The role of vitamin K-dependent proteins in tissue calcification

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**THE ROLE OF VITAMIN K-DEPENDENT PROTEINS
IN TISSUE CALCIFICATION**

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THE ROLE OF VITAMIN K-DEPENDENT PROTEINS IN TISSUE CALCIFICATION

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ter verkrijging van de graad van doctor aan de
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*How can we heal the wounds of the world
If we cannot heal our own?
And where does this peace on earth begin
If not in the home?*

R. Ferrell

aan mijn ouders

*voor Liane
Li-Juan, Paul en Lisa*

ABBREVIATIONS

B-AP	bone-specific serum alkaline phosphatase
BGP	bone Gla protein, osteocalcin
CBB	coomassie brilliant blue
DBS	4-diazobenzenesulfonic acid
DDT	dithiothreitol
Gla	γ -carboxyglutamic acid
Glu	glutamic acid
HBC	hydroxyapatite binding capacity of serum osteocalcin (measure of Gla-content)
HPLC	high performance liquid chromatography
irOC	serum immunoreactive osteocalcin
irOC _{free}	serum immunoreactive osteocalcin with a low affinity for hydroxyapatite (undercarboxylated osteocalcin)
irOC _{bound}	serum immunoreactive osteocalcin with a high affinity for hydroxyapatite (carboxylated osteocalcin)
Menaquinone	vitamine K ₂
MGP	matrix Gla protein
M _r	molecular weight
OHPro	hydroxyproline
PG	proteoglycan
PGP	plaque Gla protein
Phylloquinone	vitamin K ₁
PIVKA	protein induced by vitamin K absence of antagonists
PVDF	polyvinylidene fluoride
RIA	radioimmunoassay
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
T-AP	total serum alkaline phosphatase
QAE	quaternary amino ethyl

CONTENTS

1	Introduction	9
1.1	General introduction	11
1.2	Vitamin K status	17
1.3	Vitamin K and bone metabolism	20
1.4	Vitamin K and atherosclerotic calcifications	26
1.5	Introduction to this thesis	29
2	Serum osteocalcin as a marker for vitamin K-status in pregnant women and their newborn babies	41
3	Effects of vitamin K and oral anticoagulants on urinary calcium excretion	53
4	Vitamin K induced changes in markers for osteoblast activity and urinary calcium loss	65
5	Efficiency of phylloquinone (vitamin K ₁) absorption from spinach in human volunteers	77
6	A population-based study in women with or without atherosclerosis	85
6.1	Vitamin K intake and osteocalcin levels	87
6.2	Vitamin K status and bone mass	99
7	The isolation of γ -carboxyglutamic acid (Gla)-containing proteins	111
7.1	A specific colorimetric staining method	113
7.2	Gla-proteins in human atherosclerotic calcifications	121
8	Summary / samenvatting	137
	Dankwoord	150
	Publications	153
	Curriculum vitae	155

INTRODUCTION

1.1 GENERAL INTRODUCTION

Discovery of vitamin K and vitamin K antagonists

In 1929 Henrik Dam reported the development of a bleeding disorder in chickens when fed a cholesterol depleted diet.¹ The bleeding symptoms were caused by a deficiency of a fat-soluble factor, which appeared to be essential for the maintenance of a proper blood coagulation. He named this antihaemorrhagic factor vitamin K (according to *Koagulation* in the German spelling).² Interestingly, the symptoms of the 'sweet clover disease' which prevailed among cattle in Canada and the American mid-west in the 1920's, showed many similarities with the bleeding disorder described by Henrik Dam.³ Improperly cured hay clover was found to be the cause of the disease. It was only several years later, however, that the connection with vitamin K could be made. In 1941 the responsible component in improperly cured hay clover was identified as 3,3'-methylene-bis-(4-hydroxycoumarin) (trade name: dicumarol). It appeared that dicumarol functions as an antagonist of vitamin K.⁴ Several substituted 4-hydroxycoumarins were synthesized and are now widely used as anticoagulants (warfarin, phenprocoumon or acenocoumarol)⁵ or rodenticides.⁶ Their use has led to the discovery that plasma of anticoagulated man and cows contains abnormal, biologically inactive forms of several blood coagulation factors.^{7,8,9} Although the occurrence of these so called PIVKA's (Protein Induced by Vitamin K Absence or Antagonists) in plasma was already postulated in 1963 by Hemker *et al.*,^{7,10} it took an additional ten years to understand the biochemical reason for their abnormal behaviour. In 1974 Stenflo discovered that the functionally inactive form of the blood coagulation factor prothrombin was lacking an unusual amino acid: γ -carboxyglutamate (Gla).¹¹ The highly negatively charged Gla appeared to be involved in the Ca^{2+} -dependent phospholipid binding, necessary for the biological activity of various blood coagulation factors.^{12,13}

Synthesis of Gla-proteins

It is now clear that vitamin K is essential for the carboxylation of certain protein bound glutamic acid (Glu) residues into γ -carboxyglutamic acid (Gla) (Figure 1). Hence 'vitamin K-dependent' proteins are called Gla-proteins. The formation of Gla takes place during the posttranslational modification phase of protein synthesis.^{12,13} Although dietary vitamin K is exclusively present as vitamin K quinone (K), it is its reduced form (vitamin K hydroquinone or KH_2), that acts as the coenzyme for the vitamin K-dependent carboxylase (Figure 1). During the

Figure 1. The vitamin K cycle: vitamin K is first reduced to KH_2 , then oxidized to KO . During the oxidation step glutamic acid (Glu) is reduced to γ -carboxyglutamic acid (Gla). Finally KO is further reduced to K . 1 = NADH-dependent reductase, 2 = dithiol-dependent K-reductase, 3 = dithiol-dependent KO -reductase.

carboxylation reaction KH_2 is converted to vitamin K epoxide (KO). In its turn, KO is reduced to K and subsequently to KH_2 by the action of two reductases (Figure 1). This recycling mechanism is very effective, explaining the rather low daily requirement for vitamin K. The exact nature of the reductases is not known yet. *In vitro* studies have shown that both the K- and KO -reductase are dithiol (dithiothreitol or thioredoxin)-dependent and they are both strongly inhibited by coumarin-derivatives.^{14,15} A blockade of (one of) the reductases ultimately leads to a cessation of the carboxylation reaction. The liver, however, contains a NADH-dependent K-reductase which is not inhibited by vitamin K-antagonists, and which exclusively reduces K and not KO . Therefore, bleeding symptoms due to an intoxication with coumarins can be counteracted by high doses of vitamin K, leading to the appearance of KO in plasma. There is some evidence from *in vitro* and *in vivo* studies that the osteoblast in bone tissue lacks the NADH-dependent K-reductase.¹⁶ Feeding animals with vitamin K and warfarin effectively blocks the carboxylation of the bone Gla-protein osteocalcin, whereas their blood coagulation factors are maintained at a normal level.^{17,18,19}

All Gla-proteins are secretory ones and are characterized by an intracellular precursor form possessing a "pre" and "pro" sequence. The hydrophobic pre-sequence is required for translocation across the rough endoplasmic reticulum where it is subject to further posttranslational modifications such as pre-peptide cleavage, γ -carboxylation, disulphide bond formation, glycosylation, etc. The

Table 1: Classification of Gla-proteins

Class	Occurrence or site of synthesis	Characterised
Blood coagulation	Liver	Coagulation factors II, VII, IX and X
	Liver, endothelial cell, megakaryocyte	Proteins C, S and Z
Calcified tissues	Bone	Osteocalcin, Matrix Gla-protein (MGP) and Protein S
	Atherosclerosis	Osteocalcin, MGP and Plaque Gla-protein (PGP)
	Kidney stones	Kidney Gla-proteins
Venoms	Snake	
	Snail	
Miscellaneous	Various tissues, fibroblast and endothelial cell	'GAS-6'
	Spermatozoa	
	Urine	Nefrocalcin

pro-sequence serves as a recognition signal for the vitamin K-dependent carboxylase.^{12,13} Structural elements required for recognition by carboxylase are thought to be the highly conserved amino acid residues at the positions -16 (Phe) and -10 (Ala/Gly).²⁰ The Gla-domain itself contains a sequence which is found in all mammalian Gla-proteins known thus far: Gla-X-X-X-Gla-X-Cys, but its relevance for the enzyme-substrate interaction is not clear at this time.²¹

Occurrence of Gla-proteins

Milestones in the field of vitamin K research have been the discovery of Gla in prothrombin¹¹ and the development of an *in vitro* assay system for vitamin K-dependent carboxylase enzyme from liver microsomes.²² These developments have provided a means with which to search for vitamin K-dependent proteins by analysing various tissues for the presence of the vitamin K-dependent carboxylase enzyme and proteins for the presence of Gla.¹³ This has led to the discovery that the vitamin K-dependent carboxylase enzyme is present in a wide variety of tissues such as the liver, bone,²³ kidney,²⁴ placenta, pancreas, skin,²⁵ spleen, lung, testis²⁶ and the vessel wall.^{27,28} In addition, the carboxylase enzyme has

been detected in cultured cells such as hepatocytes, renal tubule cells, fibroblasts, endothelial cells and various tumour cell lines.²⁹ Despite the widespread distribution of the vitamin K-dependent carboxylase, the actual number of well characterized Gla-proteins has been surprisingly low (Table 1).

The majority of the currently known Gla-proteins found in plasma belong to the Gla-proteins involved in blood coagulation: FII, VII, IX, X and the proteins C, S and Z. Because they are mainly synthesized in the liver they are commonly referred to as the hepatic Gla-proteins. Gla-proteins belonging to the extra-hepatic group have been found in a wide variety of calcified tissues, such as bone (osteocalcin,^{30,31} matrix Gla-protein,³² protein S^{33,34}), dentine³⁵ (osteocalcin), calcium containing renal stones (kidney Gla-protein,³⁶), heterotopic and cardiovascular calcifications³⁷ (osteocalcin, plaque Gla-protein³⁸) and coral.³⁹ The second group of extra-hepatic Gla-proteins comprises a group of venoms from snakes and snails and finally a miscellaneous group including Gla-proteins identified in spermatozoa,⁴⁰ snake,^{41,42} urine (nefrocalcin⁴³) and snail⁴⁴ venoms and a Gla-protein involved in growth arrest ('GAS-6').⁴⁵ Except for osteocalcin and MGP, the extra-hepatic Gla-proteins have not been characterized to a molecular levels, which hampers further research as to their function.

Various forms of vitamin K

Compounds possessing 'vitamin K activity' are defined as compounds with anti-haemorrhagic activity in animals fed a vitamin K-deficient diet. They have a 2-methyl-1,4-naphtoquinone ring structure in common but differ in length and the degree of saturation of their aliphatic side chain at the 3-position (Figure 2). Based on their origin, three main vitamin K compounds can be distinguished. Phylloquinone or vitamin K₁ (2-methyl-3-phytyl-1,4-naphtoquinone) is found in plants and has the same phytol side chain as chlorophyll. The second K vitamer is produced by certain bacteria and consists of a large group of compounds known as menaquinones or vitamin K₂ (2-methyl-3-multi-prenyl-1,4-naphtoquinone). The menaquinones (MK-n) are distinguished from each other by the varying number (n) of isoprene residues in their unsaturated aliphatic side chain. Natural menaquinones range from MK-4 to MK-13. Finally, the synthetic homologue menadione (vitamin K₃) contains no side chains (2-methyl-1,4-naphtoquinone). Menadione should be regarded as a provitamin as it is first converted to menaquinone.⁴⁶ Its bisulfite derivative is water soluble and mainly used in animal foods as a vitamin K compound. In newborn babies its application has shown to lead to haemolysis and kernicterus, possibly due to the high reactivity of the

Figure 2. Chemical structures of phyloquinone (vitamin K1), menaquinone (vitamin K2) and menadione (vitamin K3)

unsubstituted 3-position. Its use in humans is therefore not recommended.^{47,48,49} If not further specified the term vitamin K will be used to describe a compound with vitamin K activity.

Sources

For humans the main source of phyloquinone is from green vegetables where it is found in tight association with chlorophyll. It is striking to note that there is relatively little data concerning the phyloquinone content of different food products. The limited number of published values vary widely, probably due to differences in detection methods, sampling size and sampling method. Variations due to differences in soil and growth conditions, geographical differences and time of harvesting have also been described.⁵³ Various ways of processing such as heating, freezing and gamma-irradiation do not markedly influence phyloquinone levels in food.^{50,51} Vegetables with the highest vitamin K₁ concentration are green leafy vegetables such as spinach, kale, cabbage and broccoli (30-1200 µg/100g edible portion).^{52,53}

To date, there have only been a few reports on the menaquinone content in different food products.^{54,55,56} Significant amounts of menaquinones occur in fermented food products like cheese, yoghurt (30 µg/100g)⁵⁶ and the Japanese natto which is very rich in MK-7 (900 µg/100g).⁵⁴ Although the colon harbours considerable amounts of menaquinones producing bacteria (eg. *E.Coli*, *B.Fragilis*),⁵⁷ it is highly questionable whether they are absorbed from this site.^{58,59,60,61} Supporting data comes from Shearer et al. who describe that in

Table 2: Mean fasting plasma phylloquinone levels in various groups

Subjects	Plasma phylloquinone levels (ng/ml)	
	Mean	Median
Healthy newborns (cords)	≤ 0.02	0.02
Healthy adults (fasting)	0.41	0.37
Healthy adults (non-fasting)	0.66	0.53
Pregnant women (at term)	2.00	
Hypercholesterolaemia	0.76	0.71
Hypertriglyceridaemia	2.55	1.97
Mixed hyperlipidaemia	4.60	1.90
Diabetes (normal lipids)	0.62	0.35
Diabetes (raised lipids)	0.88	0.72
Haemodialysis patients	0.74	0.41

Partly adapted from ref. 64

human volunteers variable amounts of menaquinones (MK6 to MK10) are present in endoscopically obtained ileal juice. These menaquinones were mainly membrane-bound, however, since they could be removed from the ileal juice by centrifugation of the sample. Their bioavailability, therefore, must be regarded as being low.⁵⁶

Absorption, transport and storage

There is no information on the efficacy with which vitamin K is liberated from different food products. It has been suggested that the net absorption from food varies from 40-70%.⁶² It is to be expected that this variations will be depended upon the source: the extraction of phylloquinone from vegetables, where it is tightly bound to the chloroplast membranes, will be less efficient than from dairy produce.⁶³ Studies performed with radiolabeled purified phylloquinone indicate that up to 80 % of an oral dose is absorbed.⁶⁴

The absorption of phylloquinone and menaquinone is thought to be comparable to that of the other fat soluble vitamins. Although some studies concerning the mechanism of absorption are frequently quoted, their relevance is highly questionable due to the non-physiological conditions used.⁶⁵ Both bile salts and pancreas lipase are necessary components for the intraluminal solubilisation and subsequent absorption.⁶⁴ Their importance is illustrated by the finding that par-

ticularly patients with cholestatic disease (impaired bile salt secretion) and to a lesser extent those with pancreas insufficiency, are prone to developing vitamin K-deficiency.⁶⁶ After its uptake by the intestinal mucosal cells, vitamin K is incorporated into chylomicrons, secreted into the lymph, after which it reaches the systemic circulation.⁶⁴ Phylloquinone is mainly transported in the triglyceride rich lipoproteins.^{67,63} Higher plasma levels are generally found in non-fasting than in fasting subjects.^{67,68} Very high plasma phylloquinone levels are found in various lipid disorders (Table 2).⁶⁸ Recently it has been suggested that plasma phylloquinone levels are genetically influenced by the apolipoprotein E genotype, an important determinant of the chylomicron-remnant clearance rate.⁶⁹ Whether the extremely high vitamin K-levels observed in the lipid disorders are representative of their tissue vitamin K-status remains to be investigated.

Presently available data indicates that the major vitamin K stores are located in the liver. Remarkably, the majority (> 90%) of the human liver vitamin K store consists of long chain menaquinones and not phylloquinone.^{58,68} The bio-availability of the high hepatic menaquinone store is uncertain, since it has been shown that a vitamin K-deficiency can be induced within a few weeks with a dietary restriction of phylloquinone.⁷⁰ Also in bone tissue vitamin K (menaquinones and phylloquinone) levels comparable to those in the liver have been described.⁷¹ Data about their bioavailability, however, is lacking.

1.2 VITAMIN K STATUS

Requirements of vitamin K

The minimal daily requirement of vitamin K has been estimated at 1-2 $\mu\text{g}/\text{kg}$ body weight. This estimation, however, is based on the minimal dose needed for the secretion of fully carboxylated coagulation factors.^{72,70} The daily vitamin K intake reported in the literature varies from 50-500 $\mu\text{g}/\text{day}$, depending on the characteristics of the population studied.^{67,72,70} Clinical vitamin K-deficiencies characterized by abnormal coagulation tests do not occur in the general healthy population. The daily nutritional vitamin K intake is therefore generally regarded as sufficient, as far as the blood coagulation factors are concerned.

Plasma vitamin K levels

At the present time the low menaquinone levels still pose detection problems. Therefore the assessment of plasma vitamin K levels is generally based on phyloquinone levels. However, the extent to which the plasma phyloquinone levels reflect one's tissue vitamin K-status is debated. As discussed earlier, the use of plasma phyloquinone levels as indicator of one's vitamin K status is limited by the marked influence of various lipids (notably the triglyceride-rich lipoproteins) and non-fasting state on circulating levels. The extremely low phyloquinone levels in newborn babies may be partly explained by the very low plasma lipoprotein levels at birth, and underestimate the vitamin K stores in the tissues.⁵⁶ It has been suggested therefore, that the ratio of plasma phyloquinone to plasma triglyceride would be a better index of vitamin K status than plasma phyloquinone alone.^{67,63} Presently available data show that it is only in a limited number of cases (e.g. hospitalized patients with a poor nutritional status) that low phyloquinone levels as such may be indicative for impaired vitamin K reserves.⁶³

Descarboxy-prothrombin

Another way to define a person's vitamin K status may be through the detection of partially or non-carboxylated Gla-proteins, the concentration of which should decrease after vitamin K supplementation. Traditionally, descarboxy-prothrombin has been used as a marker to detect a state of vitamin K-deficiency. Several tests for the detection of descarboxy-prothrombin have been described, the most sensitive one being based on monoclonal antibodies which specifically recognize descarboxy-prothrombin.^{73,74} Descarboxy-prothrombin is generally not found in the plasma of the apparently healthy population, but can be measured after severe dietary restriction of vitamin K for a few weeks.^{75,76} Using monoclonal antibodies to detect descarboxy-prothrombin, only very low concentrations are found in 20-89% of the healthy newborn babies.^{73,77}

Descarboxy-prothrombin levels have been found in subjects with acute hepatitis and cirrhosis. Vitamin K-supplementation did not decrease the descarboxyprothrombin levels, however, indicating that these subjects had an impaired vitamin K-dependent carboxylation in the liver, but no vitamin K-deficiency.⁷⁸ In subjects with a hepatocellular carcinoma, descarboxyprothrombin is frequently found and may be used as a tumor marker.⁷⁹

Descarboxy-osteocalcin

Since it has been shown that the liver preferentially extracts phyloquinone from the circulation,^{80,81} it must be questioned whether the hepatic coagulation factors are the most sensitive markers for vitamin K-deficiency. It is therefore conceivable that an impaired vitamin K-status will sooner be apparent in extra-hepatic tissues resulting for instance, in an undercarboxylation of the extra-hepatic bone Gla-protein osteocalcin.

Osteocalcin is currently the most extensively studied extra-hepatic Gla-protein. This can largely be attributed to its relatively easy isolation and the availability of several detection kits.^{82,83} Osteocalcin has the calcium dependent and Gla-mediated (thus vitamin K-dependent) property to bind to hydroxyapatite crystals. Carboxylated osteocalcin can thus be distinguished from its undercarboxylated form by the reduced affinity of the latter for hydroxyapatite. Some researchers have described procedures based on column chromatography to distinguish between the carboxylated and undercarboxylated osteocalcin levels. These methods, however, are laborious, time consuming and require rather high serum osteocalcin concentrations.^{84,85} A much more practical procedure has been described and validated by Price *et al.* It is an *in vitro* hydroxyapatite binding assay for detecting serum carboxylated and undercarboxylated osteocalcin.^{18,86} Due to the ability of most immunoassays to recognize both forms of serum immunoreactive osteocalcin (irOC), a fraction with a low affinity for hydroxyapatite (irOC_{free} or undercarboxylated osteocalcin) can be distinguished from a fraction with a high affinity (irOC_{bound} or carboxylated osteocalcin) for hydroxyapatite (Figure 3).¹⁷ IrOC_{bound}, expressed as a percentage of total serum osteocalcin is called the hydroxyapatite binding capacity of osteocalcin (HBC) and is regarded as a measure for osteocalcin Gla-content and for vitamin K status.⁸⁹ In young, healthy adults the HBC values range between 70 and 80%, and decreased values are found in subjects with an impaired vitamin K-status.⁸⁹

The first clinical studies using the hydroxyapatite assay, showed an impaired carboxylation of serum osteocalcin in subjects treated with coumarin-derivatives.^{19,87,88} One year later, Knapen *et al.* reported the occurrence of high levels of undercarboxylated osteocalcin in apparently healthy postmenopausal women, despite the presence of normal coagulation factors. Vitamin K supplementation significantly reduced the HBC levels to premenopausal values, suggesting that an impaired vitamin K status, which was not apparent in blood coagulation factors, was present in these women.⁸⁹ Since these initial publications, several other investigators have used the hydroxyapatite binding assay as a measure for vitamin K status, confirming the results of Knapen *et al.*^{69,88,90,91,92} Recently a study was published showing that a subclinical vi-

Figure 3. Total serum immunoreactive osteocalcin (total irOC) can be divided in a fraction with a low ($irOC_{free}$) or high ($irOC_{bound}$) affinity for hydroxyapatite.

tamin K-deficiency can be induced in human subjects, without affecting blood coagulation factors.⁹³ This further supports the view that blood coagulation factors may not be sensitive enough to detect a mild vitamin K-deficiency.^{59,94}

1.3 VITAMIN K AND BONE METABOLISM

At this time three Gla-proteins have been identified in bone tissue: osteocalcin (also known as bone Gla protein or BGP),^{30,31} matrix Gla protein (MGP)³² and, most surprisingly, protein S.³⁴ Although all three proteins are produced by the osteoblast, MGP and protein S are also synthesized by other cells (see below).

Osteocalcin

Structure

Osteocalcin is the most abundant non-collagenous protein in mature bone.^{30,31} It is a small (5.7 kDa) Ca^{2+} -binding protein of 49 amino acids, containing 3 Gla residues at positions 17, 21 and 24. The molecule consists of two antiparallel α -helical structures ('Gla'-helix from residues 16-25 and the 'Asp-Glu' helix from residues 30-41), stabilized by a disulphide bond between the positions 23 and 29. This Ca^{2+} induced helical conformation is dependent on the presence of the Gla

residues and important for the absorption of osteocalcin to hydroxyapatite.⁹⁵ The biosynthesis of osteocalcin is regulated by the vitamins D and K. Vitamin D stimulates the transcription of mRNA,^{96,97} whereas vitamin K is involved in the posttranslational formation of Gla residues.

Circulating levels

After its synthesis by the osteoblast, about 80% of the newly synthesized osteocalcin is incorporated in the extracellular bone matrix and the remaining part is released into the circulation where it is assessable for detection.^{83,86,98} Circulating osteocalcin is rapidly metabolised by the liver and kidney, and cleared by the kidney (half life time in rats: 5 min).^{86,99} Serum osteocalcin levels are influenced by a variety of factors, including age, gender, renal function, medication (corticosteroids, anticonvulsant therapy, oral anticoagulants) and time of blood sampling (time of day and season) (for review see ref:82).^{83,97} Serum osteocalcin levels can be used as a specific marker for bone formation.^{82,101} Bone histomorphometric studies have shown that serum osteocalcin levels correlate with bone formation in several disease states.^{100,101,102,103} It has frequently been observed, however, that serum osteocalcin levels do not always correlate with serum levels of the more commonly used bone formation marker alkaline phosphatase.^{103,104,105} An explanation may be found in the hypothesis that these two bone formation markers reflect different aspects of osteoblast differentiation: alkaline phosphatase being an early marker and osteocalcin being a late marker of osteoblast differentiation.

Detection assays

Initially, detection of serum immunoreactive osteocalcin (irOC) levels was mainly based on competitive radioimmunoassays (RIA), using bovine osteocalcin as tracer, standard and immunogen.¹⁰⁶ The successful use of bovine or ovine osteocalcin is possible due to their high sequence homology with osteocalcin of human origin. Recent developments are the use of osteocalcin from human origin, the use of nonradioactive tracers¹⁰⁷ or enzyme-based immunoassays (EIA) and sandwich based assays (for review see ref:83).^{108,109} The published normal and pathological plasma osteocalcin values vary widely.^{83,110,111} Several explanations have been given: differences in populations studied, assay format and species source of standard. One of the most important determinants, however, may be the different ability of the various antisera to additionally detect osteocalcin fragments.^{109,112,113,114,115}

Several reports have indicated that varying amounts of circulating osteocalcin fragments are present in patients with chronic renal failure,^{109,112} in patients

with Paget disease¹¹³ and in sera from healthy adults.¹¹³ It has been proposed that their presence is the result of release during the process of bone resorption,¹¹⁵ generation in the circulation or occurring in vitro after blood sampling.¹¹⁶ It has been shown recently, that fragments resulting from proteolytic cleavage at the peptide bounds 19-20 and 43-44, can be detected in human plasma.^{106,116} A large N-terminal midfragment (residues 1-43) has been claimed to represent the majority (76%-80%) of what was earlier regarded as the circulating 'intact' osteocalcin molecule. The data from these authors showed that this N-terminal midfragment is generated from proteolytic degradation of the intact molecule in the circulation and reflects formation rather than resorption.¹¹⁶

Function

Despite the large amount of research performed until now, the precise function of osteocalcin is still a matter of speculation. Evidence for a role in the regulation of bone mineralization are its abundance and specific association with the mineralized matrix, its appearance in bone tissue coinciding with the mineralization of bone and its ability to strongly inhibit hydroxyapatite crystal formation³¹ and hydroxyapatite precipitation from supersaturated solutions.^{31,117} Animal studies do not, however, unequivocally indicate an important role in bone matrix structure (see paragraph: vitamin K antagonists). Another possibility is that osteocalcin may be of importance for the maintenance of bone mass and bone remodelling by acting as a mediator of bone resorption: osteocalcin may act as a chemoattractant for osteoclasts and osteoclast progenitors by promoting osteoclastic differentiation.^{118,119,120}

Matrix Gla-protein (MGP)

MGP is a small (9.6 kDa) Gla-protein containing 5 Gla residues. Despite its small size and hydrophilic amino acid composition, it is almost water-insoluble. MGP is unique among the known Gla-proteins, in the sense that it lacks a propeptide. In contrast to osteocalcin which is associated with the mineralized bone matrix, MGP is strongly associated with the organic bone matrix.³² MGP is not specific for bone and cartilage,¹²¹ but occurs in a wide variety of tissues including lung, heart, kidney, liver, spleen and vessel wall.¹²² In spite of its wide tissue distribution, substantial amounts of protein MGP levels have only been identified in the extracellular matrix of bone and cartilage. MGP mRNA transcription is regulated by vitamin D and retinoic acid.^{123,124} The function of MGP is still unknown. In bone tissue, MGP is mainly expressed early in bone development, before the onset of mineralization.¹²⁵ It has been suggested that it

may clear extra-cellular calcium, thus protecting against tissue calcification in soft tissues.¹²² The retinoic acid dependent stimulation suggests that MGP could also be involved in the regulation of some aspects of cell growth and differentiation.

Protein S

Protein S is a single polypeptide chain molecule with an apparent molecular mass of 69 kDa containing 11 Gla residues. It is synthesized by hepatocytes, megakaryocytes and endothelial cells. In plasma about 50% is non-covalently bound to C4b-binding protein, a protein involved in the complement system.¹²⁶ The other half is present in a free form and functions as a cofactor for activated protein C in the inactivation of the activated coagulation factors V and VIII.

Based on their observation that two children with a congenital protein S-deficiency suffered from severe osteopenia. Pan *et al.* suggested that protein S may have a role in bone metabolism.¹²⁷ This view was recently supported by Maillard *et al.* who showed that protein S is synthesized and secreted by osteoblasts and could be identified in the organic bone matrix.³⁴ In addition, these authors showed that another component of the protein C pathway, thrombomodulin, is synthesized by osteoblasts. It was shown that protein C can be activated at the cell membrane of the osteoblast. Interestingly, thrombomodulin expression is regulated by several mediators of osteoblast differentiation (1,25(OH)₂D₃, retinoic acid, IL1 and TNF α). It has been hypothesized that thrombomodulin, protein S and C may be involved in regulating bone resorption by modulation of the plasmin system.¹²⁸

Vitamin K antagonists

The abundant presence of Gla-protein in bone tissue, raises the question of whether the use of vitamin K-antagonists could be harmful for bone tissue. Human Warfarin Embryopathy, also known as the Fetal Warfarin Syndrome (FWS), is a well known result of coumarin use during the first trimester of pregnancy. Typical features are nasal hypoplasia and stippled epiphyses, extremity shortening and vertebral abnormalities.¹²⁹ Although it cannot be excluded that these symptoms are the result of a direct toxic effect of warfarin on bone, an important role of vitamin K in normal bone development is supported by the description of a child with an inborn deficiency of the vitamin K epoxide reductase, showing the same bone abnormalities associated with FWS.¹³⁰ In adults on the other hand, long-term oral anticoagulant therapy has never been associated with overt bone defects and only a few studies have investigated bone

mass in these patients. The results of these studies are not consistent: three studies found no differences,^{131,132,133} whereas two other study groups reported a decreased bone mass in subjects on long-term warfarin therapy.^{134,135} A drawback of these studies is their case-control design and the sometimes limited number of subjects included.^{131,132}

Initial animal studies regarding the effects of warfarin were hindered by the concomitantly induced bleeding symptoms. A breakthrough was the finding of Price *et al.* that simultaneously giving warfarin and vitamin K counteracted the bleeding symptoms, but not the effects of warfarin on bone tissue.¹⁷ All the animal studies investigating the effects of warfarin therapy (or dietarily induced vitamin K deficiency) have shown substantial reductions in osteocalcin or Gla-content of bone tissue (up to 2% of that of normal bone). The effects on bone morphology or histology, on the other hand, are not consistent and differ depending on the species of the animals used, their age and the duration of the treatment period. This can be explained, in part, by the fact that the critical periods for various aspects of skeletal development vary in the different animals and often occur in the early postnatal period.^{18,136}

Warfarin treatment for 6 weeks of chicken embryo's or newly hatched chickens did not reveal morphological bone defects.¹³⁷ A recent study using a mild dietarily induced vitamin K-deficiency on laying hens, and developing or young growing chicks for up to 28 weeks, did not show any effects on the gross or histological morphology of the bones either.¹³⁸ On the other hand, by treating fetal rat pups during the gestational age of day 8 to 22, Feteih *et al.* observed growth plate abnormalities such as an increased calcification and widening of hypertrophic zones, and disorganisation of the hypertrophic cells.¹³⁹ Treating rabbits for 3 months with warfarin, did not reveal any effects on bone morphology, bone histology, bone strength, mineral and protein content.¹⁴⁰ The same results were obtained when rats were treated from birth for 2 month. When the treatment was prolonged (up to 8 months), however, two studies described bone abnormalities consistent with those observed in the human FWS (fusion of growth plates, cessation of longitudinal bone growth, maxillonasal hypoplasia, etc).^{136,141} Pastoureau *et al.* described that, in contrast to the rat studies, treatment of newborn lambs with warfarin + vitamin K for 3 month, induced a severe osteopenia (30% reduction in bone mass). The effects could be ascribed to a decrease in resorption and a more pronounced decrease in bone formation.¹⁴²

Effect of vitamin K in cell culture and animal studies

Most experimental work investigating the relationship between vitamin K and bone metabolism has been performed in Japan, using menaquinone-4 (menatetrenone). These studies all indicate that vitamin K stimulates bone formation and reduces bone resorption. Using several osteoblastic cell lines, two independent groups showed that menaquinone-4 suppressed the proliferation of osteoblastic cells and stimulated bone forming activity as measured by the increased cellular excretion of alkaline phosphatase, osteocalcin, and enhanced mineral deposition.^{143,144}

In one rat study it was demonstrated that a dietarily induced vitamin K-deficiency leads to hypercalciuria. The high urinary calcium excretion was corrected by phyloquinone treatment within 7 days.¹⁴⁵ The effect of menaquinone-4 on bone loss was investigated in two rat studies. In the first study, bone loss was induced by ovariectomy. Vitamin K significantly suppressed the decrease in bone density, bone strength, calcium and hydroxyproline content of the femoral, respectively.¹⁴⁶ In a second study it was shown that vitamin K reduced prednisolone-induced bone loss. This was apparent through the diminished reduction in bone length, bone strength, calcium and hydroxyproline content of the femur.¹⁴⁷ Studies performed with human osteoblastic cells and mouse calvaria organ cultures indicate that the suppressive effects of vitamin K on bone resorption can be explained in at least two ways. Besides a dose dependent inhibition of the powerful bone resorbing agent prostaglandin E_2 ,^{148,149} vitamin K has also been shown to inhibit bone resorption induced by parathyroid hormone and by 1,25-dihydroxyvitamin D_3 .¹⁴⁹

Human intervention studies

The presence of a marginal vitamin K-status in postmenopausal women is suggested by studies which have shown that circulating vitamin K levels are reduced in elderly,^{67,150} and osteoporotic women (with a hip fracture).^{151,152,153} Therefore, vitamin K-intervention studies have been mainly performed in elderly women. The results of the vitamin K-supplementation studies are in line with the findings of the cell culture and animal studies described above. One of the first studies indicating that vitamin K influences calcium metabolism was a report from Tomita *et al.*, who showed that vitamin K-treatment reduces calcium excretion in postmenopausal patients.¹⁵⁴ By studying postmenopausal women living in a convent, Knäpen *et al.* showed that vitamin K reduces urinary calcium excretion, but this was only observed in those with a high fasting urinary calcium loss. It was also shown that another marker of bone resorption, urinary hydroxyproline,

decreased significantly upon vitamin K-treatment. An unexpected finding was that the majority of the women had low serum immunoreactive osteocalcin (irOC) levels, as well as a low Gla-content of their irOC (apparent in their low HBC levels).⁸⁹ This situation is similar to that of subjects using vitamin K-antagonists.^{19,88} Additional support for the view that the postmenopausal women had a subclinical vitamin K-deficiency was the fact that vitamin K-treatment improved the low HBC levels to premenopausal values. The occurrence of high levels of undercarboxylated osteocalcin in postmenopausal women has been confirmed by others⁹¹ and recent evidence even indicates that high levels of undercarboxylated osteocalcin are associated with a six-fold increased risk for developing a hip fracture.¹⁵⁵ Further support for the view that vitamin K influences bone metabolism comes from two Japanese studies which show that high doses of menaquinone significantly reduce the loss of bone mass or even increase bone mass in haemodialysis or osteoporotic patients.^{156,157}

Side effects of vitamin K supplementation

As mentioned earlier, vitamin K₃ or menadione is not recommended for use in humans, due to its haemolytic effects.^{47,49} Although intramuscular (and not oral!) phylloquinone supplementation to newborn babies has been linked to an increased risk of childhood cancer,¹⁵⁸ several other studies have failed to confirm these findings.^{159,160,161} In adults, on the other hand, toxic effect of vitamin K supplementation have not been described despite the very high dosage used in several trials.^{156,157,162}

1.4 VITAMIN K AND ATHEROSCLEROTIC CALCIFICATIONS

Mechanism of atherosclerotic calcification: related to osteogenesis?

Atherosclerosis is the underlying cause of cardiovascular disease, accounting for the majority of deaths occurring in industrialized countries.¹⁶³ The atherosclerotic process is the result of an excessive inflammatory-fibroproliferative response to various forms of injuries to the endothelium and smooth muscle cells of the artery wall. This elicits a chronic, inflammatory response characterized by invasion of leucocytes and accumulation of cholesterolesters. A wide variety of cells participate in this complex process, including smooth muscle cells, platelets, macrophages and endothelial cells. These cells are involved in the

production of different cytokines, various regulatory growth factors and extracellular matrix proteins such as elastin, collagen and proteoglycans.^{164,165,166}

Although much insight has been gained in the process of plaque formation, the understanding of the calcification process in the atherosclerotic vessel wall is still incomplete. Atherosclerotic calcification is regarded as a form of *dystrophic* calcification, which occurs in degenerating tissues, in the presence of normal serum calcium and phosphate levels. In contrast, *metastatic* calcification occurring in injured tissue is always associated with elevated calcium and/or phosphate levels.¹⁶⁷ It has been reported that calcification is initiated in extracellular membrane-bound vesicles. These vesicles are thought to be derived from degraded smooth muscle cells and are morphological similar to the matrix vesicles found in bone and cartilage.^{168,169} In addition, several extracellular matrix components present in the plaque, such as elastic fibers,¹⁷⁰ proteoglycans and collagen type I,¹⁶⁶ have been suggested to contribute significantly to the calcification process.

Several lines of evidence seriously challenge the view that atherosclerotic calcification is a degenerative process, resulting from a mere passive precipitation of calcium salts.¹⁶⁷ It has been shown, for instance, that the atherosclerotic plaque is associated with the same proteins and ultrastructural components as in bone tissue. Atherosclerotic calcifications are occasionally even described as real ossifications with several morphological and histological similarities to bone.^{171,172} Biochemical and X-ray diffraction studies have confirmed that the mineral phase in the plaque consists of hydroxyapatite, similar to that found in bone.^{173,174} Further support comes from studies which have shown that several bone-associated proteins are present in the atherosclerotic lesions. These include type I collagen which is predominantly found in bone, whereas normal aortic tissue contains collagen type III,¹⁷⁵ osteopontin¹⁷⁶ and osteonectin.¹⁷⁷ Of interest are the reports that a potent osteogenic differentiation factor, bone morphogenetic protein-2a (BMP2a),¹⁷⁸ is present in human atherosclerotic plaque and that cells isolated from the intimal artery wall are able to produce this growth factor *in vitro*. Furthermore, these cells have osteoblast like properties: they are able to produce and secrete alkaline phosphatase, collagen type I, osteocalcin, osteonectin and osteopontin and can also form multicellular nodules which undergo spontaneous hydroxyapatite calcification. Of particular interest is that the rate of mineralization of these nodules can be induced by two abundant constituents of the plaque: TGF- β 1 and 25-hydroxycholesterol.¹⁷⁹ Based on these findings it can be hypothesized that certain stimuli in the vessel wall can induce the expression of BMP2a, leading to osteogenic differentiation of certain cells present

in the intima. These osteoblast-like cells are able to produce several (Gla-containing) bone matrix proteins as well as hydroxyapatite mineral. According to this view, vessel wall calcification must be regarded as an active, highly regulated process, similar to osteogenesis.^{180,179,181}

Gla-proteins and atherosclerotic calcifications

Soon after the initial report in 1975 that Gla is present in bone tissue,³⁰ it was also identified in several sites of ectopic calcifications, such as calcium containing skin exudates, calciumcontaining renal stones and the calcified atherosclerotic plaque.^{31,37,36} The amount of Gla in atherosclerotic calcifications has been shown to be especially associated with the mineralized parts of the atherosclerotic lesions and to be associated with disease activity.^{37,182} A considerable amount of Gla, up to 60% of that found in bone, is present in the calcified atherosclerotic plaque.³¹ Only up to 20% of the total Gla present seems to be extractable by EDTA demineralization, suggesting a tight association of the majority of the Gla-proteins with the non-mineralized extracellular matrix. Since 1976, several Gla-proteins have been reported in calcified atherosclerotic lesions. The reported molecular weights vary widely: 6-10,^{183,184,185} 80 kDa,¹⁸² or 23 kDa.³⁸ Some of these Gla-proteins appear to be similar to osteocalcin,^{31,183,184} whereas others are apparently an artifact of the isolation procedure (atherocalcin, 80 kDa).¹⁸⁶ Osteocalcin has been found in trace amounts in the calcified plaques and accounts only for a small fraction of the total Gla levels present.^{182,184,186} Although several *in vitro* studies suggest that osteocalcin can be produced by cells from the (atherosclerotic) vessel wall,^{179,187} osteocalcin mRNA levels have not yet been detected in the plaque or vessel wall. The indications that osteocalcin accounts for only a small fraction of the total Gla (0.05%),¹⁸⁴ make it less probable that it is locally produced in the atherosclerotic lesions. Based on the presently available data, the possibility that osteocalcin present in the plaque is absorbed from the blood stream cannot be excluded. Recently, mRNA, as well as protein levels of a known Gla-protein have been identified in the human atherosclerotic plaque: namely MGP.¹⁸⁸ MGP mRNA levels were detected in vascular smooth muscle cells and macrophages, especially associated with the lipid-rich calcifying regions. Of interest is that in the human calcified plaque, MGP is localized in matrix vesicles, similar to the situation found in bone tissue.¹⁸⁹ MGP has already been detected in the vessel wall, but it is the first Gla-protein for which a local production in the calcified atherosclerotic plaque has been established.

The role of vascular Gla-proteins is still unknown. *In vitro* studies show that osteocalcin as well as MGP, are potent inhibitors of hydroxyapatite forma-

tion.^{31,121} Also other, as yet unknown, Gla-proteins in the calcified plaque have been shown to strongly inhibit precipitation of calcium salts from supersaturated solutions.¹⁹⁰ Osteocalcin is known to have chemotactic properties towards macrophages, monocytes and osteoclasts.^{118,119,120} Since macrophages have an important role in the pathogenesis of the plaque,¹⁶⁴ it is conceivable that osteocalcin may have a mediating role in the migration of these cells towards the plaque. The recent detection of abundant levels of MGP in the calcified plaque, and its localization in matrix vesicles similar to that in areas of enchondral bone formation, makes it tempting to hypothesize that its function may be similar to that in the cartilage growth plate of the rapidly growing fetus:¹²¹ it may act as a local mineralization inhibitor, preventing or retarding the calcification process in the plaque.

1.5 INTRODUCTION TO THIS THESIS

Using the extra-hepatic Gla-protein osteocalcin, our group has previously found that postmenopausal women may have a marginal vitamin K-status.⁸⁹ This subnormal vitamin K-status is mainly apparent in extra-hepatic tissues. It is not known whether the prolonged presence of a marginal vitamin K-status has pathological consequences. Preliminary data suggests, that a poor vitamin K-status may affect calcium metabolism.^{154,89} Both in bone and in calcified atherosclerotic plaques Gla-proteins have been detected. These Gla-proteins may have a regulatory role in the mineralization processes both in bone and the calcified atherosclerotic plaque.

In the thesis we have investigated the latter hypothesis in several ways. Using the extra-hepatic Gla-protein osteocalcin as a marker for vitamin K-status, we have measured how the vitamin K-status is related to various markers for calcium metabolism by performing vitamin K supplementation studies with phylloquinone. Secondly, the association of vitamin K-status with the presence of atherosclerotic calcifications in the abdominal vessel wall and bone mass was investigated in a population-based study. Finally, we have attempted to isolate Gla-proteins which are present in the calcified atherosclerotic plaque.

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SERUM OSTEOCALCIN AS A MARKER FOR VITAMIN K-STATUS IN PREGNANT WOMEN AND THEIR NEWBORN BABIES

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SUMMARY

Osteocalcin (bone Gla-protein) is a vitamin K-dependent protein synthesized by osteoblasts. Its hydroxyapatite binding capacity (HBC) is generally used to estimate the Gla-content of circulating osteocalcin. Here we have used the HBC of serum osteocalcin as a marker for the vitamin K status in pregnant women and their offspring. For all cases investigated the HBC values in the cord samples were substantially lower than in the corresponding maternal ones. Babies from mothers who had been treated with vitamin K during the last 6 weeks prior to delivery, had significantly higher HBC values than those from a placebo group. The results presented in this paper are indicative for a generally occurring vitamin K deficiency in newborns. At delivery the HBC in untreated women was low as well. In both the placebo- and the vitamin K-group a good correlation was found between the HBC values in paired samples from mother and child. Whether the maternal HBC value may be used as a prenatal marker for estimating the fetal vitamin K status remains to be seen.

INTRODUCTION

Vitamin K is required for the synthesis of γ -carboxyglutamate (Gla) residues in a wide variety of proteins.¹ Gla-containing proteins are found in blood plasma (e.g. some blood coagulation factors) as well as in bone tissue (osteocalcin and matrix Gla-protein). During periods of suboptimal vitamin K status the various Gla-proteins enter the bloodstream in an undercarboxylated form, which means that the number of Gla residues per molecule is below normal, and may even become zero.² In this paper we will refer to the term vitamin K deficiency in those cases in which at least one of the Gla-containing proteins is undercarboxylated due to an inadequate vitamin K status.³ It is well known that newborns may readily develop a bleeding tendency related to vitamin K deficiency. Risk factors are: maternal drug ingestion,^{4,5} low dietary intake of vitamin K (generally related to breast-feeding),^{6,7} as well as fat malabsorption.⁸ Sometimes, however, vitamin K deficiency is also seen unexpectedly, in previously healthy newborns.⁹ A suboptimal vitamin K status is associated with the so called hemorrhagic disease of the newborn (HDN), a phenomenon with a low incidence (1 per 4 000 babies), but with a high morbidity and mortality.¹⁰ To prevent HDN, the prophylactic administration of vitamin K to all newborns is propagated in most Western countries and Japan.

Without doubt the circulating levels of vitamin K in newborns are low,¹¹ but evidence that plasma and tissue concentrations of vitamin K are correlated is conflicting.^{12,13} Moreover, the minimal dose of vitamin K required for full carboxylation of all Gla-proteins is also unknown. With the help of various blood coagulation tests it has been shown that in the newborn the level of the vitamin K-dependent coagulation factors is low (40-60% of the adult values), but data from experimental animals strongly suggest that this can be explained by a low level of transcription of mRNA.^{14,15} More solid evidence for vitamin K deficiency would have been supplied by the identification of non or partly carboxylated Gla-proteins, and until now almost all efforts have been focused on the detection of descarboxy-prothrombin.^{16,17}

It may be questionable whether coagulation factors are the best choice to establish a subject's vitamin K status: the liver is capable of extracting vitamin K preferentially and efficiently from the blood stream,¹² so that given a marginal vitamin K status the extrahepatic tissues will be the first ones to experience vitamin K deficiency. During recent years we have therefore successfully used the bone Gla-protein osteocalcin as a marker for vitamin K status.¹⁸ By measuring the immunoreactive osteocalcin (iroc) before and after adsorption to hydroxyapatite, its hydroxyapatite binding capacity (HBC) may be calculated, and this is

generally regarded as a measure for the protein's Gla-content.¹⁹ In the study presented in this paper we have used the HBC of iroc in cord serum to assess the vitamin K status of newborns; in addition we have measured the effect on this marker of vitamin K administration to the mothers during the last six weeks of pregnancy.

MATERIALS AND METHODS

Study design

At the 34th week of their pregnancy healthy women were asked to participate in our study. To detect a difference in HBC of 15%, it was calculated that at least 17 participants would be required per group (estimated SD: 12%, two-tailed p value < 0.05 with a power of 90%).²⁰ All women had passed an uneventful course of pregnancy and used no drugs known to interfere with haemostasis or vitamin K status. The participants were randomly assigned to receive an oral dose of either vitamin K₁ (Konakion®, 1 mg/day) or a placebo until delivery. Only women with a treatment period of at least 4 weeks were included in the study. With regard to their mean age and clinical characteristics there were no significant differences between both groups. Konakion was obtained in drop bottles (10 mg/ml) from Hoffmann-La Roche (Basle, Switzerland). At inclusion in the protocol venous blood samples were drawn from the mothers, at delivery samples were taken both from the mothers and from the umbilical vein, immediately after clamping of the cords. The study protocol was approved by the Medical Ethical Committee of the University Hospital, and a written informed consent was obtained from each participant. In all cases the course of the pregnancy remained uneventful, and the babies were born at term by normal vaginal delivery with Apgar scores > 8 at 1 min. According to this protocol 24 women and their babies could be evaluated in the vitamin K-treated group, and 20 in the placebo group.

Blood handling

Blood was taken by venipuncture and used for the preparation of citrated plasma and serum. Plasma was prepared by collecting the blood in 10% (v/v) of 0.1 M trisodium citrate, followed by centrifugation for 15 min at 2000 x g. Serum was prepared by incubating the blood in plastic tubes at room temperature for 2 h, and centrifugation for 15 min at 2000 x g. All samples were immediately subdivided into 0.5 ml aliquots and frozen at -80 °C until further use.

Coagulation assays

Prothrombin concentrations were assessed with a one-stage assay, and the sum of prothrombin + descarboxy-prothrombin with a two-stage assay after activation with *Echis carinatus* venom.²¹ If only prothrombin is present in the sample, the ratio between these two tests should be around 1 (normal range: 0.9-1.1), if the ratio is < 0.9 this is generally regarded as a strong indication for the occurrence of descarboxy-prothrombin; with this method the detection limit for descarboxy-prothrombin is about 5 µg/ml.²²

Osteocalcin

Serum iOC concentrations were assessed with the radioimmunoassay kit from Incstar (Stillwater, MN, Ill), and the HBC was determined after adsorption of 0.5 ml of serum with 50 mg of hydroxyapatite (BDH, Poole, UK). The HBC of serum osteocalcin is expressed as the percentage bound after adsorption to hydroxyapatite (normal adult range: 70-80%).¹⁸

Vitamin K₁

Serum vitamin K₁-levels were determined by high performance liquid chromatography, followed by electrochemical detection as described by Hart *et al.*²³

Statistical analysis

Changes within and between treatment groups were analyzed with the two-sided paired and unpaired Student's *t*-test, respectively. Linear regression analysis was performed to investigate the relationship between two variables. Analyses were performed using the software package SPSS/PC+ version 3.1 (SPSS Inc., Chicago, Ill). All data presented are expressed as the means ± S.E.

RESULTS

Maternal samples

Pregnant women entered our study six weeks before the estimated day of delivery, and were randomized into two groups: one receiving vitamin K₁ and the other a placebo. In the maternal plasma samples the one- and two-stage prothrombin concentrations were within the normal range in both study groups, and descarboxy-prothrombin could neither be detected at the start of the study nor at delivery (data not shown). In the sera the following parameters were tested: iOC, the HBC of serum iroc, and the vitamin K₁-concentration. At the start of the experiment there was no statistical difference between these markers in both

Table 1: Effect of vitamin K treatment in pregnant women and their babies

Sample origin	irOC (ng/ml)	HBC (% adsorbed)	vitamin K (pg/ml)
a. Normal adult range	2-7	70-80	100-1000
maternal starting values			
b. Vitamin K group	2.33 $\pm$ 0.31	73.1 $\pm$ 1.7	n.d.
c. Placebo group	1.87 $\pm$ 0.17	70.5 $\pm$ 3.7	n.d.
difference b/c	N.S.	N.S.	
maternal values at delivery			
d. Vitamin K group	2.38 $\pm$ 0.26	76.6 $\pm$ 1.9	2512 $\pm$ 758
e. Placebo group	2.04 $\pm$ 0.25	65.5 $\pm$ 3.7	328 $\pm$ 111
difference d/e	N.S.	p < 0.02	p < 0.03
in cord serum			
f. Vitamin K group	6.31 $\pm$ 0.49	53.2 $\pm$ 2.2	43.2 $\pm$ 2.2
g. Placebo group	6.61 $\pm$ 0.57	35.6 $\pm$ 2.7	< 20
difference f/g	N.S.	p < 0.0001	p < 0.01

The normal adult ranges for the various markers are from refs. 8, 9, 17, 22 and 23. N.S. stands for not significant, n.d. for not determined. Further details are given in the text.

groups (Table 1, lines b and c). At delivery the mean HBC value in the vitamin K group was significantly higher ($p < 0.02$) than the placebo group (Table 1, lines d and e). In the latter group 11 out of 20 samples were below the normal adult range. As was to be expected, the circulating vitamin K levels in the vitamin K-treated group were high and reached about two- to three-fold times the upper normal adult level. In the placebo group the circulating vitamin K₁-levels were low but within the normal adult range for all participants.

Cord samples

As in the maternal plasma samples, no descarboxy-prothrombin could be detected in the cord plasmas with the classical coagulation tests (data not shown). In cord serum (Table 1, lines f and g) the irOC levels were higher than those of the mothers, which is consistent with the high osteoblast activity in the rapidly growing fetal bones. No correlation was found between the irOC levels in maternal

Figure 1. Correlation between the HBC values in mothers and their newborn babies. The data presented are from the vitamin K-treated (●) and from the placebo (○) group. The straight line was fitted with a computer program equipped for linear regression calculations, with $r = +0.59$ and $p < 0.0003$.

and cord blood samples, which is in accordance with earlier reported data.²⁴ In all cord serum samples the HBC values were lower than in the maternal ones, with extremely low values for the cord sera from the placebo group. Significantly higher HBC values ($p < 0.0001$) were found in the cord samples from the vitamin K-treated group, but in only one of the 24 cases did it reach the normal adult range. In the placebo- as well as the vitamin K-treated group the HBC of the newborns appeared to be significantly correlated with the maternal values (Figure 1, $r = 0.59$, $p < 0.0003$). In all cord samples from the placebo group the vitamin K concentrations remained below the detection limit (20 pg/ml), whereas in the vitamin K₁ group they were not more than about 40 pg/ml (see Table 1, last column). The latter value is well below the normal range, and over 60 fold lower than the corresponding maternal values.

DISCUSSION

Whether a general vitamin K-deficiency is common in all healthy newborn babies is a matter of debate. By definition, a marker for vitamin K-deficiency would be the occurrence of undercarboxylated vitamin K-dependent proteins. Additional confirmation that the defect is not due to an impaired carboxylation enzyme system, should then be obtained by correction of the amount of undercarboxylated proteins after vitamin K-administration. Using the classical coagulation tests we have not been able to detect descarboxy-prothrombin in either maternal or cord plasma samples. This is consistent with the results of other studies in which incompletely carboxylated prothrombin has only rarely been found and if so, usually in trace amounts.²⁵ With more sensitive tests others have demonstrated the presence of minute amounts of descarboxy-prothrombin in 21-89 % of the newborns investigated,^{16,17} but these low levels may probably be regarded as physiological rather than as pathological. This view is supported further by the observation that trace amounts of descarboxy-prothrombin could also be detected in most healthy adult plasma samples.²⁶

Here we report that in a group of 20 untreated newborns the HBC values were extremely low in all cases, reaching values comparable with those generally found in patients on oral anticoagulant therapy.²⁷ In 11 of the 20 corresponding maternal samples we found HBC values below the normal adult range, suggesting that at least some of these women were subclinically vitamin K deficient. This is consistent with the observation of Blanchard *et al.*,¹⁷ who found descarboxy-prothrombin in 71% of the mothers at delivery. In a second group (n = 24) the mothers were treated daily with 1 mg of vitamin K during the last 6 weeks of pregnancy. With this treatment the HBC in cord serum increased significantly, but in only one out of the 24 cases did it reach the normal adult range. Several mechanisms may underlie the poor response of fetal osteocalcin to maternal vitamin K-ingestion: a) the fetal vitamin K status is sufficient, but its carboxylase system is still immature and unable to maintain the production of fully carboxylated osteocalcin; b) the dosage of vitamin K₁ which was applied in this study is too low to ensure full carboxylation of irOC of the babies in utero; c) even after six weeks of maternal vitamin K₁-intake the placental transport of the vitamin is insufficient to ensure full carboxylation of the extrahepatic Gla-proteins. The frequently postulated increased requirements for vitamin K during fetal and neonatal development (e.g. because of the osteoblast activity) may contribute to the latter possibility.

We have tried to discriminate between these possibilities by the direct analysis of the vitamin K₁-concentration in the maternal sera and in the corresponding

cord samples. In the placebo-treated group the vitamin K₁-levels were undetectable. Even in the K₁-treated group the vitamin concentration in cord serum was extremely low (i.e. 2.5-fold below the lower normal adult level and 60-fold below the corresponding maternal values), despite the high vitamin K₁-levels in the maternal sera. This demonstrates once more the substantial placental barrier for vitamin K, which was also reported by other authors,^{28,29} and suggests that the low HBC values found in newborns most probably result from their suboptimal vitamin K-status. The very low HBC values found in all cord sera of the placebo group indicate that the occurrence of substantial amounts of descarboxy-osteocalcin is common in newborns. Because at best trace amounts of descarboxy-prothrombin are detectable in only a fraction of all cord plasmas,^{16,17} we conclude that descarboxy-osteocalcin is a more sensitive marker for vitamin K deficiency of the newborn than is descarboxy-prothrombin.

The significant correlation between the HBC of the mothers and their babies in the vitamin K₁- as well as the placebo-group (Figure 1) indicates that in general the vitamin K-status of the babies improved with a better vitamin K-status of the mothers, and that their osteoblastic vitamin K-dependent carboxylase system had sufficiently developed to react appropriately. At this time we have no reason to suppose that a different situation would exist in the liver. Therefore the coupling between the maternal and neonatal HBC values may open up the possibility to use the maternal HBC during the last weeks of pregnancy as a marker for the vitamin K-status of the fetus. This may be of particular importance for pregnant women known to be at risk for developing vitamin K-deficiency, such as those using anticonvulsant and tuberculostatic drugs or certain antibiotics.^{4,30,31} Vitamin K-administration to these women is not uncommon, but the doses required to prevent perinatal haemorrhages are unknown. By using the maternal HBC as a marker, the dose/effect relation of vitamin K-administration could be measured, so that the maternal HBC could be maintained in the normal adult range. In this way, as suggested by the data in this paper, the fetal vitamin K-status will be improved. Further research will have to demonstrate whether this is accompanied by a decrease in the risk for HDN.

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EFFECTS OF VITAMIN K AND ORAL ANTICOAGULANTS ON URINARY CALCIUM EXCRETION

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SUMMARY

In a subgroup of postmenopausal women vitamin K induced a decrease of the urinary calcium loss. This effect was significant ($p < 0.0001$) in the so-called fast losers of calcium (calcium/creatinine ratio > 0.5). To find out whether vitamin K antagonists would have an opposite effect, a study was started among 141 persons on long-term oral anticoagulant therapy. In this population the number of fast losers was recorded, and compared to that in a group of age- and sex-matched non-treated controls. Notably in young men the fraction of fast losers was significantly higher in the anticoagulant-treated group than in the control group (25 vs 0%, $p < 0.02$). Differences between treated and non-treated groups may also be found in other markers for calcium and bone metabolism, notably in serum osteocalcin concentration and in urinary hydroxyproline excretion. The conclusion of our study is that oral anticoagulant treatment must be regarded as a potential risk factor for a high loss of urinary calcium.

INTRODUCTION

It is well known that vitamin K functions as a cofactor in the posttranslational conversion of glutamate residues into gamma-carboxyglutamate or Gla.^{1,2,3} The only known function of these Gla-residues is that they form Ca^{2+} -binding groups in the proteins to which they belong. Hence all Gla-containing proteins are Ca^{2+} -binding ones. Well known examples of Gla-proteins are those involved in blood coagulation.⁴ The most abundant Gla-protein in man, however, is the bone protein osteocalcin.^{5,6} Other Gla-proteins occurring in mineralized tissues are matrix Gla-protein,⁷ plaque Gla-protein⁸ and proteins found in renal stones⁹ and in ectopic calcifications of the skin.¹⁰ Although the precise function of these mineral-bound Gla-proteins is not clear at this time, it has frequently been suggested, that they may play a role in calcium metabolism.

Recently, we have studied a group of postmenopausal women who were recruited from the religious inhabitants of the convents near Maastricht. It was found that the Gla-content of their circulating immunoreactive osteocalcin was much lower than that in premenopausal subjects.¹¹ Upon vitamin K administration the osteocalcin Gla-content in the premenopausal women remained unaltered, but in the postmenopausal ones a clear and significant increase was observed. In parallel, it was found that vitamin K induced a substantial decrease of urinary calcium excretion, notably in those women who may be characterized as fast losers of calcium.

Obviously the inhabitants of a convent may not be representative of the population in general. Moreover the number of participants was too low to accurately define the groups of responders and non-responders to vitamin K treatment. Therefore we have started a number of trials among randomly selected civilians. The results of these investigations are presented in this paper and demonstrate that both vitamin K as well as vitamin K antagonists may affect the urinary calcium excretion.

MATERIALS AND METHODS

Chemicals and drugs

Vitamin K₁ (Konakion®) was obtained in drop-bottles (10 mg/ml) from Hoffmann-La Roche (Basle, Switzerland). Patients were anticoagulated with acenocoumarol (Sintrom®) from Ciba-Geigy (Basle, Switzerland). All chemicals used for laboratory techniques were of analytical grade or better.

Laboratory techniques

Urinary calcium levels were established with an atomic absorption spectrophotometer (Perkin-Elmer, Norwalk, Conn.); creatinine and serum alkaline phosphatase were assayed with commercial kits from Hoffmann-La Roche using a Cobas centrifugal analyzer. Hydroxyproline (OHPro) concentrations were assessed with the Hypronosticon kit (Organon, Oss, The Netherlands). All concentrations were calculated in millimolar values. The urinary calcium and OHPro values are expressed as the calcium/creatinine ratio (Ca/creat) and the OHPro/creatinine ratio (OHPro/creat) throughout this paper. Immunoreactive serum osteocalcin concentrations (irOC) were assessed by radioimmunoassay (Incstar, Stillwater, Minn, Ill) and the hydroxyapatite binding capacity (HBC) of serum osteocalcin was measured as described earlier.¹¹ The intra- and inter-assay variations in the osteocalcin measurements were less than 10% and 5% respectively.

Patients

Oral anticoagulant-treated patients were selected from the thrombosis services in Heerlen and Maastricht after obtaining a written informed consent. Subjects were included when they had been on long-term (> 12 months) and stable oral anticoagulant treatment, with INR values within the desired range during more than 75% of the treatment period. Exclusion criteria were: recent bone fractures or bone pathology, hepatic or renal dysfunction, and the use of drugs known to affect the calcium and/or bone metabolism.

Other participants

Apparently healthy subjects were selected during the same period from the inhabitants of Maastricht by means of a call in the local newspaper. The same exclusion criteria as mentioned above were used for these participants.

Urine collection and blood sampling

To exclude dietary influences, urine was collected during the last 2 h of an overnight (16 h) fasting period.¹² The urine was brought to pH 1 by adding a small amount of 6 N HCl, and frozen immediately until use. After the sample collection had been completed, all samples were tested in a serial way within one week. Fasting blood samples were obtained between 8.00 and 10.00 h in the morning.

Study design

In the first trial the effect of vitamin K on urinary calcium excretion was investigated in 125 postmenopausal women who were randomly assigned to oral treatment with either vitamin K (1 mg/day, $n = 85$) or placebo ($n = 40$) for 1 month. In a second study, 143 subjects on long-term and stable oral anticoagulant treatment were compared with an age- and sex-matched control group. The participants selected for this trial were subdivided into four groups, each containing 25-57 patients. The age of the participants was either between 25 and 45 years or between 60 and 80, and in both categories men and women were studied separately. In all participants the following markers for calcium metabolism were measured: serum iOC, HBC and alkaline phosphatase, and the urinary Ca/creat and OHPro/creat ratios. All protocols were approved by the local ethics committee.

Statistical analysis

The software package SPSS/PC⁺ version 3.1. (SPSS Inc., Chicago, Ill) was used for the statistical analysis and the 0.05 level of significance was chosen. Differences in proportions of subgroups were analyzed using the chi-square test. The continuity correction according to Yates was applied when appropriate.¹³ The data of the vitamin K treatment were analyzed by the two-sided paired Student's *t*-test. Highly skewed data were analyzed with the Main-Whitney U test and all data are expressed as the mean $\pm$ SE.

RESULTS

Effect of vitamin K on urinary calcium excretion, definition of subgroups

In an earlier study among a selected group of women we found a significant effect of vitamin K on urinary calcium excretion.¹¹ To test the universality of this observation, and to define more precisely the subgroup(s) in which the effect of vitamin K is to be expected, healthy postmenopausal women were randomly assigned to either vitamin K or placebo treatment for one month. In the vitamin K-treated group as well as the placebo-treated one, the mean urinary Ca/creat ratio were similar, with starting values of 0.35 (± 0.04) and 0.35 (± 0.04), respectively. Also, the mean values within the groups had remained unchanged after one month of treatment with either vitamin K or placebo: 0.33 (± 0.03) and 0.34 (± 0.04), respectively. Inspection of the data revealed, however, that some subgroups of individuals responded with a clear decrease in their urinary calcium excretion. According to their initial Ca/creat ratio, participants were therefore

Figure 1. Vitamin K-induced changes in the urinary calcium excretion. Participants were treated for one month with either placebo (A) or vitamin K (B). The observed change in the urinary calcium/creatinine ratio is plotted against the initial values.

divided into subgroups the values of which increased with intervals of 0.1. The data for the vitamin K- treated group (Figure 1B) show that a significant decrease in the urinary calcium excretion is only found in groups with a mean starting Ca/creat ratio higher than 0.5 (mean decrease: -0.20 ; paired t -test: $p < 0.0001$). A similar effect was absent in the placebo group (Figure 1A). Participants with a very low initial Ca/creat ratio (the intervals 0-0.1 and 0.1-0.2), on the other hand, showed a slight but significant vitamin K-induced increase of their urinary calcium loss (mean increase: $+0.06$; paired t -test: $p < 0.0001$). No significant effect was found on the mean calcium loss in participants with a Ca/creat ratio in the 3 intervals between 0.2 and 0.5. On the basis of these results the participants could thus be classified into three groups: positive responders, non-responders, and negative responders to vitamin K treatment, and it is at least striking that the limits found for these three categories almost precisely coincide with those generally used to define slow, normal and fast losers of urinary calcium.¹² In the rest of this paper we will use a Ca/creat ratio of 0.5 as the lower limit for fast losers of urinary calcium.

Figure 2. Effect of oral anticoagulants on serum osteocalcin (iOC). Details of the experimental set-up are given in the Materials and Methods section. The closed bars represent the subjects on long-term, stable anticoagulant therapy, the open bars are the controls. All data are expressed as the mean $\pm$ SE. A: serum concentrations of immunoreactive osteocalcin (iOC). B: hydroxyapatite binding capacity (HBC) of the iOC. *: $p < 0.03$; **: $p < 0.02$.

Effect of coumarin derivatives on markers for calcium metabolism

The observed vitamin K-induced changes in calcium metabolism justify the question of whether vitamin K antagonists (i.e. coumarin derivatives used for oral anticoagulant therapy), may have an opposite effect. This was investigated in a cohort study in which subjects on long-term and stable oral anticoagulant treatment were compared with an age- and sex-matched control group. In all patient groups the circulating iOC was significantly lower than in the corresponding controls, with a notable exception of the postmenopausal women in whom the serum iOC was low both in treated and untreated subjects (Figure 2A). In all anticoagulant-treated groups the HBC of the circulating osteocalcin was significantly lower than in the controls (Figure 2B). In Figure 3 we have plotted the percentage of fast losers in the various groups. It turned out that in all anticoagulated groups except that of the elderly women, the fraction of fast losers was higher than in the respective control groups. In the control groups of the young and elderly men we even observed no fast losers at all. The elevated number of fast losers reached the level of statistical significance only in the group of the young men (chi-square test: $p < 0.02$). No significant differences were found

Figure 3. Effect of oral anticoagulants on urinary calcium excretion. The number (nr) of fast losers in each group is expressed as a percentage of the total number of participants in that group. Differences between the anticoagulated (closed bars) and control (open bars) groups were computed with the chi-square test. *: $p < 0.02$.

between the mean serum alkaline phosphatase in the patient and the corresponding control groups (data not shown). On the other hand, a good correlation was observed between the urinary hydroxyproline and calcium excretion in the group of anticoagulated young men ($r = 0.52$, $p < 0.004$, see Figure 4), as well as in both groups of postmenopausal women ($r = 0.31$, $p < 0.0005$ for both groups).

Figure 4. Correlation between the urinary OHPro and calcium excretion in anticoagulated young men. The straight line was fitted with a computer program equipped for linear regression calculations. Both variables were related according to the equation $y = 0.009 + 0.022x$, with $r = 0.52$ and $p < 0.004$.

DISCUSSION

The first link between vitamin K-status and bone metabolism was made by Pettifor & Benson in 1975,¹⁴ who found that oral anticoagulants, administered during the first trimester of pregnancy, induced severe bone malformations in 30 % of the fetuses. The discovery of the vitamin K-dependent proteins osteocalcin (or bone Gla-protein) and matrix Gla-protein in bone tissue^{5,6,7} further contributed to speculations about a putative role of vitamin K-dependent proteins in the regulation of calcium phosphate deposition in bone tissue. Although both clinical and experimental data suggest a specific role for Gla-proteins in the mineralization of rapidly growing bone tissue, the precise function of each of these proteins remained unclear until now. On several occasions it has been proposed that, besides their effect on developing bone tissue, the bone Gla-proteins may be important for the maintenance of adult bone as well, but experiments with rats on long-term oral anticoagulant treatment did not prove this hypothesis satisfactorily.¹⁵ Moreover it remains questionable whether the results obtained with experimental animals may be extrapolated to man.

In a previous paper¹¹ we have demonstrated that vitamin K administration to post menopausal women may reduce the urinary calcium excretion, notably among the fast losers of calcium. Here we describe a second trial, in which we have substantially increased the number of participants, so that we could define more accurately the Ca/creat ratio above which an effect of vitamin K could be seen. It turned out that a vitamin K-induced decrease of urinary calcium excretion was statistically significant in all groups characterized by a Ca/creat ratio > 0.5 . We have used this figure, therefore, as the lower limit for subjects to be identified as 'fast losers' in our study among anticoagulated patients.

It is well known that coumarin derivatives antagonize the effect of vitamin K on proteins involved in blood coagulation. If these drugs would also counteract vitamin K action in processes related to calcium metabolism, one might expect them to induce an increase of the urinary calcium excretion in fast losers, or to increase the fraction of fast losers among anticoagulated subjects. A complicating factor in this kind of investigation is the difficulty to find sufficient numbers of participants shortly prior to their starting oral anticoagulant therapy, who can be checked in a longitudinal study before and during the first year of treatment. We have therefore chosen for a cross-sectional study, enabling us the statistical analysis of patients on stable, long-term anticoagulant therapy. Postmenopausal women were selected for the study described in the first part of this paper, because the fraction of fast losers of urinary calcium is known to be high in this group.¹²

Preliminary data from our laboratory indicate that the effect may not be restricted to postmenopausal women, however, but may be found in other fast losers as well (M.H.J. Knapen, unpublished results). In our study among anticoagulated patients we have therefore recorded the numbers of fast losers in four groups differing with respect to age and sex. The data thus obtained were compared to those from age- and sex-matched control groups. An elevated number of fast losers was found in both groups of anticoagulated men as well as in anticoagulated premenopausal women, only in young men the difference with the control group was statistically significant, however. These data indicate that coumarin derivatives may induce a disturbance of the calcium metabolism, manifesting itself in an elevated number of fast losers of urinary calcium.

No significant effects of vitamin K antagonists were found in the group at risk for developing osteoporosis: the postmenopausal women. It is well known that at the onset of the menopause, factors like the change of the hormonal status may induce substantial alterations in the calcium and bone metabolism; apparently these factors outweigh the effect of coumarin derivatives. Moreover, the large number of fast losers in the post menopausal control group may obscure relatively small anticoagulant-induced effects like those found in men and younger women. Of special interest is the group of young men which showed a significant increase in the number of fast losers. As was the case in both groups of postmenopausal women, a good correlation between the fasting urinary calcium and OHPro excretion ($r = 0.52$, $p < 0.004$) was also found among the anticoagulated young men, indicating that a high urinary calcium excretion was accompanied with a high bone resorption (Figure 4). A long-term increased bone resorption may eventually lead to a decrease of the bone mass, a well known risk factor for developing osteoporosis.¹² Additional indications for a possible harmful effect of oral anticoagulants on bone tissue are provided by two recent studies which both found a decreased bone mineral content in patients on long-term anticoagulant therapy.^{16,17}

As yet nothing can be said about the mechanism which underlies a putative regulation of urinary calcium excretion by vitamin K. The data on osteocalcin show that, like vitamin K, also coumarin derivatives can reach the osteoblasts in bone tissue. Their effect may well be explained by an intracellular accumulation of osteocalcin precursors, although a direct inhibition of the osteoblast activity by vitamin K antagonists cannot be excluded at this time. An altered osteoblast activity could affect the balance between bone formation and resorption leading to an increased calcium loss from bone tissue. It can not be excluded that other (still unknown) vitamin K dependent proteins found in bone tissue (e.g. MGP), could also be involved in this process. Because vitamin K-dependent carboxylase has

also been found in renal tubule cells¹⁸ and membrane-bound Gla-containing proteins have been identified in the renal cortex,¹⁹ it is also possible that the changes in urinary calcium excretion described in this paper are the result of alterations in renal rather than in bone Gla-proteins. The data presented in this paper justify the start of more elaborate, longitudinal trials in which the urinary calcium excretion and various markers for bone metabolism are measured in humans before and during the first year of treatment with either vitamin K or vitamin K antagonists.

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VITAMIN K-INDUCED CHANGES IN MARKERS FOR OSTEOBLAST ACTIVITY AND URINARY CALCIUM LOSS

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SUMMARY

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

INTRODUCTION

Vitamin K functions as the coenzyme for γ -glutamylcarboxylase, a microsomal enzyme mediating the posttranslational carboxylation of protein-bound glutamate into γ -carboxyglutamate (Gla) residues.^{1,2,3} Gla-containing proteins are found in body fluids (plasma, urine),^{4,5} and in calcified tissues (bone, hardened atherosclerotic plaques);^{6,7,8} well-known examples are the vitamin K-dependent blood coagulation factors and the bone Gla-proteins osteocalcin (OC) and matrix Gla-protein (MGP). In all these proteins the only known function of Gla is the binding of calcium ions. Blood coagulation is the only process in which the role of Gla-proteins has been well established, and in which their function has been described to a molecular level. Accumulating evidence indicates, however, that calcium metabolism is a second physiological process in which Gla-proteins are involved.⁹

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

MATERIALS AND METHODS

Subjects

Apparently healthy, physically active women were recruited via a local newspaper call. All had normal serum calcium levels, as well as normal liver and renal functions. Exclusion criteria were malnutrition, a history of recent fractures or metabolic bone disease, and medication known to affect calcium and bone metabolism or vitamin K status. Solubilized vitamin K₁ (Konakion®) was obtained from Hoffmann-La Roche (Basle, Switzerland) and distributed among the participants in drop-bottles

containing 20 mg/mL, the placebo was constituted of the same solvent as the vitamin K preparation. One drop of the vitamin (1 mg) or placebo was taken orally by mixing it with tap water immediately before drinking it. The study was approved by the local ethics committee, and informed consent was obtained from all subjects, according to the institutional guidelines.

Tests

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

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

Data analysis

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

RESULTS

Effects of vitamin K on circulating osteocalcin: relation with age

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

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

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

Table 1: Osteocalcin markers before and after vitamin K treatment in women younger than 50 years ($n = 67$) and older than 50 years ($n = 78$)

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

All results are given as the means $\pm$ S.E. P_{age} stands for the significance of the difference of one marker between two age groups (2-sided unpaired Student's *t*-test), P_{treat} stands for the significance of the difference before and after vitamin K treatment in one age group (2-sided paired Student's *t*-test).

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

In Figure 1C we have plotted the ratio between irOC_{free} and irOC_{bound} as a function of age. Before treatment with vitamin K this ratio amounted to $0.39 (\pm 0.03)$ in women younger than 50 years, but it increased to $0.67 (\pm 0.03)$ after the age of 50. The difference between both age groups is significant at $p < 0.005$. After treatment of all women with vitamin K the irOC_{free}/irOC_{bound} ratios of the younger women remained unchanged, but those of the elderly participants decreased to values which approached the premenopausal values.

To obtain more information about the nature of irOC_{bound} and irOC_{free} five serum samples from different subjects were dialyzed against buffered saline (pH 7.4) for 1

Figure 2. Prolonged treatment of postmenopausal women (50-85 yr) with vitamin K. Fast (open symbols) and normal (closed symbols) losers of urinary calcium were assembled and treated with either vitamin K (A) or placebo (B). *: $P < 0.05$, **: $P < 0.005$. All data are given $\pm$ SE.

h at 4 °C. The molecular weight cutoff value of the dialysis membrane was 3.5 kDa, which was found to be sufficient for preventing the loss of intact osteocalcin. Identical samples were incubated in plastic tubes at 4 °C for the same period, and it was ascertained that both $\text{irOC}_{\text{bound}}$ and $\text{irOC}_{\text{free}}$ remained unchanged in these control samples. In the dialyzed samples at least 50 % of the $\text{irOC}_{\text{free}}$ had disappeared within 1 h, whereas the $\text{irOC}_{\text{bound}}$ had remained unchanged (data not shown). It seems, therefore, that in elderly women at least a major part of the circulating $\text{irOC}_{\text{free}}$ has a reduced size.

Correlation between serum osteocalcin and other markers for bone metabolism

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

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

ones), and 53 in the normal losers group (28 vitamin K- and 25 placebo-treated subjects).

After 1 month of treatment the mean urinary calcium loss in the vitamin K-treated group of fast losers had decreased, whereas no effect was found in the normal losers group (Figure 2). Also, in both placebo-treated groups the urinary calcium loss remained constant during the trial. In the fast losers groups the difference between vitamin K- and placebo-treated subjects was significant ($p < 0.01$) after 1 month, continued during the two subsequent months ($p < 0.005$, mean decrease: 30 %), and gradually reversed after the treatment had stopped. In all groups the creatinine concentration in the samples had remained constant during the entire study period, and changes in the calcium/creatinine ratio were solely due to variations in the calcium excretion. These data demonstrate, therefore, that the effect of vitamin K administration on urinary calcium loss is most prominently seen among the fast losers of urinary calcium.

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

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

DISCUSSION

In the literature there is no consensus about the relation between serum irOC and age. Whereas some authors report a continuous rise in irOC in women older than 40,^{13,14} others claimed this marker to be constant¹⁵ or to decrease at increasing age.¹⁶ In this paper we demonstrate that in adult women the serum level of total serum irOC gradually increases until the age of about 55 years, but it sharply decreases in the age groups older than 60. Further analysis showed that the curve for total irOC was composed of two apparently independent variables: irOC_{free} and irOC_{bound}. Whereas irOC_{free} exhibited an age-dependent increase which was most prominent between 50 and 60, the irOC_{bound} remained nearly constant until the age group 60-65, and then sharply decreased at later ages. The ratio irOC_{free}/irOC_{bound} remained low until the age of 45, but nearly doubled immediately afterwards (Figure 1C). Vitamin K administration only affected the serum levels of irOC_{bound}. The rate at which this effect took place (within 14 days), and the fact that the vitamin K-induced changes of irOC_{bound} were strongly correlated to changes in the serum B-AP

suggest that the circulating $\text{irOC}_{\text{bound}}$ originates from de novo synthesis by the osteoblasts. This has been proposed earlier for total serum irOC .¹⁷ Moreover, the effect of vitamin K on B-AP suggests a direct effect of this vitamin on osteoblast activity. Similar effects of vitamin K have recently been reported for osteoblastic cells *in vitro*, which also responded to the vitamin with an increase of alkaline phosphatase production.¹⁸ The physiological importance of these observations is unclear at this time.

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

It is well known that around the onset of the menopause the urinary calcium excretion and the loss of bone mass may increase considerably.²⁰ It has been observed that around menopause $\text{irOC}_{\text{free}}/\text{irOC}_{\text{bound}}$ ratio increases, and that there is a correlation between $\text{irOC}_{\text{free}}$ and urinary calcium loss; both suggest that $\text{irOC}_{\text{free}}$ is somehow related to bone degradation. Here we want to put forward the hypothesis that rather than total irOC , the serum concentration of $\text{irOC}_{\text{bound}}$ is a marker for osteoblast activity, whereas serum $\text{irOC}_{\text{free}}$ levels reflect osteoclast activity. If so, the ratio between $\text{irOC}_{\text{free}}/\text{irOC}_{\text{bound}}$ forms a marker for the metabolic state of bone, and it may become an important diagnostic tool to identify subjects at risk for developing osteoporosis. Although in most women older than 50 years this ratio reacted strongly on vitamin K administration, only in fast losers of calcium did the vitamin have a significant effect on urinary calcium loss. This demonstrates that vitamin K status is not directly correlated with calcium excretion, but that low nutritional intake or poor intestinal absorption of vitamin K may be risk factors for increased urinary calcium loss.

As can be seen in Figures 2 and 3, the effect of vitamin K on $\text{irOC}_{\text{bound}}$, B-AP, and calcium/creatinine ratio was maximal after 3 months of treatment. It is also clear that for all three markers the effect was still detectable 3 months after the treatment had been stopped. Because the plasma half-life time of pharmacological doses of vitamin K is about 2 hours, we assume that at least part of the vitamin administered

in our experiments had been stored in phospholipid membranes or adipose tissue and set free therefrom during the first few months following the treatment period.

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

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**EFFICIENCY OF PHYLLOQUINONE (VITAMIN K₁) ABSORPTION
FROM SPINACH IN HUMAN VOLUNTEERS**

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SUMMARY

The human vitamin K requirement is not precisely known, the minimal requirement is, however, often assumed to be between 0.5 and 1 μg per kg body weight. In this paper we have addressed the question of the extent to which intestinal absorption of vitamin K₁ (phylloquinone) is influenced by the form in which the vitamin is consumed. The experimental group was formed by five healthy volunteers who received the phylloquinone after an overnight fast. On the first day of three successive weeks the participants consumed 1 mg of phylloquinone, either in the form of a pharmaceutical preparation (Konakion®), in the form of spinach + butter, or as spinach without additives. It turned out that circulating phylloquinone levels after consuming spinach with and without butter were substantially lower (7.5 and 24.3 fold, respectively) than those after taking the pharmaceutical concentrate. Moreover the intestinal absorption of phylloquinone from the vegetables was 1.5 times slower than from Konakion®. It is concluded that the absorption of membrane-bound vitamin K is extremely poor and may depend on food additives, notably fat. The bioavailability of nutritional vitamin K is much lower than what is generally assumed, and depends on the form in which the vitamin is ingested. These new insights may lead to a revision of the recommended daily intake for vitamin K.

INTRODUCTION

Vitamin K is a group name for a number of closely related compounds which may serve as a cofactor for the mammalian microsomal enzyme γ -glutamylcarboxylase. This enzyme catalyses the posttranslational conversion of glutamate into γ -carboxyglutamate (Gla) residues at well-defined sites in a limited number of proteins.^{1,2} If not specified further the term 'vitamin K' will be used for all compounds possessing cofactor activity for γ -glutamylcarboxylase. Gla-containing proteins have a regulatory function in blood coagulation,³ bone metabolism,⁴ and possibly also in cell growth.⁵ Potential sources of vitamin K are nutrition^{6,7,8} and the intestinal flora,⁹ but accumulating data suggests that the bioavailability of menaquinones produced in the colon is very poor.^{10,11,12} It seems that it is only after oral ingestion, that the various forms of vitamin K are absorbed, notably in the small intestine.

The main dietary sources of vitamin K are green vegetables (which exclusively contain phyloquinone) and dairy produce, in which a mixture of phyloquinone and menaquinones is found.^{6,7,8,13} In this respect it is important to notice that a) green leafy vegetables contain about 100 times more vitamin K than does cow's milk,⁶ and b) that in green plants vitamin K is tightly bound to the thylakoid membranes of the chloroplasts,¹⁴ whereas in dairy produce it is dissolved in the fat fraction. In the case of a normal bile function it may be assumed that the intestinal absorption of vitamin K from dairy produce is nearly complete. The extent to which phyloquinone is extracted and absorbed from green vegetables is, however, less clear. In this paper we describe the development, in human volunteers, of a standard protocol for measuring the intestinal absorption of vitamin K from cooked spinach, and investigate how the simultaneous consumption of fat affects this process.

MATERIALS AND METHODS

Experimental design

The three different vitamin K regimens described below were given on the first day of three successive weeks. Five healthy volunteers (3 men and 2 women, 25-45 years old) entered the experiments after an overnight fast. All five volunteers included in our experiment were subjected to the same nutritional regimen starting on the same day. In each case an oral dose of 1 mg of phyloquinone was taken at 8 a.m., either in the form of a detergent-solubilized pharmaceutical concentrate (Konakion[®], week 1), from 227 g of spinach + 25 g of butter (week

2), or from 227 g of spinach without further additives (week 3). At noon of the experimental day one meal was allowed, which was low in both fat and vitamin K (toast, marmalade, peeled bananas and apples, orange juice and water). Food restriction was continued until 10 h after vitamin K ingestion. Blood (10 ml) was taken by venipuncture to prepare serum every hour during the first 10 hours of the experimental day, and the next morning ($t=24$ h). The serum samples were subdivided into 1 ml aliquots and kept frozen and protected from light until serial vitamin K determination. The protocol of this study was approved by the Medical Ethics Committee of the University Hospital / Faculty of Medicine, University of Limburg, Maastricht.

Materials

Detergent-solubilized phylloquinone (Konakion[®]) was from Hoffmann-La Roche (Basle, Switzerland), spinach was purchased as a commercially prepared, deep-frozen product. Menaquinone-6 (MK-6) was a kind gift from Roche. Silica Sep-pack cartridges were purchased from Millipore (Milford, MA). All solvents and chemicals were of the highest purity commercially available.

Vitamin K analysis

Serum vitamin K was determined as reported by,¹⁵ with fluorometric detection after electrochemical reduction as proposed by Thijssen *et al.*¹⁶ In short, after adding an internal standard (MK-6, 1 ng per ml of serum), sera were extracted twice with hexane, and the pooled hexane phases were evaporized, dissolved in a small volume of hexane, and fractionated on a silica sep-pack column. The fraction containing vitamin K was then purified further on a semi-preparative silica straight phase column (Phase Separations, Deeside, U.K.) by high performance liquid chromatography (HPLC). Quantitative analysis of vitamin K was performed by isocratic chromatography on an Econosphere C₁₈ reversed phase column (Alltech, Deerfield, IL) in a mixture of 88.5% (v/v) methanol, 10% (v/v) isopropanol, 1.5% (v/v) water and 0.045% (v/v) tetramethylammonium octahydrotriborate. The effluent of the column was reduced with a model 5010 analytical cell (Environmental Sciences Associates, Bedford, MA), and analyzed with a Jasco 821-FP spectrofluorometric detector (Separations Analytical Instruments, Hendrik Ido Ambacht, The Netherlands). For the extraction of vitamin K from spinach, 10 g of cooked vegetables (wet weight) were mixed with 1 ml of acetone and ground with 0.5 g of sand. Subsequently 100 ml of acetone was mixed with the homogenate and shaken for 30 min. After the addition of 100 ml of water, the solution was extracted three fold with 100 ml of hexane; the pooled hexane fractions were evaporated and dissolved in 1 ml of hexane. Quantitative analysis

of phylloquinone in food extracts was performed by HPLC with postcolumn reduction and fluorescence detection, according to the same procedure as described for serum. All measurements were made in duplicate, and mean values are used throughout this paper.

RESULTS

Before starting the experiment, the various sources of vitamin K were checked for their phylloquinone content. The deep-frozen spinach used in the study was found to contain $4.41 \mu\text{g}$ of phylloquinone per gram (wet weight), so that 227 grams of spinach are equivalent to 1 mg of phylloquinone. Butter contained 60 ng of phylloquinone per gram; hence the contribution of phylloquinone in the 25 g of butter (week 2) to serum vitamin K levels will be regarded as negligible. Finally also the phylloquinone content of Konakion® was checked, and found to be 20.0 mg/ml.

Vitamin K was administered to the participants as a single oral dose, and in all cases circulating phylloquinone levels had returned to their starting levels after 24 h. Nevertheless a wash-out period of 6 days was scheduled between the three

Figure 1. Intestinal absorption of vitamin K from different sources. One mg of phylloquinone was given as a single, oral dose to five fasting volunteers. Sources of phylloquinone were either Konakion (●), spinach + butter (○) or spinach without additives (▲). The data at each time point are given as the means of the five individual values $\pm$ SE. Further details are given in the text.

Table 1:
Effect of vitamin K from different sources on serum phylloquinone levels

Subject	Konakion		Spinach + butter		Spinach - butter	
	AUC	(%)	AUC	(%)	AUC	(%)
A	139.7	100	18.3	13.1	2.6	1.9
B	97.0	100	11.9	12.3	4.4	4.6
C	90.4	100	7.9	8.8	3.1	3.4
D	54.8	100	11.5	21.0	6.0	10.9
E	52.5	100	8.2	15.6	1.9	3.7
Mean	86.9	100	11.6	13.3	3.6	1.4

The letters A through E represent the five participants in the study. AUC stands for the area under each individual absorption curve, the AUC values for both spinach regimens are expressed as a percentage of the corresponding Konakion experiment.

experimental days. During each of the experimental days one meal (noon) with low fat and vitamin K content was allowed. It is assumed that vitamin K absorption was not influenced by this meal. For each participant the serum phylloquinone concentration was established with 1 h intervals, and the mean values ($\pm$ SE) are given in Figure 1. It is striking that very similar curves were obtained for all subjects: peak values were at 4 h (Konakion[®]) and 6 h (spinach) after vitamin K ingestion, with only some interindividual variations in the peak heights. The absorption of phylloquinone from vegetables was slower than that from Konakion[®] by a factor 1.5. Butter had only an effect on the peak height, not on the time at which the maximal serum concentration was reached.

The total amounts of phylloquinone absorbed were calculated for each participant from the Area Under Curve (AUC) between 0 and 10 h after the start of the vitamin K ingestion, and the data obtained for spinach (both in the presence and absence of butter) were expressed as a percentage of the data for Konakion[®]. As can be seen in table 1, the mean absorption of phylloquinone from spinach alone is 4% from that of Konakion[®], whereas the effect of simultaneous consumption of butter improves this figure with no more than a factor 3. At 24 h after vitamin K ingestion the serum phylloquinone levels had decreased to the starting levels, irrespective of the peak height the day before. This shows that nutritional effects

are nihilated after an overnight fast, which is consistent with the short half-life time of vitamin K in the circulation (2 h).

DISCUSSION

From the data presented in this paper it is clear that phyloquinone is readily absorbed from Konakion[®], with a peak time of 4 h. This is in accordance with previous observations of Shearer, who found a peak time between 2 and 4 h.¹⁷ The later peak time for phyloquinone from spinach indicates that its absorption from plant cells is a more time-consuming process, which may be influenced by digestive factors, such as the rate at which phyloquinone is extracted from the cellular membranes. The concomitant intake of fat may increase the phyloquinone peak height, but obviously does not influence the rate of absorption. The effect of fat is probably due to its stimulation of bile secretion, which is known to be important for the absorption of hydrophobic compounds.¹⁷

Our data also indicates that the mean intestinal absorption of dietary phyloquinone is only a small fraction of the total phyloquinone content which may be extracted from the food using organic solvents.⁶ As was reported earlier, the efficiency of intestinal absorption of phyloquinone from Konakion[®] is about 80%.¹⁷ Based on this figure it may be calculated from our data that less than 10% of phyloquinone present in green vegetables is absorbed in the digestive tract. Furthermore it should be taken into account that data from our lab and elsewhere indicate that the colonic absorption of menaquinones produced by the intestinal flora is extremely poor.^{10,11,12} It may be concluded, therefore, that the relative effect of nutritional menaquinones is probably higher than generally assumed. More information about food contents and bioavailability of nutritional menaquinones is needed, however, to substantiate this conclusion. Our data demonstrate that the bioavailability of phyloquinone is substantially less than assumed up until now, and that the fat content of the meal is an important determinant for phyloquinone absorption.

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**A POPULATION-BASED STUDY IN WOMEN
WITH OR WITHOUT ATHEROSCLEROSIS:**

- 6.1 VITAMIN K INTAKE AND OSTEOCALCIN LEVELS**
- 6.2 VITAMIN K STATUS AND BONE MASS**

**A POPULATION-BASED STUDY IN WOMEN WITH OR WITHOUT
ATHEROSCLEROSIS:
VITAMIN K INTAKE AND OSTEOCALCIN LEVELS**

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SUMMARY

Protein-bound γ -carboxyglutamate (Gla) has been demonstrated in calcified atherosclerotic plaques. Vitamin K is required for the formation of Gla-residues in proteins. As the biological activity of Gla-proteins appears to be strictly dependent on the presence of the Gla-residues, vitamin K status may be an important factor in the development and progression of atherosclerotic calcifications. We studied the association of vitamin K status as assessed by nutritional vitamin K intake and measurements of two circulating immunoreactive osteocalcin (irOC) fractions, with aortic atherosclerosis in a population-based study of 113 postmenopausal women. Women with calcified lesions ($n=34$) had a $42.9 \mu\text{g}$ lower mean age-adjusted dietary intake of vitamin K /day [95 % CI -6.6 to 92.5] than those without calcifications ($n=79$). Atherosclerotic women had higher irOC levels with a low affinity for hydroxyapatite (irOC_{free}): age-adjusted difference of 0.32 ng/ml [95 % CI 0.03 to 0.61]. In addition, the high affinity irOC levels expressed as a percentage (hydroxyapatite binding capacity, HBC) were 5.12 % [95 % CI 1.32 to 8.92] lower in women with calcifications. Our study indicates that women with aortic atherosclerosis have a reduced vitamin K status as reflected by a lower nutritional vitamin K intake, an increased irOC_{free} level and a reduced HBC level. An impaired vitamin K status in subjects with atherosclerosis is compatible with the view that vitamin K or Gla-containing proteins are involved in the development of calcifications of the vessel wall.

INTRODUCTION

Cardiovascular disease is caused by an interplay between thrombosis and advanced atherosclerotic vessel wall changes.¹ The development of atherosclerotic lesions is a slow process in which gradual thickening of the vessel wall occurs, and localized atherosclerotic plaques may arise.² Plaques may further develop into non-calcified and calcified lesions. Recently proteins containing the aminoacid γ -carboxyglutamate (Gla), such as osteocalcin and plaque Gla protein (PGP), have been demonstrated in calcified atherosclerotic plaques and in calcified aortic valves,^{3,4,5,6,7} whereas in normal arterial wall and in non-mineralized lesions Gla-proteins are found only at trace levels.^{8,9} The presence of Gla is strongly associated with the amount of calcium in these calcified lesions, suggesting an involvement of Gla-proteins in vascular Ca^{++} -deposition.⁴ Recent studies have indicated that both osteocalcin and matrix Gla protein (MGP) may play a role in the atherosclerotic calcification process.^{10,11} Vitamin K is required for the carboxylation of certain protein bound glutamate residues into the strongly negatively charged aminoacid Gla.^{12,13,14} As the biological activity of Gla-proteins appears to be strictly dependent on the presence of the Gla-residues,^{13,14} vitamin K status may be an important factor in the development and progression of atherosclerotic calcifications.

The assessment of vitamin K status has generally been based on coagulation tests. These tests, however, have a maximum sensitivity only during a severe deficiency state.¹⁴ Recently, we have shown that even in mild levels of vitamin K-deficiency, the Gla-content of serum osteocalcin levels reflects the vitamin K status.^{15,16,17,18} Therefore, we regard the circulating osteocalcin Gla-content as the most sensitive marker available for measuring the current vitamin K-status. Atherosclerosis is the result of long-lasting pathophysiological processes probably due to unfavorable conditions during many years. In the present study we have investigated whether a long term low vitamin K intake as estimated by a food frequency questionnaire is associated with aortic atherosclerosis. Subsequently the current vitamin K status as indicated by the serum osteocalcin Gla-content was related to the presence of atherosclerotic calcifications in a population-based study among postmenopausal women.

METHODS

Subjects

In 1985, 855 women, aged 55 - 75, participated in a population-based study on determinants of osteoporosis and atherosclerosis. All subjects were examined radiographically for calcified deposits in the abdominal aorta.¹⁹ These calcified deposits have been shown to represent intimal atherosclerosis^{20,21} and have been associated with cardiovascular disease at several sites^{22,23} and with cardiovascular mortality.^{24,25} In 1990, 300 of the 855 randomly selected women were invited to take part in our study, of whom 256 (85.3 %) consented to participate. Subjects using vitamin K antagonists were excluded. Advanced aortic calcifications were present in 34 subjects. Measurements of the vitamin K status were performed in this group as well as in 79 women randomly selected from the cohort of women without aortic calcifications.

MEASUREMENTS

Abdominal aorta

Atherosclerosis of the abdominal aorta was assessed using a lateral X-ray of the lumbar spine (T12-S1), on which the presence of calcified deposits was determined. Calcifications were considered to be present when linear densities were clearly visible in an area parallel and anterior to the lumbar spine (L1-L4).¹⁹ All films were examined by two independent observers without knowledge of the risk factor status of the subjects. The severity of the atherosclerosis was graded according to the length of the involved area (≤ 1 cm, 2-5 cm, 6-10 cm, > 10 cm). The third and fourth classes were combined as advanced aortic atherosclerosis, whereas the first class was considered as no aortic atherosclerosis.²¹

Dietary vitamin K intake

The daily vitamin K intake was determined using a food frequency questionnaire, with emphasis on food products with a high known vitamin K content such as vegetables, dairy products. The calculation of vitamin K content of various food items was based on data kindly provided by Dr. M.J. Shearer (Haemophilia Centre, St Thomas Hospital, London) corresponding to published values.^{26,27} The average vitamin K content of each food item was multiplied by the amount and frequency of the reported use of the food item. The data thus obtained were summarized and used as an estimate of the individual daily vitamin K intake.

Anthropometric variables

Height and weight were measured without shoes and with indoor clothing. Body mass index (kg/m^2) was calculated. The circumference of the waist and hip were measured and the waist-to-hip ratio was calculated.

Vitamin K status

Non-fasting blood samples were collected by venipuncture. For the preparation of serum, blood samples were left at room temperature for 1 hour, briefly centrifuged, and immediately frozen at -80°C . Serum immunoreactive osteocalcin (irOC) levels were measured by radioimmunoassay (Incstar, Stillwater, MI) before (irOC_{total}) and after (irOC_{free}) extraction with 100 mg/ml hydroxyapatite.¹⁸ IrOC_{bound} was calculated from the difference between irOC_{total} and irOC_{free}. The hydroxyapatite binding capacity (HBC) was calculated as the percentage of irOC_{bound} relative to the irOC_{total} level, based on the Gla-dependent affinity of osteocalcin for hydroxyapatite.^{18,28} By measuring irOC before and after extraction of serum with hydroxyapatite, two fractions of serum irOC (differing in Gla-content) may be distinguished: irOC with low (irOC_{free}) or that with high (irOC_{bound}) affinity for hydroxyapatite.²⁹ The inter- and intra-assay coefficients of variation of irOC_{total} were 10 % and 5 %, respectively. In the present study irOC_{free} and the HBC levels were used as overall estimates of the Gla-content of circulating irOC levels.

Data analysis

Mean levels of vitamin K intake and osteocalcin fractions were compared between subjects with and without atherosclerotic calcifications in the abdominal aorta using Student t-tests. Multiple linear regression was used for analysis of differences across groups adjusted for variables such as age and anthropometric variables. The differences are presented with a 95 % confidence interval (CI). Analyses were performed using BMDP statistical package.

RESULTS

General characteristics of women with and without calcifications of the abdominal aorta are presented in Table 1.

Vitamin K intake

The mean age-adjusted vitamin K intake was lower among subjects with atherosclerosis of the abdominal aorta than among those without atherosclerosis, with a

Table 1: General characteristics of the study population

	Total population	Aortic Calcifications		
		Absent	Present	P-value
Number	113	79	34	
Age (years)	66.6 (4.6)	66.1 [0.46]	67.8 [1.0]	0.07
Body mass index (kg/m ²)	26.1 (3.1)	26.5 [0.34]	25.4 [0.55]	0.07
Waist-to-hip ratio	0.84 (0.06)	0.84 [0.007]	0.84 [0.009]	0.81

P-value refers to differences between groups with and without aortic calcifications. Values are expressed as means with standard deviations in parenthesis, and standard errors in brackets.

Table 2:

Indicators of vitamin K in postmenopausal women with and without abdominal calcifications

	Aortic calcifications		Mean difference	Age adj. difference
	Absent	Present		
Number	79	34		
Vit K intake (μ g/d)	243.6 (15.32)	189.9 (15.1)	-53.7 [-103.6, -3.8]	-42.9 [-92.5, 6.6]
irOCt (ng/ml)	4.70 (0.15)	4.70 (0.28)	0.01 [-0.56, 0.57]	0.19 [-0.34, 0.73]
irOCfree (ng/ml)	1.93 (0.08)	2.16 (0.15)	0.23 [-0.07, 0.53]	0.32 [0.03, 0.61]
irOCbound (ng/ml)	2.77 (0.09)	2.55 (0.17)	-0.22 [-0.57, 0.12]	-0.13 [-0.46, 0.21]
HBC (%)	59.4 (0.98)	54.6 (1.77)	-4.79 [-8.49, -1.09]	-5.12 [-8.92, -1.32]

Values: means (standard errors) or [95% Confidence Intervals]. Abbreviations: irOCt = total immunoreactive osteocalcin; irOCfree = osteocalcin free; irOCbound = osteocalcin bound; HBC = Hydroxyapatite Binding Capacity of osteocalcin

difference of 42.9 μ g/day [95 % CI -6.6,92.5] (Table 2). Adjustment for differences in age, body mass index and waist-to-hip-ratio did not substantially alter the findings. The vitamin K intake according to age and presence or absence of aortic calcifications is shown in Figure 1. Vitamin K intake decreased considerably with increasing age with an average of 7.32 μ g/day per year of day [95 % CI 4.08,10.60]. While it was higher in the relatively younger women without calcifi

Figure 1. Mean vitamin K intake (SE) in postmenopausal women according to age and presence or absence of aortic calcifications. AS- = without aortic calcifications; AS+ = with aortic calcifications. ** Comparison between subjects with and without aortic atherosclerosis ($p < 0.1$).

cations, the decrease of vitamin K intake with age was present mainly in those without atherosclerotic calcifications of the abdominal aorta compared with subjects with aortic atherosclerosis: mean decrease of 10.08 ug/day per year [95 % CI 9.49,10.67] and mean decrease of 1.23 ug/day per year [95 % CI -4.69,7.15], respectively (Figure 1). In the atherosclerotic subjects the vitamin K intake was low in all groups; the relatively high value for the 70-74 age-group was caused by one extreme outlier. From Figure 1 it is clear that the difference in vitamin K intake between atherosclerotic and non-atherosclerotic subjects (as given in Table 1) are mainly due to the nutritional habits in the 60-69 age group. In this group the difference in vitamin K intake was - 75.4 µg/day [95% CI -135.4,-15.5], whereas in the 70-79 age group the difference was not significant. After adjustment for age the difference was -74.8 [95% CI -135.1,-14.6].

Serum osteocalcin fractions

The mean age-adjusted irOC_{free} level among women with aortic atherosclerosis was higher than in those without aortic lesions with a difference of 0.32 ng/ml [95 % CI 0.03,0.61] (Table 2). Women with aortic atherosclerosis had significantly lower mean age-adjusted HBC levels with a difference of 5.12 % [95 % CI 1.32,8.92]. When differences in body mass index and waist-to-hip ratio were taken into account, the associations remained of similar direction, magnitude and significance. Both irOC fractions were significantly and negatively associated with age, body mass index and waist-to-hip ratio (data not shown). A weak non-

significant association was found between dietary vitamin K intake and $\text{irOC}_{\text{free}}$ level (β -7.0, se 16.0) and HBC level (β 0.59, se 1.23).

DISCUSSION

The findings in the present study suggest that vitamin K status as measured by vitamin K intake, $\text{irOC}_{\text{free}}$, and HBC levels is reduced in older women with aortic atherosclerosis.

Before these results can be accepted, some aspects of this study need to be discussed. Firstly, the validity of roentgenographic assessment of aortic calcification in the diagnosis of advanced atherosclerosis has been studied by comparison with assessments made on necropsy material,²⁰ and the method was shown to be highly specific. In a recent comparison of radiographs and CT images of calcified aortas the calcifications were shown to be within the vascular wall of the aorta.²¹ Roentgenographically assessed aortic calcifications have been shown to be associated with cardiovascular risk factors^{19,30,31} and their presence has been found to be a predictor of coronary heart disease^{22,32} and cardiovascular mortality.^{22,24,25} Moreover, the presence of calcification in the abdominal aorta has been associated with atherosclerosis in other arterial vessels.³³

Secondly, the extent to which the 5 year interval between the assessment of atherosclerosis of the abdominal aorta and the measurement of the vitamin K status may have affected our results should be addressed. Based on earlier findings it is not likely that the calcified atherosclerosis of the abdominal aorta present at baseline would regress over a five year period.¹⁹ However, some women initially without atherosclerosis of the abdominal aorta may have developed aortic atherosclerosis during these 5 years. Assuming that a true association exists between vitamin K status and aortic atherosclerosis, this misclassification of atherosclerotic status, if present, will have attenuated the observed association. Thus, the true association may actually be stronger than observed.

The vitamin K intake in the present study is in accordance with the limited data reported by others, in which vitamin K intake varies from 50 to 500 $\mu\text{g/day}$ depending on the characteristics of the population studied.^{34,35,36} Plasma vitamin K levels have been shown to be strongly influenced by recent vitamin K intake.^{14,34} Our finding of a decrease in vitamin K intake with age may suggest that a lower dietary vitamin K intake is one of the factors accounting for the observed lower plasma vitamin K levels in elderly.³⁴ This is of particular interest because our findings indicate that women without aortic calcifications appear to initially have a higher vitamin K intake which gradually decreases and only after

the age of 75 years reaches the lower vitamin K intake level of women with aortic atherosclerosis. Since the differences were mainly found in the younger age-groups, it would be worthwhile to also investigate the nutritional vitamin K intake among atherosclerotic and non-atherosclerotic subjects aged 40-60 years.

For measuring vitamin K status at the time of the study we have not chosen circulating phyloquinone because vitamin K status is determined not only by phyloquinone, but also by menaquinones, the detection of which still raises serious problems. The main reason, however, is that the relation between circulating vitamin K concentrations and tissue vitamin K status is only poorly understood. Results from several studies support the view that the Gla-content of extra-hepatic proteins may be regarded as a sensitive indicator for the vitamin K status.^{15,16,17,18,37,38} Recently we have successfully used the HBC of osteocalcin for this purpose. The HBC-values reported here are in line with previous findings, indicating that a poor vitamin K status is common among elderly women.^{18,29} The lower HBC levels in the atherosclerotic women are consistent with their lower vitamin K intake. Only a weak non-significant association was found between the dietary vitamin K intake and the HBC levels. This finding is consistent with the view that apart from vitamin K intake other factors such as the absorption or transport of vitamin K to the tissue, plasma triglyceride and lipoprotein content, may be important as determinants of vitamin K status. Hence substantial interindividual variations may exist in the efficiency of vitamin K

Osteocalcin is probably directly involved in the vascular mineralization process.^{3,4,7} Recently, it has been shown that osteocalcin is present in the atherosclerotic plaque,⁷ and that both osteocalcin and MGP can be produced locally in the atherosclerotic plaque and may directly be involved in calcification.^{10,11} Osteocalcin may effectively inhibit the precipitation of calcium phosphate and hydroxyapatite crystal growth.³⁹ The exact function of the various Gla proteins in the atherosclerotic plaque, however, remains to be elucidated. Higher levels of $\text{irOC}_{\text{free}}$ in the atherosclerotic women than in women without aortic calcification may suggest elevated circulating levels of Gla deficient osteocalcin.^{18,28,29,38} Assuming that these Gla-deficient osteocalcin molecules are hampered in their function, i.e. the inhibition of calcium salt precipitation, the calcification process in the vessel wall may take place at an increased rate.

In conclusion, our study indicates that atherosclerotic women have an impaired vitamin K status as reflected by a lower nutritional vitamin K intake, an increased $\text{irOC}_{\text{free}}$ and a lower HBC level. An impaired vitamin K status in subjects with atherosclerosis is compatible with the view that vitamin K or Gla-containing proteins are involved in the development of calcifications of the vessel

wall. Further studies are needed to clarify the exact mechanisms involved and their potential implications for prevention and intervention.

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**A POPULATION-BASED STUDY IN WOMEN WITH AND WITHOUT
ATHEROSCLEROSIS:
VITAMIN K STATUS AND BONE MASS**

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SUMMARY

Gammacarboxyglutamate (Gla) is an uncommon amino acid formed by vitamin K action, giving vitamin K-dependent proteins calcium binding properties. Increasing evidence indicates that Gla-proteins are involved in the regulation of the calcification processes in both bone tissue and the atherosclerotic vessel wall. In a population-based study we have previously shown that in a group of 113 postmenopausal women the presence of abdominal aortic calcifications is associated with a reduced vitamin K status. In this study we investigated whether this reduced vitamin K status was also associated with differences in bone mass or circulating calciotropic hormone levels. Women with atherosclerotic calcifications had an 8% lower bone mass (mean difference of 3.15 mm², 95%CI: 0.18-6.72). No differences between the two groups of women were observed for serum intact PTH (iPTH) and serum 25-hydroxyvitamin D levels. In the atherosclerotic women (n=34) both markers for vitamin K status were associated with bone mass (irOC_{free}: $r=-0.47$, $p=0.013$; HBC: $r=0.57$, $p=0.002$), whereas no such association was found in the non-atherosclerotic women (n=79). In addition iPTH levels were significantly associated with irOC_{free} ($r=0.30$, $p=0.002$) and HBC ($r=-0.27$, $p=0.005$) in all the women tested. The atherosclerotic women in our study may be at higher risk for osteoporotic fractures as evidenced by their lower bone mass and higher irOC_{free} levels. The finding that especially in atherosclerotic women vitamin K status is associated with bone mass, supports the view that vitamin K status affects the mineralization processes both in bone and in atherosclerotic plaques.

INTRODUCTION

Osteoporosis and atherosclerosis are two major chronic causes of morbidity. Although they tend to be regarded as two independent, age-related processes, it is striking that in women the prevalence of both disorders increases dramatically after menopause.¹ Estrogen-deficiency or a derangement in Ca-metabolism have been proposed as candidates for common pathophysiological factors.^{2,3,4}

Results from recent studies suggest that atherosclerotic calcification must be regarded as a highly regulated process similar to that occurring in bone tissue.⁵ It is of interest is that in both the calcification processes vitamin K-dependent proteins are thought to be involved.^{6,7,8,9} Vitamin K dependent proteins have calcium-binding properties mediated by the posttranslationally acquired γ -carboxyglutamate (Gla) residues.¹⁰ A diminished availability of vitamin K, e.g. due to a reduced nutritional intake or impaired intestinal absorption, may result in a decreased Gla-content of the various Gla-proteins. In the latter case Gla-proteins, such as osteocalcin will be functionally defective, resulting in a reduced affinity for the hydroxyapatite bone matrix. This can be measured in serum as the serum immunoreactive osteocalcin with a low affinity for hydroxyapatite (irOC_{free}).¹¹ The high affinity osteocalcin (irOC_{bound}), expressed as a percentage of total serum osteocalcin, is called the hydroxyapatite binding capacity of osteocalcin (HBC) and is regarded as a measure for osteocalcin Gla-content and for vitamin K status.¹²

It has been suggested that even a mild vitamin K-deficiency may affect calcium homeostasis. In a number of trials, we have recently shown that markers of bone formation increase, while bone resorption markers decrease with the administration of pharmacological doses of vitamin K.^{12,13,14} These data are consistent with two Japanese studies, both of which demonstrated that vitamin K supplementation may retard postmenopausal loss of bone mass.^{15,16} We have recently shown that in a group of postmenopausal women, the presence of atherosclerotic calcifications in the abdominal aorta, considered to be a strong predictor of cardiovascular heart disease and mortality,^{17,18,19} is associated with a marginal vitamin K status.²⁰ In the present study we examined whether the reduced vitamin K status in the atherosclerotic women was associated with a reduced bone mass as well.

METHODS

Subjects

Participants were selected from a population-based study on determinants of osteoporosis and atherosclerosis.²¹ Bone mass measurements as well as the extent of calcified deposits in the abdominal aorta were determined in 1985. In 1990, 256 women consented to take part in a follow-up examination.²⁰ In brief, aortic calcification (grade 3 or grade 4) was found to be present in 34 subjects. Seventy-nine women were randomly selected from the cohort without aortic calcification (grade 0). Subjects using vitamin K antagonists were excluded.

Abdominal aorta

Atherosclerosis of the abdominal aorta was assessed using a lateral X-ray of the lumbar spine (T12-S1), from which the presence of calcified deposits was determined.²² The severity of the atherosclerosis was graded from 0 (no calcifications) to 4 (aorta outlined with calcifications).²⁰

Bone mass

Bone mass measurements was determined using Metacarpal Radiogrammetry. The outer diameter (D) and medullar diameter (d) were measured at the mid-shaft of the metacarpal bones, using a 7 times magnifying measurement loop with an accuracy of 0.01 mm. Bone mass (metacarpal cortical area) was expressed as the mean value of $D^2 - d^2$ (mm²) of the metacarpal bones II, III, and IV of both hands. The mean intra-individual standard deviation of a duplicate measurement was 1.9 mm² (4%).

Anthropometric variables

Height and weight were measured without shoes and with indoor clothing. The body mass index (kg/m²)(BMI) was calculated. The circumference of the waist and hip were measured and the waist-to-hip ratio (WH-ratio) was calculated.

Vitamin K status

Immediately after preparation, the non-fasting serum and plasma samples were frozen at -80 °C. Serum immunoreactive osteocalcin (irOC) levels were measured before (irOC_{total}) and after (irOC_{free}) extraction with 100 mg/ml hydroxyapatite.¹² IrOC_{bound} was calculated from the difference between irOC_{total} and irOC_{free}. The Hydroxyapatite Binding Capacity (HBC) was calculated as the percentage of irOC_{bound} relative to the total irOC level and used as an overall estimate of the Gla-content of circulating irOC levels. In young, healthy adults the HBC values

range between 70 and 80 %, decreased values are found in subjects with a marginal vitamin K-status.^{12,13} Inter- and intra-assay coefficients of variation of serum $\text{irOC}_{\text{total}}$ were 10 % and 5 %, respectively.

Additional measurements

Serum intact parathyroid hormone levels (iPTH) were measured with a direct immunoradiometric assay (IRMA) (N-tact® PTH SP) and serum 25-Hydroxy-vitamin D (25OHD) levels were determined using a RIA (both obtained from Incstar, Stillwater, MI).

Data analysis

Multiple linear regression was used for analysis of differences across groups with and without atherosclerosis, adjusted for confounding variables such as age. The differences are presented with a 95 % confidence interval (95%CI). Analyses were performed using the BMDP statistical package. Results are presented as the mean values ($\pm$ standard deviation), unless indicated otherwise.

RESULTS

As reported previously, no major differences were noted between the women with or without atherosclerosis with respect to their age (67.8 (5.5) and 66.1 (4.1), respectively), BMI (25.4 (3.2) and 26.5 (3.0) kg/m², respectively) or WH-ratio (0.84 (0.05) and 0.84 (0.06), respectively).²⁰ The atherosclerotic women had higher total cholesterol levels (7.56 (1.05) mmol/l) compared to the non-atherosclerotic women (6.85 (1.00) mmol/l, $p < 0.01$), whereas no difference was noted in their HDL-cholesterol levels (1.84 (0.65) and 1.74 (0.94) mmol/l, respectively).

The bone mass of the atherosclerotic and non-atherosclerotic women was 41.6 and 45.1 mm², respectively, constituting a mean difference of 3.44 mm² (95 %CI: -6.72, -0.18) or 8 % (Table). Adjustment for age slightly attenuated the difference to -3.15 mm² (95 %CI: -6.47, 0.17). In the atherosclerotic women bone mass was significantly associated with both $\text{irOC}_{\text{free}}$ ($r = -0.47$, $p = 0.013$) and HBC levels ($r = 0.57$, $p = 0.002$). No association was found in the women without atherosclerosis ($\text{irOC}_{\text{free}}$ ($r = 0.12$, $p = 0.33$) or HBC ($r = -0.14$, $p = 0.28$). Adjustment for age, BMI or cholesterol did not essentially alter these findings (data not shown).

No differences were observed in iPTH and 25OHD levels (table). 25OHD levels of 12 ng/l (considered as borderline value) and below were present in 44

Table

Parameters tested in postmenopausal women with and without calcifications of the abdominal aorta.

	Aortic calcifications		Mean difference	Age adj. difference
	Absent	Present		
Number	79	34		
irOC _{free} (ng/ml)	1.93 (0.08)	2.16 (0.15)	0.23 [-0.07, 0.53]	0.32 [0.03, 0.61]
HBC (%)	59.4 (0.98)	54.6 (1.77)	-4.79 [-8.49, -1.09]	-5.12 [-8.92, -1.32]
bone mass (mm ²)	45.1 (0.9)	41.6 (1.4)	-3.5 [-6.7, -0.2]	-3.2 [-6.5, 0.2]
iPTH (pg/ml)	41.6 (1.8)	43.7 (2.8)	2.1 [-4.2, 8.4]	2.0 [-4.4, 8.5]
25OHD (ng/ml)	15.4 (1.6)	16.8 (1.6)	1.4 [-3.1, 5.8]	1.8 [-2.5, 6.2]

Values: means (standard errors) or [95 % Confidence Intervals]. irOC = serum immunoreactive osteocalcin; HBC = hydroxyapatite binding capacity; iPTH = intact serum PTH; 25OHD = serum 25-Hydroxyvitamin D.

% of the women.^{23,24} In both groups, bone mass was positively associated with 25OHD levels (pooled data: $r=0.23$, $p=0.07$), but not with iPTH (pooled data: $r=0.10$, $p=0.36$). In the atherosclerotic women iPTH levels were negatively associated with HDL ($r = -0.42$, $p = 0.013$). This association was most marked in those with 25OHD of 12 ng/ml or lower ($r = -0.73$, $p = 0.005$): a decrease of 0.1 mmol/l HDL being associated with an increase in iPTH of 1.52 pg/ml (95%CI: 0.68 to 2.36). iPTH levels were significantly associated with irOC_{free} (pooled data: $r= 0.30$, $p = 0.002$) and HBC (pooled data: $r = -0.27$, $p = 0.005$) levels in both groups. The associations remained essentially unchanged after additional adjustments for possible confounders as age, BMI and serum cholesterol levels.

DISCUSSION

The results of our study indicate that in postmenopausal women the presence of atherosclerotic calcifications in the abdominal aorta is not only associated with a marginal vitamin K-status,²⁰ but also with a low bone mass. Atherosclerotic calcifications in the abdominal aorta have previously been shown to represent severe aortic atherosclerosis in comparisons with necropsy and CT findings.^{25,26} These calcifications have shown to be strong predictors of coronary

heart disease^{27,28} and cardiovascular mortality.^{19,18,27} Assessment of bone mass by radiogrammetry based on multiple measurements of six metacarpal bones, on the other hand, has been shown to be sufficiently precise for meaningful comparisons of bone density between individuals.²¹

A low bone mass is a well established and important risk factor for the development of osteoporotic fractures.^{29,30} Recent data strongly indicate that high $\text{irOC}_{\text{free}}$ levels are associated with an increased risk for hip fractures³¹ and that in elderly women elevated $\text{irOC}_{\text{free}}$ levels are associated with a low bone mass.³² The findings of our study that atherosclerotic women had a low bone mass and high $\text{irOC}_{\text{free}}$ levels, therefore strongly suggest that they are at increased risk for developing osteoporotic fractures.

Several studies have reported that both low bone mass and increased frequency of osteoporotic fractures are associated with atherosclerotic calcifications^{2,3,33,34,35,36} or cardiovascular deaths.^{4,37} Some have regarded the observed associations merely as being age related phenomena,^{34,35} whereas others have suggested a causal relationship.^{3,2,33,37} High levels of iPTH or 25OHD have been proposed as causally related factors, however, this is not supported by our data. The 25OHD and iPTH levels in our study are in accordance with other studies performed in The Netherlands.^{23,24} The relatively high iPTH levels in our study population are indicative of a secondary hyperparathyroidism commonly found in women of this age.^{24,38} The fact that iPTH is associated with $\text{irOC}_{\text{free}}$ levels, suggests that serum $\text{irOC}_{\text{free}}$ may result from PTH-mediated bone resorption. Although it is assumed that $\text{irOC}_{\text{free}}$ levels represent the undercarboxylated osteocalcin, there is some evidence that at least part of the circulating $\text{irOC}_{\text{free}}$ consists of osteocalcin degradation products, possibly originating from bone resorption.^{14,39} The strong association between PTH and HDL in the atherosclerotic women was unexpected. An explanation may be found in the observation that PTH is able to induce lipolysis,⁴⁰ and that the increased free fatty acid release in serum leads to higher levels of (VLDL) triglyceride and subsequent lower HDL-cholesterol levels.⁴¹ This, however, requires further confirmation.

Evidence is accumulating indicating that vitamin K stimulates bone formation and inhibits bone resorption which ultimately may lead to a decrease in bone loss.^{12,13,14,15,16} The fact that only in the atherosclerotic women both markers for vitamin K status were strongly associated with bone mass suggests that additional (as yet unknown) factors may be of importance. It has been suggested for instance that abnormalities in lipoprotein metabolism may influence vitamin K transport to bone tissue.

What may be the pathophysiological mechanism in which a reduced vitamin K-status leads to a lower bone and an increased frequency of atherosclerotic calcifications? Recent evidence strongly supports the concept that the calcification associated with atherosclerosis must be regarded as an active, highly organised process similar to that of osteogenesis.^{5,42} Gla-proteins appear to be involved in both processes.^{6,8,9} The Gla-proteins MGP (matrix Gla-protein) and osteocalcin, are both involved in the bone mineralization process. Their exact function, however, is still unclear. It has been suggested that MGP may protect against excessive tissue calcification during early bone development.⁴³ Recently, high levels of MGP mRNA and protein were demonstrated in macrophages and in vascular smooth muscle cells, present in human atherosclerotic plaques.⁹ This strongly suggests that MGP found in the plaques is not absorbed from the blood stream, but that it is produced locally at the site of these plaques. We speculate that the function of vascular MGP is similar to that in bone, i.e. that it acts as a mineralisation inhibitor. By analogy with the fetal warfarin syndrome,⁴⁴ under-carboxylation of MGP would thus lead to an excessive mineralisation of the atherosclerotic vessel wall. Osteocalcin, on the other hand, is predominantly synthesized by the more differentiated osteoblast, relatively late in bone development, concomitant with or after the onset of mineralization.⁴⁵ Also its different molecular properties and tight association with the mineral matrix suggest a different function for osteocalcin, for instance in bone remodelling or in the accumulation of bone mass.⁴⁶ In mature bone tissue, a suboptimal vitamin K-status will therefore affect the osteocalcin-mediated remodelling process ultimately leading to a net loss of bone mass.^{12,13,14,15,16}

In conclusion, we found that the presence of atherosclerotic calcifications in postmenopausal women is associated with an impaired vitamin K-status as well as a low bone mass. These findings support the view that aortic calcification and bone loss may be two related processes, with vitamin K deficiency as a common denominator. Further studies are needed to investigate the potential benefit of vitamin K-supplementation.

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THE ISOLATION OF γ -CARBOXYGLUTAMIC ACID (GLA) - CONTAINING PROTEINS

7.1 A SPECIFIC COLORIMETRIC STAINING METHOD

7.2 GLA PROTEINS IN ATHEROSCLEROTIC CALCIFICATIONS

**A SPECIFIC COLORIMETRIC STAINING METHOD FOR
 γ -CARBOXYGLUTAMIC ACID-CONTAINING PROTEINS
IN POLYACRYLAMIDE GELS**

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SUMMARY

In this paper we describe a specific staining method for γ -carboxyglutamic acid (Gla)-containing proteins in polyacrylamide gels. The procedure is based on the colorimetric detection of Gla using 4-diazobenzenesulfonic acid and has the advantage of being simple and fast (1 h). The detection limit is 9.4 pmol for prothrombin and 150 pmol for osteocalcin. It is demonstrated that Gla-proteins can be visualized independent of the presence of an excess of contaminating non-Gla-proteins. The technique may be used for screening of large number of fractions during the purification of Gla-proteins from complex protein mixtures.

INTRODUCTION

Vitamin K is an essential cofactor in the formation of the unusual amino acid γ -carboxyglutamate (Gla).¹ The vitamin K-dependent enzyme system (γ -glutamyl carboxylase) has been identified in nearly all mammalian tissues and cell types. It is at least remarkable that at this time not more than 9 different Gla-proteins have been purified and characterized to a molecular level. Apparently only low amounts of Gla-proteins are produced in other tissues than liver and bone. Therefore, sufficiently sensitive techniques will have to be developed to allow for their detection. The discovery of Gla has been hampered for a long time by its decarboxylation under the acid conditions generally applied for protein hydrolyses and subsequent amino acid analysis. In 1977 it was described that this problem could be overcome simply by applying alkaline conditions for the hydrolysis of proteins.² Since then several methods have been described varying from precolumn derivatization followed by HPLC detection³ to incorporation of ^3H into the γ -carbon of Gla.^{4,5} These methods, however, are all relatively time-consuming and only applicable to hydrolysates. In 1981 Pecci and Cavallini reported a colorimetric method based on the conversion of Gla to a proline derivative using acetaldehyde.⁶ Colorimetric assays using 4-diazobenzenesulfonic acid (DBS) have been described for the detection of Gla in protein hydrolysates as well as in solutions and Western blots.^{7,8} We have adapted the latter method⁸ and have made it directly applicable for SDS-PAGE gels. Our procedure for the detection of Gla-proteins is quick and simple and may be used, for example, for the rapid screening of large numbers of fractions during protein purification.

MATERIALS AND METHODS

Materials

4-Diazobenzenesulfonic acid was obtained from FLUKA. Trizma base, dithiothreitol (DTT) were both purchased from Sigma. Sodiumnitrite, EDTA, NaOH, acetate buffer, and citrate were products from Merck. Electrophoresis reagents and protein molecular weight standards were from Bio-Rad.

Bovine prothrombin was a kind gift from Mrs. J. Govers (University of Limburg, Maastricht). Bovine osteocalcin was purified as previously described.⁹ The QAE-Sephadex extract of human calcified atherosclerotic plaques was prepared as described previously.¹⁰ A barium citrate extract was made from human plasma in order to concentrate the various Gla-proteins present.¹¹

Procedures

Gel electrophoresis was performed under reducing conditions according to Laemmli, on 15% polyacrylamide gels in the Bio-Rad minigel system.¹² Proteins were blotted from the gels on PVDF membranes (Applied Biosystems) in a Bio-Rad Mini Trans-Blot Cell system.

DBS was washed by repeated suspension and centrifugation in ice-cold acetone until no major color change was observed in the supernatant. The DBS was dried and used for the DBS staining solution containing 8.5 mM DBS, 6.4 mM NaNO₃ in 2 M acetate buffer (pH 4.6) as described by Nishimoto.⁸ Immediately after gel electrophoresis the gels were washed three times for a period of 5 min in 50% methanol, 10% acetic acid to fix the proteins and to remove SDS. The gels were then stained in approximately 50 ml DBS staining solution under gentle agitation for 2 h at room temperature and in the dark. Subsequently, the staining solution was decanted and the gels were incubated in developing solution consisting of 24 ml 12.5 M NaOH and 3.8 ml 0.5 M EDTA. After gentle shaking in the dark for at least 30 min, the wet gels were ready to be photographed. Relatively weak red color staining, although readily visible in the gel, were rather faint after being photographed. For this reason black and white photographs were taken on a high-contrast film (Technical Pan obtained from Kodak). For the preservation the gels were dried on filter paper backing using a gel dryer, both obtained from Bio-Rad. It should be noted that a rapid crystallization of NaOH salts can be prevented by sealing the dried gel in plastic.

RESULTS AND DISCUSSION

Establishment of optimal staining conditions

Initially, the visibility of the specific red Gla staining was hampered by a yellow-red background staining. Thorough washing of the DBS with cold acetone before use and washing of the gel prior to the DBS-staining procedure appeared to be an important step for reducing the background color of the gel. After the addition of the developing solution the gel tends to shrink, which slightly increases the color intensity. Long incubation periods (> 2 h) are not recommended as they lead to an expansion of the gel. For photographic recording we recommend photographing of the wet gel immediately after a developing period of 0.5 - 1 h. Thereafter, the gel can be dried on paper and preserved (in the dark) for at least 12 weeks, without notable loss of color.

The optimal conditions for the detection of Gla-proteins in polyacrylamide gels were assessed in a (1:1 w/w) mixture of two purified Gla-proteins:

Figure 1. DBS staining of a (1:1 w/w) mixture of purified prothrombin (FII) and osteocalcin (OC) in a 15% polyacrylamide gel, dried on paper. The numbers above each lane depict the amount ($\mu\text{g}/\text{lane}$) of each protein loaded on the gel.

prothrombin (M_r 72 kDa, 10 Gla residues) and osteocalcin (M_r 5.8 kDa, 3 Gla residues). Gla-proteins stain red, whereas proteins not containing Gla obtain a yellow color with the DBS-staining method. The detection limit of the yellow-stained non Gla-proteins, as assessed with bovine serum albumin, is approximate-

Figure 2. Specificity of the DBS staining in two protein mixtures. A is a Coomassie blue-stained gel; lane 1: 1:10 diluted plasma; lane 2: QAE-Sephadex bone extract. B is stained according to the DBS-method; lane 1: 1:10 diluted plasma; lane 2: QAE-Sephadex bone extract; OC = osteocalcin.

Figure 3. DBS staining of extracts from plasma and human calcified atherosclerotic plaques. Lane 1: barium citrate plasma extract; lane 2: as in lane 1, but unreduced gel. $\rightarrow$ = low chain FX; lane 3: QAE-Sephadex extract of human calcified atherosclerotic plaques. $\blacktriangle$ = Gla-proteins of approximately 10, 18, and 27 kDa.

ly 5 μ g (data not shown). A false-positive staining appearing as a diffuse area in the low molecular region was observed with β -mercaptoethanol but not with DTT (10 mM) as a reductant. The detection limits of prothrombin and osteocalcin were 9.4 pmol (0.68 μ g) and 150 pmol (0.87 μ g), respectively (Figure 1). This is comparable to the results reported for the Western blot procedure (osteocalcin: 1 μ g or 170 pmol).⁸ The detection limits found for prothrombin and osteocalcin are not comparable, although the molar amount of Gla in prothrombin is 3.3 times higher than that in osteocalcin. This apparent discrepancy obtained with DBS staining has been reported earlier.⁷

Specificity of the DBS staining

The specificity of the DBS staining is demonstrated best when applied to several complex protein samples. A QAE-Sephadex bovine bone extract showed one Gla-containing (osteocalcin) protein after staining with DBS (Figure 2B, lane 2). Coomassie staining showed a major band at the same position, but several other non-Gla-containing protein bands to be present (Figure 2A, lane 2). DBS-staining of human plasma (1:10 diluted), on the other hand, did not show any Gla-containing protein bands to be present (Figure 2B, lane 1). In order to enrich the plasma sample in Gla-proteins, adsorption to barium citrate and subsequent elution was performed. A barium citrate extract of human plasma showed the existence of a prominent Gla-protein at approximately 72 kDa, whereas a faint red staining was observed at 25 kDa (Figure 3, lane 1). Additional analysis with

specific antibodies showed a positive reaction of the 72 kDa-band with antiprotrombin and of both the 25-kDa and 60-kDa with antifactor X (data not shown). The fact that in a non-reducing gel the 25-kDa protein band had migrated to 60 kDa (Figure 3, lane 2) was an indication that this band was the light chain of the blood coagulation factor X. Finally, DBS staining of a QAE-Sephadex extract of human calcified atherosclerotic plaques showed the presence of at least three Gla-proteins of approximately 27, 18, and 10 kDa (Figure 3, lane 3).

CONCLUSIONS

In this paper we describe a method for specific staining of Gla-proteins in polyacrylamide gels. Our method has several advantages over the DBS staining procedure recently described for Western blots. First, in blots the optimal blotting time for each protein varies with the molecular weight of the protein, which may drastically influence the efficiency of the protein transfer, notably in complex mixtures. With our method there is no loss of (unknown amounts of) proteins, which makes the evaluation of heterogenous Gla-proteins of different molecular weights much easier. Second, in contrast to the blots, the gels can be preserved for at least 12 weeks without notable color loss by drying the gels on filter backing paper. The gel staining method is also much quicker (1 h staining instead of overnight). Finally, non Gla-proteins ($> 5 \mu\text{g}/\text{band}$) are also detected with our staining procedure, but in a different color.

With this technique we have shown the occurrence of several Gla-containing proteins in barium citrate enriched plasma, as well as in QAE-Sephadex extracts from bone and human calcified atherosclerotic plaques. The DBS-gel staining method appears to be a rapid and sensitive method for the assessment of Gla-proteins in crude protein mixtures.

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GAMMA-CARBOXYGLUTAMIC ACID (GLA)-CONTAINING PROTEINS IN ATHEROSCLEROTIC CALCIFICATIONS OF THE HUMAN VESSEL WALL

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SUMMARY

Vitamin K-dependent or γ -carboxyglutamic acid (Gla) containing proteins are probably involved in the mineralization process in the atherosclerotic vessel wall. Although several new Gla-proteins have been identified in atherosclerotic calcifications, none of them have been purified to homogeneity. In this study we describe the presence of protein-bound Gla of approximately 10, 18 and 27 kDa in the calcified atherosclerotic plaque. The isolation and characterization of these proteins is greatly hampered by considerable losses during each fractionation. An explanation may be found in the concomitant extraction of interfering components such as negatively charged proteoglycans.

INTRODUCTION

The unusual calcium binding amino acid γ -carboxyglutamate (Gla) was initially shown to be present in blood coagulation factors.¹ A few years later, it was revealed that Gla residues are not confined to proteins involved in blood coagulation but are also present in a number of proteins located in sites of physiological and pathological mineralizations including those found in bone tissue, calcium containing renal stones and skin exsudates, calcified heart valves, and the calcified atherosclerotic plaque.^{2,3,4,5}

During the vitamin K-dependent carboxylation reaction, certain protein-bound glutamate residues are converted into Gla.⁶ The Gla residues seem to be essential for the biological activity of Gla-proteins, and their presence substantially contribute to the calcium binding capacity of these proteins. The function of Gla-proteins associated with atherosclerotic calcifications is not precisely known. Interestingly, the bone Gla-proteins osteocalcin and MGP have both been identified in atherosclerotic calcifications.^{7,8,9} Since in bone tissue these proteins are thought to be involved in the regulation of the calcification process, it is tempting to assume a similar function in the atherosclerotic vessel wall.^{2,9} Recent studies indicate that also other (non-Gla containing) matrix proteins normally found in bone tissue, are actively involved in the plaque mineralization process.^{9,10,11}

Gla-proteins reported to be present in atherosclerotic calcifications vary widely in their molecular range: 6-10,^{7,8,12} 80 kDa,¹³ or 23 kDa.¹⁴ Whereas some of these Gla-proteins were shown to be similar to osteocalcin,^{2,7,8} others apparently were an artifact of the isolation procedure (atherocalcin, 80 kDa).¹⁵ The three Gla-proteins found in bone tissue, osteocalcin, MGP and protein S¹⁶ are well characterized. The isolation of Gla-proteins found in the calcified plaque, on the other hand, has been hampered by poor reproducibility and very low yields of various fractionations. The study presented here was undertaken to improve the isolation and characterization of the Gla-proteins present in the EDTA-soluble fraction of the calcified plaque.

METHODS

Materials

Guanidine hydrochloride (guanidine HCl), aprotinin, and human serum albumin (HSA), p-hydroxymercuribenzoic acid, phenylmethylsulfonyl fluoride (PMSF), 6-aminocaproic acid EDTA, Trizma® base and Bis-Tris were obtained from Sigma (Saint Louis, MO). 6-Aminocaproic acid and PEG 20000 were purchased

from Fluka Chemica (Buchs, Switzerland). QAE-sephadex, Mono Q HR 5/5 and Protein G Superose HR 10/2 columns were from Pharmacia (Uppsala, Sweden). Benzamidine hydrochloride was a product of Janssen Chimica (Beerse, Belgium) and urea was from Merck (Darmstadt). Spectrapor dialysis tubing (molecular weight cutoff, 3.5 kDa) was purchased from Spectrum Industries (Los Angeles, CA). *O*-Phthaldehyde (OPA) was obtained from Pierce (Rockford, Ill) and acetonitrile (HPLC grade) was from Rathburn (Walkerburn, Scotland). All chemicals used were of analytical grade. Purified human prothrombin was kindly provided by Mrs T. Janssen (University of Limburg, Maastricht), polyclonal antibodies against FX and FII purified by affinity chromatography were a gift from dr. T. Lindhout (University of Limburg, Maastricht).

Extraction of calcified plaques

Human atherosclerotic aortas were obtained at autopsy and frozen within 12 h after death (-80 °C). None of the donors had been treated with oral anticoagulants. The investigation protocol had been approved by the University Hospital Ethics Committee. For a standard extraction procedure 60-120 aorta's were used, which resulted in approximately 100g calcified material. The procedure consisted of thawing of the aorta's, careful dissection of the calcifications from surrounding non-mineralized tissue, and pooling of all the calcified plaques. The plaques were extensively washed in buffer A (0.02 M Tris, 0.05 M NaCl, pH 7.5), delipidated 3 times in acetone, chloroform/methanol (2:1, v/v)¹⁷ and once in acetone (each washing step lasted 12 hrs at room temperature), air-dried and subsequently milled to powder in liquid nitrogen using a Spex impact Mill. Proteins were extracted during 2-3 days at 4 °C, by continuously stirring the powder in buffer B (1.0 M EDTA, 0.2 M KCl, pH 8.0) including protease inhibitors (0.01M benzamidine HCl, 0.05 M 6-aminocaproic acid, 10 KIU aprotinin, 0.01 M PMSF and 0.01 M *p*-hydroxymercuribenzoate). After centrifugation (1500 g, 30 min, 4 °C) of slurry, the non-solubilized material was subjected to at least two more extraction procedures. Pooled EDTA-extracts were diluted 20 fold in distilled water (4 °C) and absorbed batchwise to QAE-sephadex preswollen in buffer A (10 ml of preswollen slurry per liter diluted extract). The absorbed proteins were eluted with 1.0 M NaCl in buffer A, dialysed extensively in buffer C (0.02 M Bis-Tris, 0.05 M NaCl, pH 7.0), concentrated in 30% PEG, buffer C and dialysed again. Finally, protein concentration was determined according to Sedmak and Grossberg.¹⁸ The solution thus obtained will be referred to as the plaque extract.

Column chromatography

Chromatography was performed using a Separations HPLC High Precision pump, Model 480. The plaque extract was applied on a Mono-Q anion-exchange column, equilibrated with buffer C, elution was performed with a linear gradient from 0.05 to 1.0 M NaCl in buffer C. Elution profile was monitored at 280 nm using a UV-detector (Separations, Model 759A absorbance detector).

Gla-determination

For the detection of Gla, samples were subjected to alkaline hydrolyses for 16 hrs in 2.0 M KOH, *in vacuo* in sealed glass tubes, at 110 °C. The method used is based on a precolumn derivatization with OPA and subsequent HPLC separation (Separations, High Precision Pump Model 300) using a Nucleosil 5B column (Gimex, Netherlands) at 47 °C as described by Kuwada and Katayama.¹⁹ The identity of Gla was determined by comparing the elution time with that of purified Gla and the disappearance of the Gla-peak after acid hydrolyses. For the determination of the Gla-concentration, a reference curve of known amounts of purified Gla was used. The purification procedure of Gla-proteins was monitored by assessing the Gla/Glu ratio of the hydrolysed sample: samples enriched in Gla-proteins demonstrated an increase in the Gla/Glu ratio. Immunoreactive osteocalcin levels were determined with the radioimmunoassay (RIA) kit from INCSTAR (Stillwater, MI).

Digestive enzymes

Trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), neuraminidase type III (EC 3.2.1.18) and alkaline phosphatase from bovine intestinal mucosa (EC 3.1.3.1.) were from Sigma (Saint Louis, MO). Chondroitinase ABC protease free (EC 4.2.2.4) and hyaluronidase type V (EC 3.2.1.35) were purchased from Boehringer Mannheim.

Trypsin digestion was performed in 0.05 M Tris buffer, pH 8.5 at 1:1000 (w/w), 1:100 (w/w), 1:20 (w/w) for varying time intervals up to 4 hrs, at 37 °C. Chymotrypsin digestion was performed in 0.05 M Tris, pH 8.0 at 1:1000 (w/w), 1:100 (w/w) or 1:20 (w/w) at 37 °C for up to 4 hrs. For both enzymes the effect of an incubation period of 24 hrs at 37 °C was also evaluated. Hyaluronidase digestions were performed (100 U/mg) under several conditions: in buffers of 0.05 M sodium acetate pH 5, and 0.05 M sodium phosphate pH 6, 7 or 8 for 1 h at 37 °C in the presence of a protease inhibitor (0.01 M 6-aminocaproic acid) and/or 0.1 % SDS. All other enzymatic digestions were used according to the manufacturer's description.

Antibodies

Polyclonal antibodies were raised in rabbits by emulsifying the protein in Freund's complete adjuvant (Difco Laboratories, Detroit, USA) and immunizing the animals with several intradermal and one intraperitoneal injection. Booster injections in Freund's incomplete adjuvant were given at 3 week intervals. IgG was purified from immunized sera by protein G affinity chromatography.

Gel electrophoresis

Gel electrophoresis was performed according to Laemmli²⁰ or Shägger²¹ using the Bio-Rad Mini Gel System. Slab gels were either 15% or 10% and stained with Coomassie Brilliant Blue (CBB), Silver Stain Plus, DBS (4-diazobenzene-sulfonic acid) or Stains All (all from Bio-Rad). A specific colorimetric (DBS) staining for Gla-proteins in polyacrylamide gels was performed as described previously.²² The Stains All staining was performed according to Campbell *et al.*²³ with the following modification: immediately after electrophoresis the gel was fixed in fixing solution (50% (v/v) methanol, 10% (v/v) acetic acid) over night at room temperature, followed by extensive washing in freshly made fixing solution. This staining procedure has been optimized for the blue-staining of Ca^{2+} -binding proteins, while other proteins stain red. For the staining of glycoproteins the Periodic acid-silver staining according to Dubray was used.²⁴ Electroblotting was performed on PVDF membrane (ProblottTM, Applied Biosystems) in a Bio-Rad mini transblot cell system. Electroblotted (glyco)proteins were stained with the 'DIG Glycan/protein double labeling' kit (Boehringer Mannheim). Preparative gel electrophoresis was performed with the Prep Cell, Model 491 obtained from Bio-Rad (Richmond, CA). A 10 % resolving gel (0.375 M Tris, pH 8.8) was casted using a 37 mm ID column and led to polymerize overnight. The 4% separating gel consisted of 0.125 M Tris, pH 6.8, the elution buffer consisted of 0.02 M NH_4HCO_3 , pH 7.5.

Protein sequencing

Samples were electrophorised in a 15% SDS-polyacrylamide gel, using PDA (Bio-Rad) instead of bisacrylamide. The proteins were electrophoretically transferred on a PVDF membrane in 90 min, 150 mA. The transfer buffer consisted of 0.01 M CAPS (Aldrich, Milwaukee) at pH 9-11, 10% methanol. Blotted proteins were sequenced using an Applied Biosystems Protein Sequencer, Model 473A.

RESULTS

Optimalisation extraction procedure

The extraction procedure was performed according to Gijsbers *et al.*¹⁴ with the following modifications: the calcified plaques were delipidated once in acetone, three times in chloroform/methanol (2:1 v/v) and finally once more in acetone. With this procedure the lipids on the surface of the plaques were removed. The addition of 4-6 M Guanidine-HCl or 6 M Urea to the extraction-buffer did not lead to an increased yield or purification of Gla-proteins i.e. an increase in the absolute amount of Gla or Gla/Glu ratio present in (fractions of) the plaque extract and was therefore omitted. Also delipidation of the plaques with freon did not have a favourable effect. The grinding procedure of the plaques with a Spex impact Mill, generally lead to a 5-7% loss of plaque material. The protein yield of the plaque extract was $0.30 \text{ mg} \pm 0.10 \text{ (SD) protein/g plaque}$, with a mean concentration of $1.57 \pm 0.22 \text{ nmol Gla}/\mu\text{g protein}$ or $0.48 \pm 0.02 \mu\text{mol Gla/g plaque}$. The Gla/Glu ratio varied from 0.05-0.07 (Figure 1).

Column chromatography

When the plaque extract was applied to a Mono-Q anion-exchange column, no significant separation in distinct protein peaks was observed (Figure 2). About 80% of all protein-bound Gla was equally distributed in the two main peaks. In addition, substantial losses of Gla (up to 60% !) were noticed after chromatography, without an increase in Gla/Glu ratio in any of the eluted fractions. In order to improve the purification procedure a wide variety of columns were evaluated: ion-exchange (Mono-S), size exclusion chromatography (Superose-12, TSK (SW 2000/3000), Superdex 75), hydroxyapatite, hydrophobic interaction (Protein C4) and reversed phase columns. All of them essentially had the same result, with a chromatogram more or less similar to Figure 1. Several other techniques also

Figure 1. A typical chromatogram of the Gla-analysis of the plaque extract. Gla elutes at 13.97 min and Glu at 9.14 min. The Gla/Glu ratio of this sample was 0.06.

Figure 2. Mono-Q anion exchange chromatography of the plaque extract. Samples were eluted with a linear gradient from 0.05 to 1.0 M NaCl in buffer C. The percentages in the figure denote the distribution of Gla present in various fractions, indicating that Gla was mainly present in the two major peaks.

failed to improve the purification of Gla-proteins: the addition of chaotropic agents such as 4-6 M Guanidine HCL or 6 M Urea to buffer A and protein samples; variations in pH of the mobile phase; using a gradient of 0 - 1.0 M CaCl_2 as eluent; or variation in the slope of the gradient, using continuous and discontinuous gradients.

SDS-polyacrylamide gel electrophoresis

When analyzing the plaque extract on SDS-polyacrylamide gel, a prominent smear was visible in the whole lane of the plaque extract after Coomassie Brilliant Blue (CBB) (Figure 3A) or silver staining. In addition, a few discrete protein bands were visible: the most prominent one of 67 kDa and a weaker staining was observed at approximately 27 kDa (Figure 3A). Electrophoresis in a native or non-reducing gel did not alter the findings of both staining methods, neither did analysis in the presence of 4-6M Urea (data not shown). The DBS-staining method²² revealed the presence of three Gla-proteins of approximately 10, 18 and 27 kDa (Figure 3C).

The extraction procedure applied is expected to extract negatively charged Ca^{2+} -binding proteins. We therefore used the cationic carbocyanine dye "Stains All", which stains strong Ca^{2+} -binding proteins, sialic acid-rich proteins and heavily phosphorylated proteins in blue, whereas other proteins are stained in red.²³ A red protein band was visible at 67 kDa (not apparent on black and white

Figure 3. SDS-polyacrylamide gel electrophoresis of the plaque extract. A: Coomassie Brilliant Blue staining of a 12.5% gel, arrow denotes a 27 kDa protein band (arrow); B: Periodic acid staining revealing a smear of carbohydrates; the 67 kDa albumin band did not stain; C: 15% polyacrylamide gel stained with the DBS-staining method, due to the different gel percentage and addition of 12.5 M NaOH, the proteins bands are positioned differently, the three arrows indicate Gla-proteins of approximately 27, 18 and 10 kDa, respectively.

photography), covered by a blue smear extending across the whole lane. In addition at least three blue-stained bands were visible between approximately 40 and 60 kDa (Figure 4A). While digestion with alkaline phosphatase²⁵ did not affect the staining pattern, digestion with neuraminidase²³ completely abolished the blue smear (Figure 4B). This indicated that the blue smear did not contain heavily phosphorylated proteins but mainly consisted of sialic-acid rich proteins.²³ The presence of sialic acid residues was confirmed by staining the electroblotted plaque extract with the DIG-glycan detection kit after mild oxidation with sodium metaperiodate (0.01 M at 0 °C for 20 min.) (data not shown). Periodic acid-silver staining of the plaque extract confirmed that the smear contained substantial amounts of carbohydrates (Figure 3B). The 67 kDa band (later shown to be human albumin) did not stain, indicating that it lacked carbohydrates. These staining procedures have thus shown that the smear covering the whole lane consists of sialic acid rich glycoproteins or proteoglycans.

Several other enzymatic digestions were tested and evaluated on SDS-polyacrylamide gel by CBB, silver or DBS-staining: trypsin, chymotrypsin, CNBr, hyaluronidase,²⁶ chondroitinase ABC.²⁷ None of these digestions, however, lead to the disappearance of the background smear or improved DBS-staining of the Gla-proteins. Neither were there improvements found, by the addition of detergents (0.1% SDS or CHAPS) to the sample prior to digestion. Attempts to precipitate the smear or Gla-proteins with acetone²⁸ and ethanol²⁹ were

Figure 4. SDS-polyacrylamide gel (15%) of the plaque extract stained with Stains All, before (A) and after (B) neuraminidase digestion.

unsuccessfull. Absorption of the plaque extract either to BaSO_4 ³⁰ or hydroxyapatite³¹ and subsequent elution, also did not lead to an improved purification of Gla-proteins. Mild delipidation with freon had no appreciable effect either.

Sequencing

A high background signal was obtained when sequencing the 27 kDa protein band. The presence of a high signal indicated that the amount of protein on the blot must have been sufficient to be sequenced. However, no additional sequencing information was obtained, which could be due to N-terminal blockade of the protein. Proteolytical (CNBr) digestion of the blotted protein to overcome this did not improve the results. Several precautions to prevent N-terminal blockade (i.e. chemicals of the highest purity, polymerization of the SDS gel on the day before use, etc) did not alter the findings either.^{32,33} The procedure we used was not responsible for a possible N-terminal blockade, since commercially obtained purified HSA could be sequenced without any problems. Sequencing of other parts of the Western blotted plaque extract revealed that: 1) the 67 kDa band could be succesfully sequenced despite the presence of a high background signal and had a N-terminal sequence identical to that of HSA; 2) the same background signal was present in all parts of the blot containing the smear-like CBB staining ; 3) the background was absent in a piece of the blot beneath the front of the lane. These

results indicate that the background smear present in the plaque extract is the source of the high background sequencing signal and that only proteins of a sufficient high concentration to overcome the interfering signal, for example the human albumin-band, can be sequenced.

Polyclonal antibodies

Several fractions of the plaque extract isolated by preparative gel electrophoresis were used to raise polyclonal antibodies: a fraction above the 27 kDa band (fraction I), the 27 kDa fraction (fraction II) and background smear between 20 and 25 kDa (fraction III). Preincubation of the IgG antibodies with HSA was performed to overcome an apparent crossreaction of the antibodies with albumin. The antibodies against fraction I and III crossreacted with a 29 kDa band, whereas those against fraction II crossreacted with a 27 kDa band. In a BaCitrated extract of human plasma the same crossreactivity was visible (data not shown). DBS-staining of the gel, however, did not reveal the presence of Gla-proteins in the BaCitrated extracts, indicating that these antibodies were not directed against a Gla-protein or that the assumed Gla-protein is present beneath the detection limit of the DBS-staining method.

Presence of other vitamin K dependent proteins

Immunoblotting of the plaque extract using antibodies against either prothrombin, coagulation factor X or osteocalcin did not reveal any crossreactivity. Trace amounts of osteocalcin (16 ng osteocalcin/mg plaque extract) could be identified by radioimmunoassay.

DISCUSSION

The atherosclerotic process is attended by complex interactions of various cell-types, including endothelial cells, platelets, macrophages and vascular smooth muscle cells (vSMC's).³⁶ Putative functions of these cells include the synthesis of various extracellular matrix components (collagen, elastin, glycoproteins and proteoglycans), often proposed to contribute to lipid accumulation and calcification of the atherosclerotic plaque.^{34,36} The presence and local production of several bone-associated proteins have recently been reported in atherosclerotic lesions. These include type I collagen, bone morphogenetic protein-2a (a potent osteogenic differentiation factor),¹⁰ osteopontin³⁵ and osteonectin.³⁶ In addition, the two most common Gla-proteins present in bone, osteocalcin and MGP, have both been identified in the atherosclerotic lesions.^{9,7,8} These Gla-proteins were

shown to be closely associated with the calcified deposits in the human atherosclerotic plaque and may be locally produced by a calcifying vascular cell,^{10,37} vSMC's or macrophages.^{3,5,9,11,35}

The analysis of Gla-proteins present in the plaque, is hampered by the difficulty of obtaining sufficient human calcified atherosclerotic plaques and by the relatively low concentration of Gla-proteins present in these lesions. It is not surprising therefore, that the purification of these Gla-proteins has proven to be a laborious task. The many attempts performed in this study have shown that the plaque extract contains at least three Gla-proteins with an apparent molecular weight of 10, 18 and 27 kDa, albumin and a heterogenous mixture of negatively charged macromolecules of a wide molecular range, rich in carbohydrates and sialic acid.

Gla-proteins

We have confirmed the presence of protein-bound Gla in calcified atherosclerotic plaques of human origin (Figure 1, 3C). The amount of Gla found (0.48 $\mu\text{mol/g}$ plaque material) is in accordance with the 0.53 $\mu\text{mol/g}$ plaque previously reported by Price.² It is striking that three Gla-proteins were visible with the DBS-staining method, whereas CBB and silver staining only confirmed the presence of the 27 kDa Gla-protein (Figure 3C). This could be due to the acidic nature of the two other Gla-proteins. It is well known that the latter two procedures may poorly stain acidic amino acids and some calcium-binding proteins.^{38,39} This phenomenon has been described for dog osteocalcin, for instance, which cannot be made visible with silver staining.⁴⁰ Another explanation may be found in components present in the plaque extract, that interfere with or obscure the subsequent (CBB and silver) staining of the two Gla-proteins. Since the DBS-method specifically stains Gla residues, interference of other (non-Gla containing) proteins is less likely to occur.²²

Several of our findings are in line with those described by Levy *et al.*⁸ who reported that 1) the EDTA-soluble extract of the human calcified plaque consists of a heterogenous extract of a wide range of macromolecules giving rise to a chromatogram virtually identical to that of Figure 1; 2) the isolation of Gla-proteins from the plaque is resistant to biochemical resolution techniques and 3) any fractionation tried was characterized by high losses of protein-bound Gla.

Other components of the plaque extract

It is still unknown whether human albumin, frequently identified in the EDTA-soluble fraction of the calcified plaque, has a role in the calcification process or is

merely the result of passive absorption from the circulation. Albumin is known to interact with other (Gla)proteins such as osteocalcin, occasionally leading to artifacts in the isolation procedure.^{12,15}

A major part of the plaque extract consists of negatively charged glycoproteins or proteoglycans with glycoaminoglycan side chains of varying lengths. Proteoglycans are a well known constituent of intimal atherosclerotic lesions and are thought to be involved in thrombosis, lipid accumulation and calcification of the atherosclerotic plaque.³⁴ These macromolecules are of a wide molecular range, appearing as a smear on SDS-polyacrylamide gel electrophoresis, and interacting with a wide variety of proteins present in the plaque extract, ultimately impeding further isolation of the Gla-proteins. We cannot exclude the possibility that, despite the use of protease inhibitors, the plaque extract also contains a mixture of (non-dialysable) protein breakdown products or protein complexes, possibly altered via non-enzymatic glycosylation or proteolysis. This view is supported by the results of the sequencing experiments which indicated that the background smear contained large amounts of negatively charged amino-acids. The previously published aminoacid composition and protein sequence of PGP may therefore be partly derived from these contaminants.¹⁴

Despite the fact that several studies have shown protein-bound Gla to be present in the atherosclerotic plaque, none of these proteins have been further characterized yet. Their putative function, therefore, remains a matter of speculation. It is noteworthy that the apparent molecular weight of the three Gla-proteins reported here, coincide with those of PGP (27 kDa), osteocalcin (18 kDa) and MGP (10 kDa). To date, however, both mRNA and protein levels of only one Gla-protein have been identified in calcified human atherosclerotic plaques, namely MGP.⁹ The fact that its specific localization in the lipid-rich calcified areas of the plaque is similar to that in cartilage ultimately undergoing endochondral ossification, strongly suggests a role in the regulation of the mineralization process. We hypothesize that similar to its role in rapidly developing fetal bones, vascular MGP may act as a mineralization inhibitor, either protecting against calcification of the vessel wall or retarding the process.

In conclusion, we have shown the presence of three discrete Gla-proteins in SDS-polyacrylamide gel with apparent molecular weights coinciding with those of PGP, MGP and osteocalcin. Their isolation, however, appeared to be hampered by the presence of contaminating sialic acid rich glycoproteins or proteoglycans. In addition, considerable losses of protein-bound Gla occurred during various isolation procedures, probably because of excessive precipitation. This study shows that conventional biochemical isolation procedures do not lead to a signifi-

cant purification of the Gla-proteins present in the calcified plaque. Other approaches are needed therefore to investigate the role of Gla-proteins in the atherosclerotic process.

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SUMMARY / SAMENVATTING

SUMMARY

Vitamin K is an essential cofactor for the formation of the unusual aminoacid γ -carboxyglutamate (Gla) which is found in a limited number of proteins. In these so called 'vitamin K-dependent' or Gla-proteins, the Gla-residues are involved in the calcium-dependent binding to negatively charged phospholipid membranes (*e.g.* the blood coagulation factors FII, VII, IX and X) or to hydroxyapatite (*e.g.* the bone Gla proteins osteocalcin and matrix Gla-protein (MGP)). In the case of an impaired vitamin K-status, the formation of Gla is hampered and the Gla-proteins are synthesized and secreted in a functionally impaired form. These so called undercarboxylated or descarboxy Gla-proteins, lack the aminoacid Gla but contain glutamate (Glu) instead (**chapter 1**). Up until now, the diagnosis of vitamin K-deficiency has mainly relied on tests measuring the functional activity of the hepatic Gla-proteins involved in blood coagulation. In using the coagulation factors as a marker for vitamin K-status, we must conclude that vitamin K-deficiency is extremely rare in the general population. There is evidence, however, that tests based on blood coagulation factors are not sensitive to a marginal vitamin K-status.^{1,2}

The studies described in this thesis are investigations into the relationship between vitamin K and aspects of calcium or bone metabolism (chapters 2,3,4), as well as the vessel wall calcification process occurring in the atherosclerotic plaque (chapters 6,7,9).

Vitamin K-status was assessed with the hydroxyapatite binding assay of serum osteocalcin. This assay is based on the 'vitamin K-dependent' property of osteocalcin to bind to hydroxyapatite via its Gla-residues. Serum osteocalcin (irOC) with high affinity for hydroxyapatite (irOC_{bound}) is assumed to represent the fully carboxylated fraction, whereas that with low (irOC_{free}) affinity is thought to represent the undercarboxylated fraction.³ The hydroxyapatite binding capacity (HBC) (irOC_{bound} expressed as the percentage of the total irOC levels), can be used as a semi-quantative measure for the Gla-content of serum osteocalcin (Figure 3, chapter 1). Low HBC levels (<70%) are commonly found in subjects with an impaired vitamin K status such as those using vitamin K-antagonists (chapter 3).

The hydroxyapatite binding assay was validated in a population at high risk for a marginal vitamin K status: the newborn babies (**chapter 2**). Pregnant women were treated with either vitamin K (1 mg/day) or a placebo during the last 6

weeks of their pregnancy. The fact that all babies from the placebo treated group had very high concentrations of $\text{irOC}_{\text{free}}$ and HBC levels as low as those in adults on oral anticoagulant therapy, strongly suggested they had a poor vitamin K status. This suggestion was confirmed by the observation that the 'vitamin K-treated' babies had significantly lower $\text{irOC}_{\text{free}}$ and higher HBC levels. Since no descarboxy-prothrombin was detectable in any of the tested cord blood samples, these results then indicate that the HBC of serum osteocalcin is a more sensitive marker for measuring vitamin K than tests based on the blood coagulation prothrombin.

In the first part of the study described in **chapter 3** we have investigated the extent to which the vitamin K-status affects urinary calcium excretion. This was done in a placebo-controlled study among postmenopausal women. Vitamin K-treatment appeared to significantly decrease the fasting urinary calcium excretion in the so called fast losers of calcium (calcium/creatinine ratio > 0.5). To investigate whether vitamin K-antagonists would have an opposite effect, we performed a case control study among patients on long-term stable anticoagulant therapy. Only the group of young men (aged 25-45) contained a significantly higher fraction (7/27) of fast losers as compared with the age-matched control group (0/25). The positive association of the fasting urinary calcium with the urinary hydroxyproline excretion ($r=0.52$, $p<0.004$) indicated that the high calcium excretion in the anticoagulated young men may be related to a high bone resorption.

Chapter 4 deals with the effect of vitamin K-treatment (1 mg/day during 2 weeks) on serum osteocalcin (irOC) levels in women aged 20-85. Since vitamin K-supplementation mainly affected irOC levels in women older than 50, a second study was performed in elderly women (aged 50-85) with a longer treatment period of 3 months. Vitamin K treatment increased the serum levels of two markers for bone formation (total irOC levels and bone-specific alkaline phosphatase), whereas a 30% decrease in fasting urinary calcium excretion was noticed in the fast losers of urinary calcium. These findings are supported by several *in vitro* studies which have shown that vitamin K stimulates osteocalcin and alkaline phosphatase excretion by osteoblastic cells⁴ and decreases bone resorption.^{5,6,7}

A surprising observation of this study was that vitamin K-treatment mainly increased $\text{irOC}_{\text{bound}}$ levels, whereas the mean $\text{irOC}_{\text{free}}$ levels remained unaffected. This is in contrast with the situation found in newborn babies (chapter 2), who had lower $\text{irOC}_{\text{free}}$ levels upon vitamin K-treatment. These findings indicate that

irOC_{free} (especially in postmenopausal women) may not only represent undercarboxylated osteocalcin, as assumed up until now.^{2,3} The results of the study described in chapter 4 indicate that irOC_{free} may also partly represent osteocalcin fragments, probably derived from the process of bone resorption. Additional support for this view has recently been published by Minisola *et al.*⁸ Of interest is that the presence of osteocalcin fragments has recently been confirmed by Garnero *et al.* who, using a set of various monospecific antibodies, showed that 64% of the total circulating immunoreactive antigens can be ascribed to osteocalcin fragments.⁹

In the vitamin K-supplementation studies we have performed thus far, we have used a dosage of 1 mg phyloquinone from a pharmaceutical preparation (Konakion®). Because of its highly soluble form, its bioavailability can be expected to be fairly good. We have therefore compared the bioavailability of 1 mg phyloquinone presented in the form of spinach (with or without butter) with that of Konakion® (chapter 5). Compared with Konakion®, the absorption of phyloquinone from spinach appeared to be substantially lower. The addition of fat to spinach, did however, enhance the amount of phyloquinone absorbed about 3-fold. This study shows that the amount of bioavailable phyloquinone as estimated from the dietary vitamin K-intake, is far less than assumed up until now. Therefore, studies are needed to investigate the bioavailability of vitamin K from various sources and the effect of other components of the diet (e.g. the amount of fat) on the absorption of vitamin K.

Besides in bone, Gla-proteins are found in the calcified lesions of the atherosclerotic plaque. An impaired vitamin K-status may therefore affect the development and progression of atherosclerotic calcifications. In the study described in chapter 6.1 we investigated the vitamin K-status in postmenopausal women with or without severe atherosclerotic calcifications. In the non-atherosclerotic women, the nutritional vitamin K-intake decreased considerably with an average of 10.08 µg/day (95%CI: 9.49-10.67), whereas in the atherosclerotic women the vitamin K-intake remained at a constant low level. Women with calcified lesions (mean intake: 189,9 µg/day) appeared to have a lower age-adjusted nutritional vitamin K-intake of 43 µg/day, constituting a difference of 18%. In addition, compared to the non-atherosclerotic women, their atherosclerotic counterparts had a poor vitamin K-status as reflected in their significantly higher irOC_{free} and lower HBC levels. These findings are in line with the hypothesis that vitamin K or Gla-proteins are involved in the development of vessel wall calcifications.

Chapter 6.2 reports the associations between the presence of atherosclerotic calcifications, bone mass and markers for vitamin K-status in the population-based study described in chapter 6. Women with atherosclerotic calcifications had an 8% lower bone mass (mean difference of 3.15 mm², 95%CI: 0.18-6.72) and in addition, both markers for vitamin K status were associated with bone mass (iOC_{free}: $r=-0.47$, $p=0.013$; HBC: $r=0.57$, $p=0.002$). The negative association of iOC_{free} with bone mass is of special interest, since recent findings indicate that high iOC_{free} levels are associated with a high risk for developing osteoporotic hip fractures.¹⁰ Thus both the lower bone mass, and the higher iOC_{free} levels in the atherosclerotic women, indicate that they may be at a high risk for osteoporotic fractures. The findings of this study support the view that osteoporosis and atherosclerosis are two related processes and that vitamin K may act as one of the common denominators in both disease states.

In **chapter 7.2** we have confirmed that substantial amounts of non-dialysable, protein-bound Gla are present in the calcified human atherosclerotic plaque. With a specific colorimetric staining method described in **Chapter 7.1**, we have been able to identify three Gla-proteins in the calcified human atherosclerotic plaque with apparent molecular weights of 10, 18 and 27 kDa. Their further isolation and characterization, however, has proven to be extremely difficult. One of the contributing factors may be the abundant presence of contaminating sialoglycoproteins or proteoglycans. This study indicates that conventional biochemical techniques are insufficient to investigate the role of vitamin K or Gla-proteins in atherosclerotic calcifications.

The initial report that a suboptimal vitamin K-status is common in postmenopausal women has been confirmed by the studies described in this thesis (chapter 3,4,6), and by reports from other investigators.^{1,10,11} In addition, evidence has been obtained that a suboptimal vitamin K-status may affect bone metabolism as well as the process of vessel wall calcifications.

The measurement of the two iOC fractions differing in their ability to bind to hydroxyapatite, has shown to be a promising tool for assessing one's (bone) tissue vitamin K-status. The additional value of this distinction is illustrated by the finding of Szulc *et al.* that iOC_{free} may be used as a marker for developing hip fractures.¹⁰ The hydroxyapatite binding assay is a semi-quantative measure and can therefore only be used as an estimate of the degree of carboxylation of serum osteocalcin. Tests are needed which have been precisely characterized with respect to the immunoreactive forms of osteocalcin that are detected, and are able to directly detect undercarboxylated iOC levels.

The finding that vitamin K-status may affect bone metabolism and atherosclerotic calcifications raises the question of whether the recommended daily allowances (RDA) for vitamin K should be reconsidered. The future use of extra-hepatic Gla-proteins (osteocalcin, MGP) may become important tools in reestablishing the RDA-values. Furthermore, studies aimed to investigate the efficacy of vitamin K (phylloquinone and menaquinone) absorption from different food products are needed.

SAMENVATTING

Vitamine K is een essentiële cofactor in de aanmaak van het ongewone aminozuur γ -carboxyglutaminezuur (Gla) dat in een beperkt aantal eiwitten voorkomt. In deze zogenaamde 'vitamine K-afhankelijke' of Gla-eiwitten, zijn de Gla-residuen betrokken in de calcium-afhankelijke binding aan negatief geladen fosfolipid membranen (zoals bij de bloedstollingsfactoren FII, VII, IX en X) of aan hydroxyapatiet (zoals bij de Gla-bevattende boteiwitten osteocalcine en matrix Gla proteïne (MGP)). Bij een slechte vitamine K-status, zal de aanmaak van Gla verstoord zijn en zullen de Gla-eiwitten aangemaakt en uitgescheiden worden in een niet-functionele vorm. Deze zogenaamde ondergecarboxyleerde of descaboxy Gla-eiwitten, bevatten glutaminezuur (Glu) in plaats van Gla (**hoofdstuk 1**). Om de diagnose vitamine K-deficiëntie vast te stellen heeft men tot nu toe hoofdzakelijk gebruik gemaakt van testen die gevoelig zijn voor de functionele activiteit van de hepatische bloedstollings Gla-eiwitten. Als we de stollingseiwitten gebruiken als merkstof voor de vitamine K-status, valt op dat een vitamine K-deficiëntie in de algemene bevolking zeldzaam is. Er zijn echter aanwijzingen dat testen gebaseerd op bloedstollingsfactoren, niet gevoelig genoeg zijn om een gering tekort aan vitamine K aan te tonen.^{1,2}

De studies in dit proefschrift beschrijven het onderzoek naar de relatie tussen vitamine K en aspecten van het calcium- of botmetabolisme (hoofdstukken 2,3,4), alsook de relatie met de verkalkingsprocessen in de atherosclerotische plaque (hoofdstukken 6,7,9).

De vitamine K-status werd onderzocht met behulp van de 'hydroxyapatiet bindings assay' van het serum osteocalcine. Deze assay is gebaseerd op de 'vitamine K-afhankelijke' eigenschap van osteocalcine om aan hydroxyapatiet te binden met behulp van zijn Gla-residuen. Het immuunreactieve serum osteocalcine (irOC) met een hoge affiniteit voor hydroxyapatiet ($\text{irOC}_{\text{bound}}$) wordt verondersteld de gecarboxyleerde fractie te zijn, terwijl de osteocalcine fractie met een lage affiniteit voor hydroxyapatiet het ondergecarboxyleerde osteocalcine ($\text{irOC}_{\text{free}}$) vertegenwoordigt.³ De hydroxyapatiet bindings capaciteit (HBC) ($= \text{irOC}_{\text{bound}}$ uitgedrukt als een percentage van het totale irOC gehalte), kan aangewend worden als een semi-kwantitatieve maat voor het Gla-gehalte van het serum osteocalcine (Figuur 3, hoofdstuk 1). Lage HBC gehalten ($< 70\%$) komen frequent voor bij diegenen met een verminderde vitamine K-status zoals diegenen die vitamine K-antagonisten gebruiken (orale antistollingsmiddelen zoals sintrom mitis* (hoofdstuk 3)).

De hydroxyapatiet bindings assay werd gevalideerd in een bekende risicogroep voor vitamine K-deficiëntie: de pasgeborenen (**hoofdstuk 2**). Zwangere vrouwen werden gedurende de laatste 6 weken van hun zwangerschap behandeld met vitamine K (1 mg/dag) of een placebo. Het feit dat alle baby's uit de met een placebo behandelde groep zeer hoge concentraties $\text{irOC}_{\text{free}}$ hadden, met HBC gehalten vergelijkbaar met die van volwassenen onder orale antistolling, suggereert heel sterk dat ze een slechte vitamine K status hebben. Dit werd ondersteund door de observatie dat de 'vitamine K behandelde' baby's significant lagere $\text{irOC}_{\text{free}}$ en hogere HBC gehalten hadden. Aangezien descaboxy-protrombine in geen van de geteste navelstreng bloedmonsters werd aangetroffen, geven deze resultaten aan dat het HBC gehalte van het serum osteocalcine een gevoeligere marker is voor het meten van de vitamine K status dan testen, gebaseerd op de bloedstollingsfactor protrombine.

In het eerste deel van het onderzoek zoals beschreven in **hoofdstuk 3**, hebben wij onderzocht in welke mate de vitamine K-status de calcium uitscheiding in de urine beïnvloedt. Het betreft een placebo-gecontroleerde studie onder postmenopauzale vrouwen. Behandeling met vitamine K leidde tot een significante daling van de nuchtere calcium uitscheiding in de urine bij vrouwen met een hoge calcium uitscheiding (bij de zogenaamde fast losers: calcium/creatinine ratio > 0.5). Of vitamine K-antagonisten juist een tegenovergesteld effect zouden hebben werd onderzocht in een case control onderzoek bij patiënten onder langdurige, stabiele antistollingstherapie. Alleen bij de jonge mannen (25-45 jr) werd een significant hoger aantal fast losers (7/27) aangetroffen dan in een leeftijds-gematchte controle groep (0/25). De positieve associatie van de nuchtere calcium uitscheiding in de urine met de hydroxyproline uitscheiding ($r=0.52$, $p<0.004$), geeft aan dat de hoge calcium uitscheiding in de geantistolde groep jonge mannen gerelateerd is aan een hoge botresorptie.

Hoofdstuk 4 beschrijft de effecten van vitamine K-behandeling (1 mg/dag gedurende 2 weken) op het serum osteocalcine (irOC) gehalte bij vrouwen van 20-85 jr. Hieruit bleek dat vitamine K behandeling hoofdzakelijk een effect had op het irOC gehalte bij de vrouwen ouder dan 50 jr. Er werd daarom een tweede, langdurige studie verricht bij oudere vrouwen (50-85 jr). Vitamine K behandeling gedurende 3 maanden leidde tot een verhoging van het serum gehalte van twee merkstoffen voor botvorming (totale irOC gehalte en het bot-alkalische fosfatase en een afname met 30% van de calcium uitscheiding in de urine bij de 'fast losers'. Deze bevindingen worden ondersteund door verschillende *in vitro* studies

die aantonen dat vitamine K de osteocalcine en alkalische fosfatase uitscheiding door osteoblast cellen stimuleert⁴ en de botresorptie doet afnemen.^{5,6,7}

Een verrassende observatie van deze studie was de bevinding dat vitamine K-behandeling hoofdzakelijk het $\text{iOC}_{\text{bound}}$ gehalte deed toenemen, terwijl het gemiddelde iOC_{free} gehalte hetzelfde bleef. Dit is in tegenstelling tot de resultaten bij de pasgeborenen (hoofdstuk 2), bij wie de iOC_{free} spiegels juist daalden na vitamine K therapie. Deze gegevens wijzen erop dat iOC_{free} (met name bij postmenopauzale vrouwen), in tegenstelling tot wat tot nu toe werd aangenomen, mogelijk niet alleen het ondergecarboxyleerde osteocalcine voorstelt.^{2,3} De resultaten van de studies beschreven in hoofdstuk 4 wijzen erop dat iOC_{free} ook voor een deel bestaat uit osteocalcine fragmenten, mogelijk afkomstig uit het botresorptie proces. Deze visie wordt ondersteund door de recente studie van Minisola *et al.*⁸. De aanwezigheid van circulerende osteocalcine fragmenten is recent bevestigd door Garnero *et al.* die, met behulp van verschillende monospecifieke antilichamen, aantoonde dat 65% van het totale immuunreactieve circulerend antigeen toegeschreven dient te worden aan osteocalcine fragmenten.⁹

In de vitamine K-suppletie studies die wij tot nu toe verricht hebben, is er een dosering gebruikt van 1 mg fyloquinon (= vitamin K₁) afkomstig van een farmaceutisch preparaat (Konakion®). Vanwege zijn hoog oplosbare vorm, is de te verwachten biologische beschikbaarheid groot. Wij hebben daarom de biologische beschikbaarheid van 1 mg fyloquinon in de vorm van spinazie (met of zonder boter) vergeleken met die van Konakion® (**hoofdstuk 5**). De absorptie van fyloquinon uit spinazie bleek substantieel lager te zijn dan die uit Konakion®. Toevoeging van vet aan spinazie, verbeterde echter de absorptie van fyloquinon uit spinazie met een factor 3. Deze studie geeft aan dat de hoeveelheid biologisch beschikbare fyloquinon, zoals die geschat wordt uit de dietaire vitamine K inname, aanzienlijk minder is dan tot nu toe werd aangenomen. Er zijn daarom studies nodig die de biologische beschikbaarheid van vitamine K uit verschillende voedingsbronnen en de effecten van andere componenten uit het dieet (zoals de hoeveelheid vet) op de absorptie van vitamine K bestuderen.

Behalve in bot, worden Gla-eiwitten ook gevonden in de verkalkte atherosclerotische plaque. Een precare vitamine K-status zou daarom de vorming en progressie van de atherosclerotische verkalkingen kunnen beïnvloeden. In **hoofdstuk 6.1** wordt een studie beschreven die de vitamine K-status onderzoekt in postmenopauzale vrouwen met of zonder ernstige atherosclerotische verkalkingen van de vaatwand. In de niet-atherosclerotische vrouwen blijkt de vitamine K-inname via de voeding aanzienlijk af te nemen met het toenemen van de leeftijd: gemiddeld

10.08 $\mu\text{g}/\text{dag}$ per jaar (95% betrouwbaarheidsinterval (BI): 9.49-10.67), terwijl de vitamine K inname in de atherosclerotische vrouwen op een constant laag niveau blijft. Vrouwen met atherosclerotische verkalkingen (gemiddelde vitamine K-inname: 189,9 $\mu\text{g}/\text{day}$) bleken een 18% (43 $\mu\text{g}/\text{dag}$) lagere vitamine K-inname (leeftijds-gecorrigeerd) te hebben. Bovendien bleken de vrouwen met atherosclerotische verkalkingen een slechtere weefsel vitamine K-status te hebben, zich uitend in statistisch significant hogere $\text{irOC}_{\text{free}}$ en lagere HBC gehalten. Deze bevindingen ondersteunen de hypothese dat vitamine K of Gla-eiwitten betrokken zijn in de ontwikkeling van vaatwand verkalkingen.

Hoofdstuk 6.2 beschrijft de associatie tussen de aanwezigheid van atherosclerotische verkalkingen, botmassa en markers voor de vitamine K status in de populatiestudie beschreven in hoofdstuk 6.1. Vrouwen met atherosclerotische verkalkingen bleken een 8% lagere botmassa (gemiddeld verschil van 3.15 mm^2 , 95% BI: 0.18-6.72) te hebben. Bovendien waren beide markers voor vitamine K status gecorreleerd aan de botmassa ($\text{irOC}_{\text{free}}$: $r = -0.47$, $p = 0.013$; HBC: $r = 0.57$, $p = 0.002$). De negatieve associatie van $\text{irOC}_{\text{free}}$ met de botmassa is met name interessant, omdat recente gegevens erop wijzen dat hoge $\text{irOC}_{\text{free}}$ gehalten geassocieerd zijn met een hoger risico voor het ontwikkelen van osteoporotische heup fracturen.¹⁰ Zowel de lagere botmassa als de hogere $\text{irOC}_{\text{free}}$ gehalten in de atherosclerotische vrouwen geven aan dat atherosclerotische vrouwen een hoger risico hebben voor osteoporotische fracturen. Deze bevindingen ondersteunen de hypothese dat osteoporose en atherosclerose twee aan elkaar gerelateerde processen zijn, waarbij een tekort aan vitamine K een belangrijke rol speelt.

In **hoofdstuk 7.2** bevestigen wij dat aanzienlijke hoeveelheden niet-dialyseerbare eiwitgebonden Gla aanwezig is in de verkalkte humane atherosclerotische plaque. Met een specifieke colorimetrische kleuring, beschreven in **Hoofdstuk 7.1**, hebben wij de aanwezigheid van drie Gla-eiwitten in de verkalkte humane atherosclerotische plaque aangetoond, met een moleculair gewicht van 10, 18 en 27 kDa. Hun isolatie en karakterisatie bleek echter extreem moeilijk te zijn. Eén van de bijdragende factoren is waarschijnlijk de overvloedige aanwezigheid van contaminerende sialo-glycoproteïnes of proteoglycanen. Deze studie geeft aan dat conventionele biochemische technieken niet voldoende zijn om de rol van vitamine K of Gla-eiwitten in atherosclerotische verkalkingen te onderzoeken.

De waarneming dat een suboptimale vitamine K status veelvuldig voorkomt bij postmenopauzale vrouwen werd bevestigd door de studies beschreven in dit proefschrift (hoofdstuk 3,4,6) en die van andere onderzoekers.^{1,10,11} Bovendien hebben wij aanwijzingen gevonden dat een marginale vitamine K-status zowel het botmetabolisme als het proces van vaatwandverkalkingen kan beïnvloeden.

De bepaling van de twee irOC fracties, met een verschillende affiniteit voor hydroxyapatiet, blijkt een veelbelovende manier te zijn om iemands (bot) weefsel vitamine K-status te bepalen. De additionele waarde van deze bepaling wordt geïllustreerd door de bevinding van Szulc *et al.* dat irOC_{free} gebruikt kan worden als risicoindicator voor een heupfractuur.¹⁰ De hydroxyapatiet bindings assay is een semi-kwantitatieve meting en kan daarom alleen gebruikt worden als een schatting van de carboxyleringsgraad van het serum osteocalcine. Er zijn daarom bepalingsmethodes nodig, die precies gekarakteriseerd zijn met betrekking tot de immuunreactieve vormen van het osteocalcine dat ze detecteren, en die tevens in staat zijn om direct het ondergecarboxyleerde osteocalcine aan te tonen.

De inzichten dat de vitamine K-status het botmetabolisme en het proces van atherosclerotische verkalkingen beïnvloedt roept de vraag op of de huidige aanbevolen dagelijkse hoeveelheid vitamine K niet heroverwogen dient te worden. Het is de verwachting dat in de toekomst het gebruik van extra-hepatische Gla-eiwitten (zoals osteocalcine, MGP en PGP) belangrijke hulpmiddelen zullen zijn om de dagelijks aanbevolen hoeveelheid vitamine K te bepalen. Studies die de efficiëntie van vitamine K absorptie (zowel fylloquinon als menaquinon!) uit verschillende voedingsbronnen bestuderen zijn bovendien aangewezen.

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CURRICULUM VITAE

De auteur van dit proefschrift werd op 18 januari 1964 geboren te Paramaribo (Suriname). Hij behaalde het diploma Atheneum-B aan het Jeroen Bosch College te 's-Hertogenbosch. In 1983 werd de studie geneeskunde aangevangen aan de Rijksuniversiteit Limburg. Van 1985 tot 1986 was hij student-assistent bij de vakgroep Interne Geneeskunde (dr. B. Schutte, Prof.dr. G.H. Blijham), waar hij onderzoek verrichtte naar de flowcytometrische detectie van oestrogeen- en progesteronreceptoren in mammatumorcellen. Het artsexamen werd behaald in 1990.

Vanaf 1990 was hij werkzaam als assistent in opleiding (AIO) bij de vakgroep Biochemie van de Rijksuniversiteit Limburg, alwaar hij onder leiding van Dr. C. Vermeer en Dr. K. Hamulyák gewerkt heeft aan het onderzoek zoals beschreven in dit proefschrift. Voor een deel van het onderzoek (The effect of oral anticoagulants on the urinary calcium excretion) kreeg hij in 1991 de 'Young Investigators Merit Traval Award' (XIIIth Congress ISTH, Amsterdam).

Sinds oktober 1994 is hij in opleiding tot internist in het Academisch Ziekenhuis Maastricht (opleider: Prof. dr. H.F.P. Hillen).