we have investigated whether human test samples might contain IgG that would interfere with the assay by binding directly to mouse IgG. To this aim, serum was analysed in various dilutions before and after adsorption onto protein G sepharose. Both curves were identical, thereby showing that the assay was not disturbed by pre-existing anti-murine IgG.

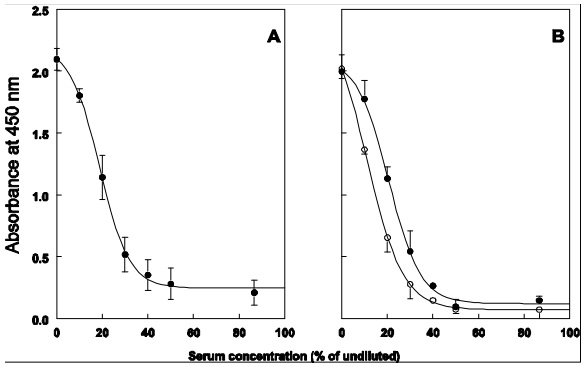

Figure 1
Dose-response curves with treated and untreated serum. A. Reference curve for MGP in human reference serum. Points are means of duplicate measurements made on 12 different days, error bars represent standard deviation. B. Controls with pooled serum: (closed circles), standard assay; (open circles), serum depleted of potential anti-MGP by adsorption onto rMGP; Points represent means of duplicate experiments.

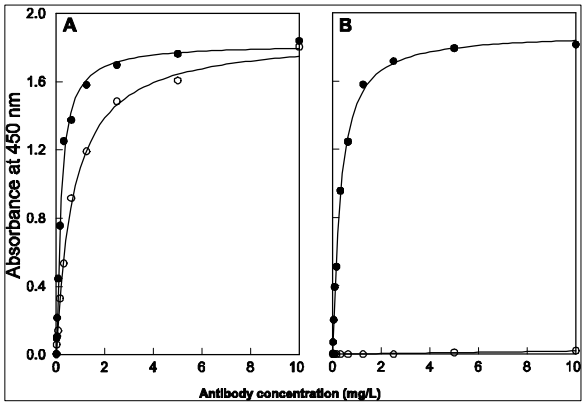

Figure 2
Reactivity of mAb$^{3-15}$ antibodies with purified rMGP (A) and rOC (B). The amount of recombinant protein on the microtiter plate was quantitated with anti-6His antibodies (closed circles), the reactivity with mAb$^{3-15}$ was tested in the same plate (open circles). In both cases staining was performed by incubation with a second antibody (rabbit anti-mouse total IgG conjugated with horse radish peroxidase) as described in Materials and Methods.

B. Sample preparation

To further evaluate the robustness of the assay, we have checked the influence of variations in the sample preparation procedure at the following steps: centrifugation speed (1,500 and 10,000 x g) during serum preparation, centrifugation (10,000 x g) after adding of mAb$^{3-15}$, freeze-thawing of the serum sample (up to 8 cycles of freeze-thawing) and incubation time (between 3 and 60 minutes at room temperature) of the serum sample with mAb$^{3-15}$. In none of these cases did the sample treatment measurably affect the observed MGP concentration.

C. Assay specificity

The mAb$^{3-15}$ used in the assay was tested for its ability to differentiate between two recombinant bone Gla-proteins: osteocalcin and MGP (both as chimeric constructs linked with 6His-DHFR). Microtiter plates were coated with either purified recombinant MGP (1 µg/well) or equimolar amounts of purified recombinant osteocalcin. Coupling efficiency of both proteins was checked with anti-6His antibodies. As is shown in Figure 2, both plates contained similar amounts of recombinant protein (A: MGP; B: osteocalcin), and mAb$^{3-15}$ reacted well with MGP, but not with osteocalcin. The species specificity of mAb$^{3-15}$ was tested further by comparing their reaction with human, rat, and murine serum. Cross reaction with rodent sera was below the detection limit (< 8.5 U/L) in all dilutions tested. Epitope specificity was tested by comparing the extent to which various synthetic peptides were capable of extinguishing the response with 10-fold diluted human serum. Under standard conditions (i.e.: when using 6.7 nmol/L of mAB$^{3-15}$) almost complete quenching of the signal was obtained by mixing the serum with 50 nmol/L of MGP$^{3-15}$, with a half-maximal effect at 5

nmol/L. No effect was observed with the peptides MGP^{63-75}, OC^{1-16} and OC^{29-43} up to concentrations of 65 µmol/L (see also Figure 3).

D. Normal range, within-day, and day-to-day variations

The 'normal range' for MGP was established in 80 apparently healthy men between 20 and 84 years of age. It was found that the mean value for serum MGP in this group was 96 ± 17 U/L. Hence the normal range (defined as the mean ± 2SD) was calculated to be between 62 and 130 U/L. No apparent age-dependence was observed for MGP in this group. Similar data were observed for elderly women (> 60 years of age), but a larger range was found in women between 20-55 years. This may be related to hormonal changes, and forms the basis for our decision that women < 60 years old were not included in the experiments presented in this paper. The time-related variability of serum MGP was established in a group of 12 healthy subjects from whom blood was taken by venipuncture on nine time points of one day, and on 4 different days at 09.00 a.m. with one week intervals. The within-day variation was calculated for each subject separately by expressing the standard deviation as a percentage of the mean of the nine time points, and amounted 11%. No distinct circadian pattern was observed (see Figure 4). The day-to-day variation was calculated in a similar way from the four samples obtained with weekly intervals, and was found to be 8%.

E. MGP in patients

The potential clinical utility of the newly developed MGP assay was tested in a pilot study among a limited number of patients. Since bone and arterial vessel wall are the major sites of MGP production, we have focussed on subjects with either bone disease (osteoporosis) or

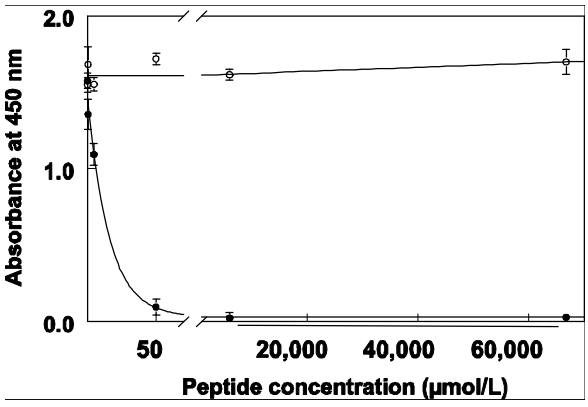

Figure 3
Reactivity of mAb^{3-15} antibodies with synthetic MGP-derived peptides. Serum was mixed with either MGP^{3-15} (closed circles) or MGP^{63-75} (open circles) before testing. Points represent means of triplicate experiments ± SD

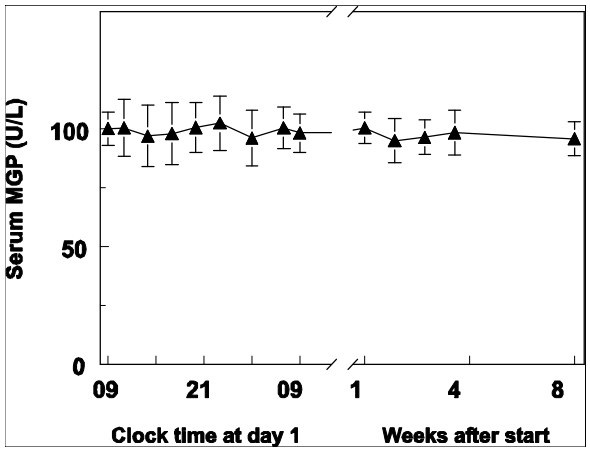

Figure 4
Absence of circadian pattern for circulating human MGP. Points represent means ± SD of twelve different subjects; nine blood samples were obtained during the first 24 hours, and 5 samples were obtained at 9 a.m. during the following two months.

vascular disease (atherosclerosis), and the data are summarized in Table 1. No correlations were found between serum MGP levels and either low or high bone mass, osteoporosis, or vascular intima thickening. On the other hand, circulating MGP was significantly elevated in subjects with advanced atherosclerosis. Also those with type I diabetes mellitus, a risk factor for atherosclerosis, circulating MGP had increased.

Table 1
Serum MGP concentrations in patients.

Condition	number	serum MGP (% of age-and sex-matched controls)
High femur BMD (mean + > 1SD)	40	98.5 ± 5.7
Low femur BMD (mean - < 1SD)	38	102.0 ± 5.2
Senile osteoporosis	28	101.8 ± 6.8
Increased intima/media thickness	43	97.3 ± 6.1
Type 1 diabetes mellitus	23	134.5 ± 8.6*
Severe atherosclerosis	26	145.8 ± 6.6*

Data are given as mean ± SE. Values indicated by an asterix were significantly different from the controls ($p<0.05$). Increased intima/media thickness was the highest quartile from a group of 200 apparently healthy elderly subjects. Subjects with high and low BMD of the femur neck were obtained from a reference population (n=250) recruited among the Maastricht population. Patients with osteoporosis, diabetes mellitus and atherosclerosis were obtained via various departments of the University Hospital Maastricht.

F. MGP-polymorphisms related to MGP serum levels

Four polymorphisms were identified in the promoter region of the human MGP gene, two of which (G-7A and the T-138C) were shown to have an important impact on the in vitro promoter activity. The T-138C variation lies in a region of the promoter which is critical for transcription in vascular smooth muscle cells because it contains a potential activating protein-1 (AP-1) binding element. Conversion of -138T to C results in altered binding of an AP-1 complex to this region. To test whether the observed variations in the promoter sequence might affect circulating MGP-levels in vivo, restriction fragment length polymorphisms analysis was performed for the T-138C and G-7A polymorphism on the samples of 156 healthy subjects in whom serum MGP had been assayed. It was found that the serum levels of MGP varied as a function of the T-138C (ANOVA, $p< 0.0001$; Kruskal-Wallis test, $p< 0.0001$) but not of the G-7A polymorphism (ANOVA, $p=0.67$; Kruskal-Wallis test, $p=0.759$). Thus, the CC variant at -138 was associated with higher mean serum levels of MGP (124.6 units/L) than subjects with TT variant (96.4 units/L). A gene dose effect is also evident, with the CT heterozygotes having intermediate values (101.9 units/L).

DISCUSSION

In this paper we report the development of an assay for human MGP, and with this assay (a so-called 'antibody-capture' ELISA) the presence of MGP-related antigen in the circulation was established. Recorded immunoreactive MGP turned out to be independent of sample preparation, and showed small within-day and day-to-day fluctuations. At this time the assay still has a number of weaknesses, inherent to a single-antibody assay. Substantial improvements may be expected from the availability of a second antibody, which will allow us to set up a 'sandwich' ELISA. The question remains what is the origin of serum MGP. The protein is known to be synthesized by the chondrocytes in cartilage, and by smooth muscle cells in the arterial vessel wall. In a first survey among a limited number of patients we have demonstrated that in severely atherosclerotic patients circulating MGP was significantly increased. In more elaborate clinical studies it should be investigated whether the severity of the disease (e.g. the extent of aortic calcification) correlates with the concentration of serum MGP, but at this time no such data are available. In collaboration with the University of Cambridge, four novel polymorphisms were identified in the promoter region of the human MGP gene. It was found that one of these polymorphisms (T-138C) is significantly correlated with serum MGP levels in human subjects. Therefore, this study strongly suggests a genetic basis to variations in MGP transcriptions and serum levels. It is interesting to hypothesize that these promoter polymorphisms may affect an individual's susceptibility to vascular or valvular calcification. It is possible that the -138C variant provides protection against tissue calcification in vascular smooth muscle cells by resulting in higher levels of MGP transcription. The results of this study have important implications for understanding the mechanisms underlying conditions that involve vascular calcification, such as atherosclerosis and aortic valve stenosis, since it strongly suggests a genetic basis for regulation of tissue calcification. In contrast to the data obtained in atherosclerotic subjects, MGP was found to be normal in all cases of bone disease tested thus far. The apparent association between serum MGP and vascular disease suggests that the circulating protein originates from the vessel wall rather than from bone.

In bone, MGP accumulates in relatively large quantities, which is why bone is the only tissue from which native MGP has been isolated thus far [9]. However, under physiological conditions MGP originating from human and bovine bone is one of the most insoluble proteins known. Comparison between its primary structure and the amino acid sequence derived from cDNA coding for MGP shows that in bone-derived MGP the last 7 C-terminal amino acids are missing [10], and it may be imagined that proteolytic cleavage of its C-terminus forms a mechanism for insolubilizing MGP by which it is retained in bone tissue. On the other hand it cannot be excluded that in bone MGP is complexed to the organic or inorganic matrix, or that it is folded in a way preventing its escape into the circulation. Because of its poor solubility it is difficult to envisage how significant amounts of MGP could be filtering from bone into the circulation.

From in situ hybridization we know that also in the arterial vessel wall MGP mRNA transcription takes place, but with the aid of immunohistochemical techniques only low levels

of MGP protein were found in the healthy vessel wall [11]. Thus, unlike bone and cartilage, healthy vessels do not retain considerable stores of MGP. The reported strong upregulation of MGP mRNA synthesis and high amounts of immunoreactive MGP at sites of atherosclerotic lesions [11-13] suggests a feed-back mechanism for local synthesis of MGP-related antigen, the Gla-content and calcification-inhibitory activity of which remains unknown. This is consistent with the hypothesis that at least part of the vascular MGP reaches the circulation and may account for the positive signal obtained in healthy subjects, and for the elevated serum values observed in atherosclerotic patients. Our hypothesis does not explain the mechanism by which circulating MGP remains in solution. One possibility is that after cellular secretion vascular MGP is processed differently than that in bone. From its primary structure it can be deduced that among the last 7 amino acids predicted by the cDNA sequence coding for the 84 residues of human MGP, five are positively charged. These 7 C-terminal amino acids are missing in MGP isolated from bone, but may be present in serum MGP. Hence the isolation and C-terminal sequence determination of serum MGP may provide evidence for its origin. An alternative explanation for the apparent solubility of serum MGP is that it may be bound to a soluble carrier protein or that it is associated with the lipoprotein fraction.

Assuming that the Gla-residues in MGP are essential either for its cellular secretion or for its calcification-inhibitory activity, poor vitamin K status could be an independent risk factor for tissue calcification. The latter hypothesis is consistent with data from Jie et al., who demonstrated in a population-based study (EPOZ) an inverse correlation between dietary vitamin K intake and the occurrence of calcified aortic lesions in elderly subjects [14]. The fact that major fractions of both osteocalcin and MGP isolated from human bone [15,16], as well as circulating osteocalcin seem to occur in an undercarboxylated form [17,18], suggests that human vitamin K requirement should not be inferred from the hepatic synthesis of fully carboxylated coagulation factors, but from the ability of non-hepatic tissues to maintain full carboxylation of locally produced proteins such as osteocalcin and MGP. All presently available data suggest that the extra-hepatic Gla-proteins are more susceptible to a reduced dietary intake of vitamin K than are the classical coagulation factors, and that present RDA values have to be redefined to ensure complete carboxylation of the extrahepatic Gla-proteins [19]. Unfortunately, the assay described in this paper does not allow for the discrimination between carboxylated and undercarboxylated MGP, which hampers full evaluation of its diagnostic value. More elaborate clinical studies are required to evaluate whether MGP total antigen may become a marker for the diagnosis or patient follow-up during the treatment of atherosclerosis.

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8 Summary and general conclusions

Samenvatting en Algemene Conclusies

SUMMARY AND GENERAL CONCLUSIONS

Vitamin K is a group name for a number of related compounds, which have in common a methylated naphthoquinone ring structure, but which vary in the aliphatic side chain attached at the 3-position. The two most important forms of vitamin K are vitamin K_1 (phylloquinone) and the group of K_2 vitamins (menaquinones). Green vegetables such as broccoli and spinach, as well as some plant oils are the major source of phylloquinone in the human diet [1-4]. Menaquinones are primarily found in meat (menaquinone-4, MK-4) and fermented foods like cheese and curds (MK-6 through MK-9) [4]. The only known function of vitamin K in mammals is that it serves as a cofactor for the endoplasmic enzyme gamma-glutamylcarboxylase. The vitamin K-dependent step is a posttranslational carboxylation reaction by which a number of well-defined peptide-bound glutamate residues are converted into gamma-carboxyglutamate (Gla) [5-7]. Gla-residues form calcium-binding groups in proteins, and are essential for the correct functionality of these proteins. During episodes of vitamin K-deficiency the carboxylation reaction cannot proceed and undercarboxylated species of Gla-proteins are released in the circulation. A general literature overview on vitamin K is given in **chapter 1**.

The classical role of vitamin K is its requirement for normal blood coagulation: six different Gla-proteins are involved in the regulation of this complex process: prothrombin, factors VII, IX, and X, and the proteins C and S [8]. More recently, it was discovered that other Gla-proteins function as regulator of bone formation (osteocalcin) [9,10], inhibitor of cartilage and vascular calcification (MGP) [11] and regulator of cell growth and apoptosis (Gas6) [12]. Gla-proteins seem to be important for bone and vascular health, and these two functions form the topic of this thesis. In the first part we describe the effects of vitamin K supplementation on bone health. Several authors have demonstrated that low serum vitamin K [13-15] and high circulating undercarboxylated osteocalcin concentrations [16-18] as well as low dietary intakes of vitamin K [19] are associated with low bone mineral density (BMD) and an increased risk for osteoporotic hip fractures. The two vitamin K intervention studies described in this thesis are the first clinical trials reported thus far in which the effects of vitamin K_1 supplementation on BMD in different risk groups are monitored. In **chapter 2** we have investigated the potential synergistic effect of vitamin K_1 and a mineral + vitamin D_3 supplement on bone loss in postmenopausal women during a 3-year treatment period. Participants received a daily supplement containing either a mixture of calcium, zinc, magnesium and vitamin D_3 or the same supplement with additional vitamin K_1; a third group received a placebo only. It turned out that the supplement containing minerals and vitamin D_3 transiently decreased the rate of bone loss. Addition of vitamin K_1 to this supplement, however, resulted in a long-term beneficial effect at the site of the femoral neck, leading to a 35% reduction of bone loss (as compared to placebo). It is to be expected that a more complete carboxylation of the bone Gla-protein osteocalcin contributes to the observed effects, but since the function of this protein on a molecular level has remained unclear thus far, the mechanism by which vitamin K promotes bone health remains a matter of speculation. It is at least encouraging that – although a complete halt of bone loss was not achieved in this study – the rate of bone loss

was substantially decreased by a simple food supplement. If this result may be extrapolated to longer treatment periods, the proposed formulation may result in a significant reduction of osteoporotic fractures. Our data have been applied by the nutrition industry, and the optimal formulation used in our study is presently marketed in several countries.
A second group at risk for rapid bone loss is formed by female endurance athletes. The high intensity and work load of training may lead to a disturbed hormonal metabolism, low endogenous oestrogen production, amenorrhoea, and loss of bone mass at young age [20,21]. Although these problems are well recognized among sport physicians, the number of prospective studies to quantify the bone loss and to monitor effect of treatment in this group is low. In **chapter 3** we report on a 2-year follow-up study among the largest cohort of female endurance athletes described thus far, in which we have quantified the rate of bone loss, and investigated the effects of oestrogen and vitamin K supplementation. Participants were recruited from Belgium, Germany and the Netherlands, and belonged to the national top in their sports. The rate of bone loss in female endurance athletes was unexpectedly high and a significant higher rate of femoral neck bone loss was found in the amenorrheic athletes compared to the eumenorrheic athletes. Oestrogen supplementation may retard, but did not prevent the bone loss. In contrast to the study in postmenopausal women, no beneficial effect of vitamin K was found in athletes. The most plausible reason for this apparent discrepancy is that bone loss is a multifactorial process in which also minerals and vitamin D play an important role. As a consequence an optimal effect of vitamin K will only be found if also the other components are supplemented. Another reason for the discrepancy between the data in postmenopausal women and female endurance athletes may be the accumulation of risk factors for bone loss in the latter group and the resulting extremely high rate of bone loss. It is at least feasible that under these conditions food supplements are inadequate tools to reverse this process, and that only medication such as bisphosphonates may prevent further damage.

In the second part of this thesis the function of vitamin K in vascular tissue was investigated. When hypothesizing about mechanisms underlying a possible relationship between vitamin K and vascular health, the most obvious protein involved is the vitamin K-dependent matrix Gla-protein (MGP), which is synthesized by the vascular smooth muscle cells in the arterial vessel wall. In experimental animals, MGP was found to be a potent inhibitor of soft tissue calcification [11], and its five Gla residues are essential for this activity [22]. Accumulating evidence suggests that in a substantial part of the population the vitamin K status of the arterial vessel wall is inadequate to support full MGP carboxylation [23], which may contribute to cardiovascular calcification. The hypothesis that subclinical vitamin K-deficiency forms a risk factor for cardiovascular disease is the basis for the second part of this thesis. Consequences of such deficiency at the site of the arteries may be vascular hardening and calcification, increasing stiffness, and loss of elastic properties. Several studies were performed to establish the potential importance of vitamin K for vascular health. In the well-known Framingham Offspring cohort we have investigated the association between vitamin K intake and intermediary markers for cardiovascular disease. In **chapter 4** we describe the

associations between reported dietary vitamin K intake, lifestyle, and serum lipid concentrations in this cohort. It turned out that participants with a reported high vitamin K_1 intake had a healthy dietary pattern and a healthy overall lifestyle. Furthermore, higher reported vitamin K_1 intakes were associated with a lipoprotein profile indicative of decreased risk for developing cardiovascular disease (high HDL-cholesterol, low LDL-cholesterol, and low ratio of total cholesterol to HDL-cholesterol). In contrast to vitamin K_1, the reported dietary vitamin K_2 intakes were associated with an overall less healthy lifestyle pattern, including higher BMI, higher intakes of total fat, saturated fat, cholesterol and meat, and lower intakes of dietary fruit and fibre. Surprisingly, reported vitamin K_2 intakes in the current study were also associated with a favourable lipid profile. The magnitude of the effect of K_2 is remarkable, because vitamin K_2 forms about 10% of the total vitamin K content in the human diet. The fact that both in healthy and unhealthy lifestyles vitamin K intake was positively correlated with a favourable serum lipid profile, is consistent with the hypothesis that low vitamin K intake is an independent risk factor in the development of cardiovascular disease. A subsequent step in investigating the importance of high vitamin K intake for vascular health was to measure the effect of vitamin K supplements on vessel wall characteristics. The term vessel wall characteristics is used to describe the elastic properties and the intima-media thickness of the vessel wall. Changes in the elastic properties of the main arteries have major implications for the development of vascular disease. Arteries, especially the larger ones such as the common carotid artery, become stiffer during ageing and arterial stiffness has been shown to be associated with cardiovascular disease [24,25]. Before investigating the effects of vitamin K supplementation on vessel wall characteristics, it was decided to monitor first in a prospective way the effects of ageing on vessel wall characteristics in healthy postmenopausal women (**chapter 5**). In this study we have measured with a 3-year interval the distensibility coefficient (DC) and the compliance coefficient (CC), both variables are generally used to describe the elastic properties of the vessel wall. With the same (ultrasound-based) technique, also the intima-media thickness (IMT) of the artery wall was measured [26-28]. The results showed a significant decrease in DC and CC of the common carotid artery as a function of time. In parallel with the decreased elasticity, we observed a significant increase of the arterial diameter and IMT of the common carotid artery. An increase in arterial diameter is the adaptation of the vessel wall to stiffening and the resulting increased blood pressure, whereas an increase of the IMT is generally regarded as one of the first steps in atherosclerosis [29,30]. The same cohort as described in chapter 2 was used to study effects of vitamins D+K supplementation on the vessel wall characteristics in postmenopausal women **(chapter 6).** Participants received either placebo, minerals + vitamin D, or minerals + vitamins D + K. For recording the vessel wall characteristics the outcome variables mentioned in chapter 5 were used. The supplement containing minerals and vitamin D did not influence any of the outcome variables. However, the supplement with additional vitamin K had a long-term beneficial effect on the elastic properties of the carotid artery, namely, DC, CC and also the blood pressure remained constant over the entire 3-year period. In contrast to the effects on vascular elasticity, no effect of vitamin K was found on the IMT. To our knowledge, this is the first intervention study showing a beneficial effect of

vitamins K_1 + D supplementation on the vessel wall characteristics. These data are consistent with a common subclinical vitamin K-deficiency, sub-optimal MGP carboxylation, and inadequate protection against cardiovascular calcification in non-supplemented subjects. Clinical trials in which the effect of vitamin K supplements on the progression of calcification is measured by more sophisticated techniques (such as computed tomography) are needed to provide conclusive evidence for the importance of vitamin K in the prevention of cardiovascular disease.
On the basis of our present knowledge, MGP seems to be the most likely Gla-protein via which vitamin K exerts its beneficial effect on the vessel wall. MGP does not exclusively accumulate in tissue, but in low concentrations it also occurs in the circulation. If the concentration and Gla-content of MGP in serum would reflect vascular MGP synthesis and Gla-content, circulating MGP might be used as a marker either for cardiovascular risk assessment, or for the diagnosis of cardiovascular disease. In this respect we differentiate between assays for total MGP, which are based on antibodies recognizing any form of MGP, and conformation-specific assays, which are based on antibodies against either fully carboxylated or undercarboxylated MGP. In **chapter 7** we describe the development of such assay for total MGP. It is shown that in patients with atherosclerosis and diabetes mellitus the serum MGP concentration is significantly increased compared to age-matched healthy controls. The assay was also used to demonstrate that certain promotor poymorphisms in the MGP gene are associated with a high or low MGP expression and parallel fluctuations in MGP serum concentrations. Although the results clearly show an association between serum MGP and cardiovascular disease, more work needs to be done before the diagnostic utility of this assay will be clear.

In conclusion, we have demonstrated that vitamin K_1 retards bone loss in postmenopausal women, but only if it is co-administered with minerals and vitamin D. We would recommend such products, therefore, for subjects at risk for bone loss (e.g. women after 50 years of age). If the effect continues during longer periods of supplementation, the threshold for osteoporotic fractures may shift to higher ages by 8-10 years, which must be regarded as a major benefit to public health and which may substantially contribute to the reduction of health care costs. The question of whether vitamin K alone is effective in counteracting high bone loss needs to be addressed since this could not be demonstrated in this thesis. Other questions remaining to be answered are: 1) are the effects of vitamins K_1 and K_2 of comparable magnitude, 2) what is the optimal dose for achieving maximal effect, 3) what is the optimal formulation of the supplement (e.g. other vitamins and minerals), and 4) is this formulation also of potential benefit for other groups at risk for high bone loss, such as endurance athletes, ballet dancers, astronauts during weightlessness, and patients with anorexia nervosa.
A second observation of potential importance was that vitamin K_1 strongly retards age-related loss of arterial elasticity. Because the vascular measurements formed the second arm of the study on bone density, it cannot be excluded that also in the vessel wall minerals and vitamins D and K have a synergistic effect. However, based on our present knowledge

this seems unlikely. Rather we would postulate that the observed effect is solely due to an improved vascular vitamin K status, resulting in a more complete MGP carboxylation and enhanced calcification inhibitory effect of this protein. Since at the dosage used no loss of elasticity was observed during the entire 3-year study, the minimal dose required for maintenance of elasticity remains to be established, and also the question of whether the effect may continue at much longer (lifelong?) intake of vitamin K. Without doubt this part of our work has the potency of a breakthrough in the primary and secondary prevention of cardiovascular disease.

The data on the utility of serum MGP as a marker for cardiovascular disease are promising but need further exploration. The assay described is a typical home-made lab test, and has been improved considerably since our first publication. In parallel with this assay for total circulating MGP antigen, assays for carboxylated and undercarboxylated fractions thereof are badly needed. Monoclonal antibodies on which such assays are necessarily based, are being developed at this time. Data presented in this thesis demonstrate the feasibility of the diagnostic approach, and form the start of an independent diagnostics line in our group. Apart from their potential importance in cardiovascular diagnostics, it may be expected that new, conformation-based assays will form a tool for the assessment of a subject's vascular vitamin K status (Glu-MGP / Gla-MGP ratio). This ratio may than be used as a guidance for the further development of functional foods and food supplements with health benefit claims in the cardiovascular field.

SAMENVATTING EN ALGEMENE CONCLUSIES

Vitamine K is de verzamelnaam voor een aantal vergelijkbare stoffen die allemaal dezelfde gemethyleerde naphthoquinon ringstructuur bezitten, maar die verschillen in de alifatische zijketen, gebonden aan de 3-positie. De twee belangrijkste vormen van vitamine K zijn vitamine K_1 (fyllochinon) en vitamine K_2 (menachinonen). De belangrijkste voedselbronnen van fyllochinon zijn groene groenten zoals broccoli en spinazie, en plantaardige oliën [1-4]. Menachinonen komen voornamelijk voor in vlees (menachinon-4, MK-4) en gefermenteerde producten zoals kaas en kwark (MK-6 t/m MK-9) [4]. Vitamine K functioneert als een cofactor voor het endoplasmatisch enzym gamma-glutamylcarboxylase. De carboxylering van specifieke glutaminezuur (Glu) residuen tot gamma-carboxy glutaminezuur (Gla) is een van de posttranslationele modificaties die worden uitgevoerd door het vitamine K-afhankelijke enzym gamma-glutamyl carboxylase [5-7]. De omzetting van Glu naar Gla is nodig voor de calcium bindende eigenschappen van Gla-eiwitten. Tijdens perioden van vitamine K–deficiëntie, kan de carboxyleringsreactie niet plaatsvinden en zullen er Gla-eiwitten in een niet-actieve, ondergecarboxyleerde vorm gesynthetiseerd worden. Een algemeen literatuur overzicht over vitamine K wordt weergegeven in **hoofdstuk 1** van dit proefschrift.

Vitamine K speelt een belangrijke rol in de bloedstolling: zes verschillende Gla-eiwitten zijn betrokken bij de regulatie van dit complexe proces: protrombine, factor VII, IX, and X, en protein C en S [8]. Meer recent zijn nieuwe Gla-eiwitten ontdekt die niet betrokken zijn bij het bloedstollingproces. Dit zijn onder andere osteocalcine (regulator van botvorming) [9,10], matrix-gla proteine (MGP) (remmer van kraabeen en vaatwand verkalking) [11] en Gas6 (regulator van celgroei en remmer van apoptose) [12]. Gla-eiwitten spelen dus ook een belangrijke rol in het bot en de vaatwand en deze functies vormen de basis van het in dit proefschrift beschreven onderzoek. In het eerste gedeelte beschrijven we de effecten van vitamine K-supplementatie op de botdichtheid. Lage serum vitamine K-concentraties [13-15], hoge niet-gecarboxyleerde osteocalcine concentraties [16-18] en lage innames van vitamine K [19], zijn geassocieerd zijn met een lage botdichtheid en een verhoogd risico op osteoporotische heupfracturen. De twee interventiestudies beschreven in dit proefschrift, zijn de eerste studies waarin de effecten van vitamine K-supplementatie op botdichtheid onderzocht worden. In **hoofdstuk 2** hebben we het mogelijke synergistische effect onderzocht van een supplement bestaande uit mineralen en de vitamines D en K_1 op het botverlies in postmenopausale vrouwen gedurende een periode van drie jaar. De proefpersonen ontvingen dagelijks een placebo of een supplement bestaande uit een mengsel van calcium, zink, magnesium en vitamine D_3 of hetzelfde supplement met extra vitamine K_1. Het bleek dat het supplement met mineralen en vitamine D_3 alleen een kortdurend effect had op de remming van het botverlies. De toevoeging van vitamine K_1 aan het supplement resulteerde echter in een langdurende bescherming tegen botverlies, waarbij het botverlies met 35% gereduceerd was ten opzichte van placebo. De verwachting is dat een meer complete carboxylering van het Gla-eiwit osteocalcine bijdraagt aan het geobserveerde effect, maar omdat de functie van het eiwit op moleculair niveau nog niet bekend is, blijft het achterliggende mechanisme via welke vitamine K het botverlies remt nog

onduidelijk. Het is op zijn minst opmerkelijk dat het botverlies gereduceerd werd door een eenvoudig voedingssupplement, hoewel een complete remming van het botverlies niet bereikt is in deze studie. Indien men deze resultaten extrapoleert naar langdurige behandelingsperioden, kan het gebruik van het onderzochte supplement resulteren in een significante reductie van het aantal osteoporotische fracturen. Onze resultaten worden gebruikt door de voedingsindustrie en het optimale supplement wordt op dit moment in verschillende landen op de markt gebracht.
Een tweede risicogroep voor het ontwikkelen van botverlies zijn de vrouwelijke atleten. De hoge intensiteit en belasting van training kan leiden tot een verstoring van de hormoonhuishouding, een lage endogene oestrogeen productie, amenorroe (het wegblijven van de menstruatie) en het verlies van botmassa op jonge leeftijd [20,21]. Hoewel deze problemen wel bekend zijn bij de sportartsen, zijn er maar weinig prospectieve studies die het botverlies in vrouwelijke atleten gekwantificeerd hebben en effecten van behandeling onderzocht hebben. In **hoofdstuk 3** beschrijven we een follow-up studie van 2 jaar omtrent een cohort van 115 vrouwelijk atleten, waarin de snelheid van botverlies gekwantificeerd is en de effecten van oestrogeen- en vitamine K-behandeling (zonder toevoeging van mineralen en vitamine D) onderzocht zijn. De proefpersonen werden gerekruteerd uit België, Duitsland en Nederland en behoorden tot de nationale top in de atletiek. De snelheid van botverlies van de femur hals was ongekend hoog in de vrouwelijke atleten en een significant hoger botverlies werd geobserveerd bij de atleten met amenorroe in vergelijking met de atleten met een regelmatige menstruatie. Oestrogeen-supplementatie verminderde wel het botverlies, maar zorgde niet voor een complete remming. In tegenstelling tot de studie in postmenopausale vrouwen, werden er geen gunstige effecten van vitamine K gevonden op de snelheid van het botverlies bij de vrouwelijke atleten. De meest voor de hand liggende verklaring voor deze schijnbare tegenstelling is dat botverlies een multifactorieel proces is waarin mineralen en vitamine D ook een belangrijke rol spelen. Als gevolg hiervan, zal een optimaal effect alleen gevonden worden indien deze componenten tegelijk met vitamine K ook gesupplementeerd worden. Een andere reden voor de discrepantie tussen de resultaten van de postmenopausale vrouwen en atleten is de accumulatie van risicofactoren voor botverlies in de atleten en het extreem hoge botverlies in deze groep. Onder deze omstandigheden is het waarschijnlijk vrijwel onmogelijk om het botverlies terug te dringen door eenvoudige voedingssupplementen en is op z'n minst medicatie (zoals bisfosfonaten) nodig om het proces stoppen.

In het tweede gedeelte van dit proefschrift wordt de rol van vitamine K in de vaatwand onderzocht. Het vitamine K-afhankelijke eiwit matrix-gla proteine (MGP) wordt gesynthetiseerd door de gladde spiercellen in de vaatwand. In experimentele proefdierstudies is aangetoond dat MGP een remmmer is van arteriële verkalking [11], en dat de vijf Gla-residuen binnen het eiwit essentieel zijn voor de activiteit van MGP [22]. Recente studies laten zien dat in een groot deel van de populatie de vitamine K status van de vaatwand onvoldoende is om volledige MGP carboxylering te bewerkstellingen [23], waardoor

het risico op arteriële verkalking verhoogd is. Mogelijk dat een suboptimale vitamine K-status, verharding, verhoogde stijfheid en verlies van elastische eigenschappen van de vaatwand tot gevolg kan hebben; dit leidt op den duur weer tot een verhoogd risico op het ontstaan van hart-en vaatziekten. Verschillende studies zijn uitgevoerd om deze hypothese verder te onderzoeken. In het bekende Framingham Offspring Cohort is de associatie tussen de vitamine K-inname via de voeding en intermediaire markers van hart-en vaatziekten bestudeerd. In **hoofdstuk 4** beschrijven we de associatie tussen vitamine K-inname, levensstijl en serum lipide concentraties in dit cohort. Uit de resultaten bleek dat personen met een hoge gerapporteerde vitamine K_1-inname een gezond voedingspatroon hadden en een algemeen gezonde levensstijl. Verder waren hoge vitamine K_1-innames geassocieerd met een gunstig lipoproteine profiel (hoog HDL-cholesterol, laag LDL-cholesterol, en een lage ratio van totaal cholesterol / HDL-cholesterol). In tegenstelling tot vitamine K_1, was de vitamine K_2-inname geassocieerd met een ongezonde levensstijl, namelijk een hoge body mass index (BMI), hoge inname van totaal vet, verzadigd vet, cholesterol, vlees en lage inname van fruit en vezel. De vitamine K_2-innames waren echter ook geassocieerd met een gunstig lipideprofiel, terwijl vitamine K_2 maar ongeveer 10% van het totale vitamine K gehalte in de voeding omvat. Het feit dat de vitamine K-inname correleerde met een gunstig lipideprofiel in associatie met zowel een gezonde als een ongezonde levensstijl is consistent met de hypothese dat een lage vitamine K inname een onafhankelijke risicofactor is voor het ontstaan van hart-en vaatziekten.

Een volgende stap in het bestuderen van de rol van vitamine K in de vaatwand was het meten van het effect van vitamine K-supplementen op vaatwandeigenschappen. De term vaatwandeigenschappen wordt gebruikt om de elastische eigenschappen van de vaatwand en de vaatwanddikte te beschrijven. Een verandering in de elastische eigenschappen van arteriën heeft implicaties voor het ontstaan van hart- en vaatziekten. Arteriën zoals de halsslagader, worden stijver met het ouder worden, en arteriële stijfheid is geassocieerd met hart-en vaatziekten [24,25]. Alvorens de effecten van vitamine K-supplementatie op de vaatwandeigenschappen werden onderzocht, werden eerst de effecten van veroudering op de vaatwand bestudeerd in gezonde postmenopausale vrouwen (**hoofdstuk 5**). Aan het begin en aan het einde van de 3 jaar-durende studie, werden met behulp van ultrasound de distensibiliteits coefficient (DC), compliantie-coefficient (CC) evenals de vaatwanddikte gemeten [26-28]. Deze variabelen worden gebruikt om de elastische eigenschappen van de vaatwand te beschrijven. De resultaten van deze studie toonden een significante daling in DC en CC als functie van de tijd. Parallel met de gedaalde elasticiteit werd een significante stijging van de arteriële diameter en de vaatwanddikte geobserveerd. Een stijging van de arteriële diameter kan gezien worden als een adaptatie proces als gevolg van de verstijving van de vaatwand en de gestegen bloeddruk, terwijl een gestegen vaatwanddikte algemeen beschouwd wordt als een van de eerste stappen van het atherosclerose proces [29,30]. Het in hoofdstuk 2 beschreven cohort is ook gebruikt om de effecten van vitamine D+K-supplementatie te bestuderen op de vaatwandeigenschappen (**hoofdstuk 6**). Het supplement bestaande uit alleen mineralen en vitamine D had geen invloed op de vaatwandeigenschappen, terwijl het supplement bestaande uit mineralen, vitamine D+K een

gunstig effect bleek te hebben op de elastische eigenschappen van de halsslagader. Gedurende de hele periode van drie jaar bleven de DC,CC en ook de bloeddruk constant; de vaatwanddikte was echter wel significant gestegen en niet verschillend ten opzichte van de placebo. Dit is de eerste interventiestudie die een gunstig effect van vitamine D+K_1 laat zien op vaatwandeigenschappen. Deze resultaten zijn consistent met de hypothese dat er in de vaatwand onvoldoende vitamine K aanwezig is voor een adequate carboxylering van MGP, dat op zijn beurt een onvoldoende bescherming tegen arteriële verkalking als gevolg heeft. Klinische trials, waarin de effecten van vitamine K-supplementatie op arteriële verkalking (gemeten door geadvanceerde technieken zoals CT) worden onderzocht, zijn nodig om bewijs te leveren dat vitamine K een belangrijke rol speelt in de preventie van hart-en vaatziekten.
Op basis van de huidige kennis, lijkt vitamine K zijn gunstige effect op de vaatwand uit te oefenen via MGP. MGP accumuleert niet alleen in weefsels, maar in lage concentraties komt het ook voor in de circulatie. Als de concentratie en het Gla-gehalte van MGP de vasculaire MGP-synthese zou reflecteren, dan kan het serum MGP gebruikt worden als een marker voor het risico op en de diagnose van hart-en vaatziekten. Hierbij dient onderscheid te worden gemaakt tussen tests voor totaal MGP, die gebaseerd zijn op antilichamen die elke vorm van MGP herkennen en conformatie-specifieke tests, die gebaseerd zijn op antilichamen die tegen ofwel alleen gecarboxyleerd dan wel niet-gecarboxyleerd MGP gericht zijn. In **hoofdstuk 7** beschrijven we de ontwikkeling van een assay voor totaal MGP. De resultaten laten zien dat bij patiënten met atherosclerose en diabetes mellitus de serum MGP concentratie significant hoger is dan bij gezonde controle patiënten. De test werd ook gebruikt om te demonstreren dat bepaalde promoter polymorfismen in het MGP gen geassocieerd zijn met een hoge of een lage MGP expressie en parallelle fluctuaties in MGP serum concentraties. Hoewel de resultaten duidelijk een associatie laten zien tussen serum MGP en hart-en vaatziekten, dient nog meer onderzoek te gebeuren voordat de diagnostische bruikbaarheid van deze test zichtbaar wordt.

Concluderend, in dit proefschrift hebben we aangetoond dat vitamine K_1 het botverlies remt in postmenopausale vrouwen, maar alleen als het samen gesupplementeerd wordt met mineralen en vitamine D. Dergelijke supplementen zijn dus aan te bevelen voor vrouwen die een verhoogd risico hebben op botverlies (vrouwen > 50 jaar). Als het effect aanblijft tijdens langdurig gebruik, dan is het treshhold niveau voor osteoporotische fracturen met 8-10 jaar verlengd. Dit heeft een belangrijke impact op de algehele gezondheid en draagt substantieel bij aan reductie van de kosten van gezondheid. De vraag of vitamine K alleen effectief is in het tegengaan van hoog botverlies dient beantwoord te worden in vervolgstudies, aangezien dit niet aangetoond kon worden in dit proefschrift. Andere vragen die nog beantwoord dienen te worden, zijn:
1) zijn de effecten van vitamine K_1 and K_2 vergelijkbaar 2) wat is de optimale dosis voor het bereiken van een maximaal effect 3) wat is de optimale samenstelling van het supplement (bijvoorbeeld welke andere vitaminen en mineralen), en 4) is dit supplement ook gunstig voor

andere risicogroepen voor botverlies, zoals astronauten, ballet danseressen and patiënten met anorexia nervosa.

De tweede belangrijke observatie in dit proefschrift is dat vitamine K_1 het verlies aan arteriële elasticiteit bij veroudering tegengaat. Het kan niet uitgesloten worden dat ook in de vaatwand mineralen en vitaminen D+K een synergistisch effect hebben. Gebaseerd op de huidige kennis lijkt dit echter onwaarschijnlijk. De hypothese is dat het geobserveerde effect vrijwel geheel te wijten is aan de verbeterde vasculaire vitamine K status, resulterend in een meer complete MGP-carboxylering en daarmee een versterkt remmend effect van arteriële verkalking door MGP. De minimale dosis die nodig is voor het behoud van elasticiteit dient vastgesteld te worden en ook de vraag of het effect aanblijft tijdens langdurig gebruik (> 3 jaar) van vitamine K supplementen is nog onbeantwoord. Zonder twijfel heeft dit onderzoek de potentie om te leiden tot een doorbraak in de primaire en secundaire preventie van hart- en vaatziekten.

De resultaten over de bruikbaarheid van serum MGP als een marker voor hart- en vaatziekten zijn veelbelovend, maar dienen verder onderzocht te worden. De beschreven assay is een typische home-made laboratorium test en dient verder ontwikkeld te worden na de eerste publicatie. Naast de test voor totaal circulerend MGP antigen, zijn assays voor gecarboxyleerd en ondergecarboxyleerd MGP antigen dringend nodig. Monoclonale antilichamen waar zulke assays op gebaseerd zijn, worden momenteel ontwikkeld. Data die gepresenteerd worden in dit proefschrift, demonstreren de mogelijkheid van een diagnostische benadering en vormen de start van een onafhankelijke diagnostische lijn binnen onze onderzoeksgroep. Naast het belang voor de cardiovasculaire diagnostiek, kan verwacht worden dat nieuwe conformatie-specifieke assays een instrument vormen voor de vaststelling van de vitamine K status van een persoon (Glu-MGP / Gla-MGP ratio). Deze ratio kan dan gebruikt worden als richtlijn voor de verdere ontwikkeling van functional foods en voedingssupplementen met gezondheid claims op het gebied van hart-en vaatziekten.

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ABSTRACTS

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CURRICULUM VITAE

Lavienja Braam werd op 5 april 1973 geboren te Berg en Terblijt. Na het behalen van het VWO B diploma aan het St. Maartenscollege te Maastricht, begon zij in 1992 met haar studie Gezondheidswetenschappen aan de Universiteit Maastricht, met als afstudeerrichting Biologische Gezondheidkunde. De afstudeerstage werd verricht aan de Katholieke Universiteit Leuven in België, waar onderzoek verricht werd naar het lipidenmetabolisme bij diabetes patiënten. In het laatste jaar van de studie volgde ze het epidemiologietracé en deed ze een extra onderzoeksstage bij het RIVM te Bilthoven, waarmee uiteindelijk de registratie tot Epidemioloog A werd behaald. In september 1997 ontving ze haar doctoraalbul Gezondheidswetenschappen en in datzelfde jaar werd Lavienja assistent in opleiding (AIO) bij de vakgroep Biochemie binnen de onderzoeksschool hart- en vaatziekten (CARIM) aan de Universiteit Maastricht. Onder leiding van Dr. C. Vermeer en Prof. Dr. J. Rosing werd onderzoek op het gebied van vitamine K verricht, waarvan de resultaten beschreven staan in dit proefschrift. Tijdens haar AIO-periode nam Lavienja nog deel aan een summerschool te Boston (USA) georganiseerd door het New England Epidemiology Institute. Een jaar later verbleef ze wederom in Boston om gedurende 4 maanden onderzoek te doen aan het Human Nutrition Research Center bij de onderzoeksgroep van Dr. S. Booth. In 1999 ontving ze de Young Investigators Award op het ISTH Congress (Washington DC, USA) en in 2000 de Fulbright Award van de Netherlands America Commission for Educational Exchange (NACEE). Aan het einde van haar AIO-periode kreeg Lavienja een verlenging van haar aanstelling binnen VitaK BV waar zij thans part-time werkzaam is als onderzoeker. Tevens is ze vanaf september dit jaar part-time werkzaam als projectleidster bij het voedingsconcern Danone te Parijs.

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