Potential effects of vitamin K supplementation on bone metabolism

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Potential Effects of Vitamin K Supplementation on Bone Metabolism

Dr. R. Smith

Abstract: This study investigated the potential effects of Vitamin K supplementation on bone metabolism in a group of postmenopausal women. The results showed that supplementation with Vitamin K significantly increased bone mineral density (BMD) and reduced the risk of fractures compared to the control group.

Personal Effects of Vitamin K Supplementation on Bone Metabolism

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Potential Effects of Vitamin K Supplementation on Bone Metabolism

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
Prof. Dr. A.C. Nieuwenhuijzen Kruseman
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op vrijdag 16 maart 2001 om 14.00 uur

door

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Discovery of the vitamins and their life saving value in the prevention and cure of nutritional deficiency diseases is one of the most important contributions of biochemistry to medicine and human welfare.

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"Be realistic. Ask for the impossible..."

Pope John Paul II

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ABBREVIATIONS

apo E	Apolipoprotein type E
BAP	Bone Alkaline Phosphatase
BGP	Bone Gla Protein; Osteocalcin
BMD	Bone Mineral Density
BMI	Body Mass Index
CTX	Collagen type I C-terminal end
DPD	Deoxypyridinoline
ELISA	Enzyme-Linked Immunosorbent Assay
F II	Prothrombin
F IX	Coagulation factor IX
F VII	Coagulation factor VII
F X	Coagulation factor X
FWS	Fetal Warfarin Syndrome
Gas 6	Growth arrest specific gene 6 product
Gla	Gamma-carboxyglutamic acid; Gamma-carboxyglutamate
Glu	Glutamic acid; Glutamate
HBC	Hydroxyapatite Binding Capacity
HCO-60	polyoxyethylene hydrogenated castor oil derivatives
HDL	High Density Lipoproteins
HDN	Haemorrhagic Disease of the Newborn
HOP	Hydroxyproline
HPLC	High Performance Liquid Chromatography
IL	Interleukins
K ₁	Phylloquinone
K ₂	Groupname for Menaquinones
K ₃	Menadione
kDa	KiloDalton
KH ₂	Vitamin K hydroquinone
KO	Vitamin K 2,3 epoxide

ABBREVIATIONS

LDL	Low Density Lipoproteins
MGP	Matrix Gla Protein
MK ₄	Menaquinone 4
MK-n	Menaquinones
NAD(P)H	Nicotinamide Adenine Dinucleotide (Phosphate) reduced form
NTX	Collagen type I N-terminal end
OC	Osteocalcin
PIVKA	Protein Induced by Vitamin K Absence
PT	Prothrombin Time
PTH	Parathyroidian hormone
RDA	Recommended Dietary Allowance
RDI	Recommended Dietary Intakes
RIA	Radio Immuno Assay
TRAP	Tartrate Resistant Acid Phosphatase
VKDBN	Vitamin K Deficiency Bleeding of the Newborn
VLDL	Very Low Density Lipoproteins

CHAPTER 1

Introduction

The purpose of this chapter is to provide a brief overview of the book's content and to introduce the reader to the concepts and terminology used throughout the text. The chapter is divided into two main sections: **INTRODUCTION** and **CONCLUSION**. The **INTRODUCTION** section discusses the importance of the book and the role of the reader. The **CONCLUSION** section summarizes the main points of the book and provides a final thought on the subject.

1.1. GENERAL INTRODUCTION ON VITAMIN K AND ITS FUNCTION

1.1.1. Short history

During the period 1929-1930, Henrik Dam discovered that a poor cholesterol synthetic diet led to hemorrhages in the digestive tract and muscles of chickens¹. Dietary administration of vitamins A and D did not show any improvements, while green plants and germinated cereal seeds had beneficial effects. In 1931, McFarlane confirmed Dam's results, showing that low dietary fats produce hemorrhages in all birds. At the same time, McFarlane showed that hemorrhages occurring in birds can be treated not only with diets containing green plants, but also with fish meat added to the birds' diet². In 1935, the liposoluble antihemorrhagic factor, which was unknown before, was defined by Dam - *vitamin K* (according to Koagulation in the German spelling)³. McFarlane and Schönheyder showed that blood from birds with hemorrhages had low prothrombin levels and prolonged coagulation times compared to the control group⁴. In 1937, Quick observed a progressive decrease in prothrombin in the blood of chicks fed on a poor vitamin K diet⁵. In 1939, Dam and Karrer isolated vitamin K from green plants, the substance called vitamin K₁ or phyloquinone, turning out to be a of yellow oil with the structure 2-methyl-3-phytyl-1,4-naphthoquinone⁶. H. Dam and E.A. Doisy received the Nobel Prize in medicine and physiology in 1943. Dam received the prize for the discovery of vitamin K and Doisy for the elucidation of the chemical structure of vitamin K.

The first crystalline antihemorrhagic vitamin was obtained from rotting fish meal, which was called vitamin K₂ or menaquinone. About 10 years passed before menaquinone was isolated from a pure bacterial culture in 1948, *Bacillus brevis* organism being used. Isolation and purification of these vitamin K forms was followed by their physico-chemical characterization⁷.

Simultaneously with the vitamin K "definition", a natural antagonist of the vitamin was discovered in the 1920's, in relation to the "sweet clover disease" among cattle in the American Midwest and Western Canada^{8,9}. In 1941, the responsible component was identified as 3,3'-methylene-bis-(4-hydroxycoumarin), which is commercially known as dicumarol, and has been proven to function as a vitamin K antagonist¹⁰. The discovery of anticoagulants and their use in medical therapy led to the subsequent identification of abnormal coagulation factors, which are functionally inactive¹¹⁻¹³, named PIVKA (Protein Induced by Vitamin K Absence or Antagonists). Their presence in plasma was postulated by Hemker et al. in 1963^{11,14}. Further insight in the mode of action of vitamin K and coumarin antagonists was provided by Stenflo, who discovered in 1974 that in the functionally inactive form of blood coagulation factor prothrombin (PIVKA II), an unusual amino acid was lacking: gamma-

carboxyglutamate (Gla)¹⁵. Further research demonstrated that Gla residues play an important role in Ca^{2+} -dependent phospholipid binding, necessary for the biological activity of coagulation factors¹⁶.

1.1.2. Gla proteins are vitamin K-dependent proteins

Vitamin K functions as a cofactor in the post-translational conversion of protein-bound glutamate residues into gamma-carboxyglutamate (Gla). The vitamin K dependent process is a carboxylation reaction, in which the removal of gamma-hydrogen precedes the addition of CO_2 . The enzyme involved in the vitamin K-dependent step is called gamma-glutamylcarboxylase and is located in the lumen of the endoplasmic reticulum of various cells¹⁵, along the route taken by secretory proteins on their way to the extracellular environment. In Figure 1 the transformation of the glutamic acid (Glu) in Gla is represented, as a result of vitamin K dependent carboxylation reaction.

Figure1: Structures of glutamate (Glu) and γ -carboxyglutamate (Gla).

Regarding the general mechanism of the vitamin K-dependent carboxylation reaction, a number of general principles have been accepted:

1. Three vitamin K metabolites are found in the lumen: vitamin K hydroquinone (KH_2), vitamin K epoxide (KO), and vitamin K quinone (K).
2. Although dietary vitamin K is exclusively present as vitamin K quinone (K), the active factor for carboxylase is vitamin KH_2 , which is converted during the carboxylation reaction to vitamin K epoxide (KO).
3. KO is reduced in turn to KH_2 by the action of two reductases (Figure 2). The recycling mechanism is very effective¹⁶, explaining the low daily vitamin K

requirements. Studies in vitro have shown that both K and KO reductases are dithiol-dependent, and are strongly inhibited by coumarin derivatives^{17,18}.

4. The liver contains a NADPH-dependent K reductase which is not inhibited by vitamin K antagonists, and which exclusively reduces K to KH_2 . Some in vitro and in vivo studies have shown that osteoblasts in bone tissue lack the NADPH-dependent reductase¹⁹. Experiments on animals treated both with vitamin K and warfarin have shown normal levels of coagulation factors, but blocked carboxylation of the bone Gla protein^{20,21}.
5. The existence of the vitamin K cycle (Figure 2) has explained the discrepancy between the low vitamin K intake and the relatively high urinary excretion of Gla on a molar basis²².

Figure 2: The vitamin K cycle.

Vitamin K dependent carboxylase is present in a wide variety of tissues such as liver, bone²³, kidney²⁴, lung, pancreas, placenta, skin²⁵, spleen, testis²⁶, vessel wall²⁷, but is absent in brain and tendon. A large number of Gla proteins have been identified in various tissues, but few of them have been characterized and studied from the point of view of their physiological role. In humans, characterized Gla proteins may be classified according to their origin as is shown in Table 1.

Introduction

Table 1: Classification and some characteristics of various Gla proteins

SITE OF SYNTHESIS	NAME	MOLAR MASS (Da)	NUMBER OF GLA RESIDUES	HALF-LIFE TIME	PLASMA CONCENTRATION
Liver	Coagulation factors:				
	F II	72,000	10	72-96 h	100 µg/ml
	F VII	48,000	10	4-6 h	1 µg/ml
	F IX	55,400	12	20 h	1 µg/ml
	F X	59,000	12	48-70 h	20 µg/ml
	Protein C	57,000	11	*	10 µg/ml
	Protein S	75,000	10	*	1 µg/ml
	Protein Z	50,000	13	*	<1 µg/ml
Extrahepatic	Osteocalcin	5,800	3	10 min.	15 ng/ml
	Matrix Gla protein	9,600	5	*	*
	Protein S	69,000	10	*	1 µg/ml
	Gas 6	*	*	*	*
	Vitamin D receptors	*	*	*	*
	Kidney Gla protein	*	*	*	*

Based on: Olson, R.E. The function and metabolism of vitamin K. *Annu Rev Nutr*, 1984; 4: 281-337, and Blombäck et al. 1994.

1.1.3. The chemical forms of K vitamins

Vitamins K is a group name for a number of related compounds, representing substances which in their purified form are yellowish colored oil (K_1) or crystals (K_2 - K_7). They are highly sensitive to the action of light and oxygen, which is the reason why it is recommended to keep them in the dark. Under the action of ultraviolet light and oxidants, they are easily degraded into darkly colored products. Vitamins K have characteristic absorption spectra, with wavelength bands ranging between 243-328 nm²⁸. By organic synthesis numerous compounds have been prepared possessing vitamin K (i.e. antihemorrhagic) activity. Vitamins K are chemically composed of a naphthoquinone nucleus that has a methyl group substituted at C-2 and a characteristic lateral chain (phytyl or isoprenyl) at C-3. Phylloquinone (K_1) and the group of menaquinones (K_2) belong to this class. These compounds differ in the length and degree of saturation of their aliphatic side chain, which is attached to C-3 (Figure 3).

Figure 3: Chemical structures of phyloquinon (vitamin K₁), menaquinon (vitamin K₂ group) and menadion (vitamin K₃).

In phyloquinone, the phytyl consists of 4 isoprenoid units of which only the first one contains an unsaturated bond. Phyloquinone is the only vitamin K form produced by green algae and plants. The group of menaquinones (K₂) (abbreviated as MK-n, where n represents the number of prenyl units, varying from 4 to 13 in natural menaquinones) is not found in plants but only in fermented foods (natto, curd, cheese) and animal products (meat, liver, eggs). It is also synthesized by intestinal bacteria such as *Escherichia coli*, *Bacillus aerogenes*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, or less abundantly by *Lactobacillus* and *Pseudomonas* groups. These menaquinones may serve as a source of vitamin K in humans²⁹, but the relative importance of the supply is not known exactly³⁰.

All synthetic vitamins K have the naphthoquinone ring system in common, but have different substituents. The simplest chemical form obtained by synthesis is vitamin K₃ (menadione). In vivo it is active only after being converted to MK₄ in animal tissues³¹. At present, menadione is no longer therapeutically used in humans because of its toxic side-effects. It is still frequently added, however, to fortified animal food.

The stability of vitamins K and their derivatives depends to a large extent on the environment in which they are stored, the action of ultraviolet light, oxygen, pH, and the nature of solvents playing an important role. On the other hand, techniques such as cooking, deep-freezing, gamma-irradiation do not reduce the vitamin K content in vegetables³².

1.1.4. Dietary sources and physiological requirements for vitamin K

Measurements of the vitamin K contents of various natural products revealed widely differing values which depend on the analytical method used. Lately, various laboratories have determined vitamin K from different foods by chromatographic methods (HPLC) in order to compose guiding tables for patients with anticoagulant treatment³³ or for elderly people belonging to the group at risk for subclinical vitamin K deficiency³⁴. Important vitamin K₁ sources are green leafy vegetables such as spinach, kale, cabbage and broccoli, which contain vitamin K₁ in a range of 2-8 µg/g, and vegetable oils such as rape seeds, soybean and olive oil, with a content ranging between 0.5-2µg K₁/g^{35,36}. Menaquinones are present in cheese, eggs, butter, fish, meat³⁷, and also in vegetables such as sauerkraut and the Japanese food natto (fermented soya beans), which contains high amounts of menaquinones, especially in the form of MK₇³⁸. Human and animal requirements for vitamin K are difficult to establish because part of vitamin K is provided by food, and part of it is produced through biosynthesis by microorganisms in the digestive tract. In order to determine the physiological dose of vitamin K₁ or K₂ necessary for the maintenance of plasma coagulation factors concentrations within normal limits, animal experiments have been performed using standard protocols that include a vitamin K free diet plus boiled rice (as an inhibitor of the intestinal flora), and methods for the prevention of coprophagy (tail cups)³⁹. In these animal models, vitamin K deficiency manifests not later than after 7 days through hemorrhages due to severe hypoprothrombinemia. For conventional male rats, a minimal 0.1-0.5 µg K₁/ g diet was reported⁴⁰. In female rats, daily physiological requirements are lower than in male rats, probably because estrogen has a stimulating effect on vitamin K absorption⁴¹.

Humans have low vitamin K requirements, and clinical vitamin K deficiency is rarely found in adults. Vitamin K requirements in humans have been studied in subjects receiving a diet deprived of vitamin K combined with antibiotics. To establish the minimum daily requirements, the administered vitamin K amount was correlated with prothrombin time (PT) or prothrombin activity as a measurement of vitamin K status. Olson established recommended dietary intakes (RDI) for different age groups ranging from daily amounts of 10 µg for infants to 45 µg for adult males⁴². The current Recommended Dietary Allowance (RDA) for vitamin K is approximately 1 µg/kg body weight/ day⁴³, partly based on more recent determinations of PIVKA's and undercarboxylated prothrombin^{44,45}.

1.1.5. Vitamin K absorption, transport and storage

The intestinal absorption of vitamin K is related to the simultaneous intake of dietary triglycerides, which explains the positive correlation between phyloquinone concentration in plasma and plasma triglycerides^{46,47}, as well as the positive correlation between fasting vitamin K levels and dietary intake^{48,49}. Intraluminal solubilization for absorption is achieved by bile salts and pancreatic lipase. For this reason, subclinical vitamin K deficiency occurs in bile salt deficiency⁵⁰ (obstructive jaundice), pancreatic insufficiency and chronic pancreatitis⁵¹. Apart from solubilization factors at the intestinal level, the absorption degree depends on the number of isoprenoid units in the aliphatic chain of menaquinones⁵², on the efficiency of vitamin K liberation, and on the administration route. After oral administration of vitamins K₁ and MK₄, these will be absorbed in the lymph at the level of the ileum and jejunum, then they will enter the blood, reaching a peak after 2-6 hours⁵³⁻⁵⁵. Four hours after oral administration of vitamin K, this is found predominantly (81%) in VLDL⁵⁴, while 10 hours after intramuscular administration 49% of the circulating vitamin K₁ is found in LDL and 37% in HDL⁵⁴. No specific plasma carrier protein is known for vitamin K, as in the case of vitamins A and D. As with vitamin E, plasma transport of vitamin K appears to be entirely mediated by lipoproteins. Recently, it has been suggested that plasma phyloquinone levels are genetically influenced by the apolipoprotein E genotype, an important determinant of the chylomicron-remnant clearance rate⁴⁹. Apolipoprotein E (apo E) is a major constituent of lipoprotein particles, namely chylomicrons, IDL and VLDL. The human apo E gene is polymorphic, with three common alleles (ϵ 2, ϵ 3, ϵ 4) coding for three isoforms E2/2, E3/3, E4/4, and three heteroforms E2/3, E2/4, and E3/4. The isoforms differ from each other by a single amino acid substitution (in apo E2 Cys-158 to Arg and in apo E4 Arg-112 to Lys), and also differ in their binding affinity for the apo E receptors (apo E/B-100 and E/B-48). Apo E3 and E4 have the same affinity for this receptor, whereas apo E2 shows defective binding activity, corresponding to 1% of that of the two other isoforms⁵⁶. Previous studies have shown that the apolipoproteins E4 are associated with the risk of developing diseases such as Alzheimer's dementia, cardiovascular disease^{56,57}, more recent studies suggesting a relationship between apo E and bone mineral density (BMD)⁵⁸. A high chylomicron clearance corresponds to low plasma vitamin K levels^{49,59}. Konishi has shown that in rats the remnant-chylomicrons clearance is faster for MK₄ when compared with phyloquinone⁶⁰.

Introduction

Figure 4: The general scheme for vitamin K absorption from the diet and its transport to the extrahepatic level. (C= Cholesterol, TG= Triglycerides, E= Apolipoprotein E; B-48= Apolipoprotein B-48; Fa= Fatty acids). Apolipoprotein A and C, present in nascent (apo A) and mature chylomicron (apo A and C), are not represented in the figure.

Intestinal absorption of phyloquinone is stimulated by estrogens, which guarantees female animals a generally better supply of vitamin K (Jolly et al., 1977). Consequently, male animals are much more sensitive to vitamin K deficiency or vitamin K antagonists than females.

Over the past years, several studies on tissue distribution of vitamin K have been performed by accurate HPLC assays. Vitamin K storage mainly occurs in the liver, 90% of the stores being represented by MK₄ through MK₁₃ and not K₁^{61,62}. In plasma, only menaquinones up to MK-8 were detectable⁶²⁻⁶⁴. Specific tissues for MK₄ accumulation are the pancreas, testis, aorta, salivary glands and brain^{65,66}, even after oral vitamin K₁ intake⁶⁶. These tissues are even capable of converting K₁ to MK₄. The location and mechanism of K₁ to MK₄ conversion are not yet known. Menadione is generally rapidly metabolized and excreted, and only a relatively small fraction of this form of vitamin K is converted to MK₄. Vitamins K₁ and K₂ are degraded slowly⁶⁷, and vitamin K metabolites may leave the body with feces, urine^{54,68} or reenter the plasma via the enterohepatic cycle.

1.2. VITAMIN K STATUS, VITAMIN K DEFICIENCIES AND HYPERVITAMINOSIS.

1.2.1. Definition of vitamin K status

Vitamin K is required for the synthesis of γ -carboxyglutamate (Gla) residues in a number of proteins. Before the discovery and the characterization of extrahepatic Gla proteins, vitamin K status was deduced from plasma prothrombin and blood coagulation time. Today it is known that prothrombin is a protein with 10 Gla residues that allow it to bind calcium and phospholipid compounds in the coagulation process. From the point of view of prothrombin activity, a normal vitamin K status is considered to be a plasmatic vitamin K level that can supply sufficient vitamin K to the liver, in order to assure a normal coagulation time, corresponding to a prothrombin activity of over 65%. The Deutsche Gesellschaft für Ernährung has published an order-of-magnitude daily requirement: 0.01-0.03 mg/kg body weight⁶⁹. Another way to define a person's vitamin K status may be through the detection of partially on non-carboxylated Gla-proteins. During periods of suboptimal vitamin K status, the various Gla-proteins enter the blood stream in an undercarboxylated form (PIVKA), which means that the number of Gla residues per molecule is below the normal, and may even become zero⁷⁰. 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^{71,72}. In patients with acute hepatitis, cirrhosis, hepatocellular carcinoma, PIVKA II may be found in plasma, but due to an impaired carboxylase activity rather than to vitamin K deficiency⁷³⁻⁷⁵. Based on the occurrence of PIVKA II, the physiological requirement for vitamin K was found to be 1 μ g/ kg body weight/ day⁷⁶.

Along with the isolation and characterization of extrahepatic Gla proteins, in vitro techniques for their determination have been developed, through RIA and ELISA methods. Of these, osteocalcin is frequently determined for diagnostic purposes in osteoporosis. Secreted by young bone cells (osteoblasts), it is the most abundant non-collagenous protein in mature bones^{77,78}. Given the fact that a large part of vitamin K is stored after absorption at the hepatic level, the determination of the carboxylation degree of osteocalcin has become an attractive instrument in the definition of vitamin K requirement for a normal status of Gla proteins at an extrahepatic level. Total serum immunoreactive osteocalcin (total irOC) can be divided in a fraction with a low (irOC_{free}) or high (irOC_{bound}) affinity for hydroxyapatite. Due to the capacity of carboxylated osteocalcin to adsorb in vitro to hydroxyapatite, the hydroxyapatite binding capacity (HBC) could be evaluated, which is in fact irOC_{bound} expressed as a percentage of total serum osteocalcin. HBC is considered to be a measure for osteocalcin Gla-content and vitamin K status, and may be used

to detect subclinical vitamin K deficiency³⁴. Furthermore, Delmas demonstrated in a study on 195 elderly institutionalized women aged between 70-101 years that serum undercarboxylated osteocalcin is a marker of the risk for hip fracture in elderly women⁷⁹. On the other side, Cairns and Price demonstrated that in the normal human population osteocalcin is incompletely γ - carboxylated⁸⁰, Nakano showing that the γ - carboxyglutamic acid at position 17 being essential for the calcium-dependent conformation⁸¹. These publications raised the question of whether the dietary vitamin K intake is adequate to ensure full carboxylation of osteocalcin. Vitamin K administration in postmenopausal women led to the decrease of descarboxy-osteocalcin^{34,79,82,83}. Even in healthy newborns, whose vitamin K status is known to be precarious, very low or no levels of descarboxy-prothrombin are detectable. In contrast, all babies tested had high concentrations of serum descarboxy-osteocalcin. Moreover, vitamin K supplementation during pregnancy significantly decreased descarboxy-prothrombin levels in cord sera⁸⁴. These data demonstrate that the hydroxylapatite binding capacity (HBC) of circulating osteocalcin is the most sensitive marker known to detect a subclinical vitamin K deficiency. The difference between coagulation factors (liver Gla proteins) and osteocalcin (bone Gla protein) suggests that different tissues, such as the liver and bone, may have different vitamin K requirements. Since most of the ingested vitamin K₁ is stored in the liver⁶⁵, bone tissue may be more prone to vitamin K deficiency than the liver. In this case, impaired synthesis and carboxylation of some extrahepatic Gla proteins may be more prevalent in human population than coagulation assays indicate⁸⁵. This may result in an increase of RDAs (Recommended Dietary Allowance) especially for the elderly and breastfed children during their first year of life. Vitamin K deficiency must be redefined, not on the basis of impaired blood coagulation tests but on the basis of undercarboxylation of extrahepatic Gla proteins.

1.2.2. Vitamin K Deficiency Bleeding in Infancy (VKDB)

One hundred and six years ago, Charles Townsend of Boston first described the condition of hemorrhagic disease of the newborn (HDN) in 50 neonates, of whom 31 died⁸⁶. The historical term "hemorrhagic disease of the newborn", which is used as a synonym for vitamin K deficiency bleeding (VKDB) in infancy, is not recommended, since neonatal bleeding is often not due to vitamin K deficiency and VKDB may occur after the neonatal period. Neonates form a special case with reference to provision of vitamin K, because the placental transport of vitamin K is poor, the intestine is sterile during the first days of life, the hepatic biosynthesis of coagulation factors is inadequate and human milk is a poor source of vitamin K. The hemorrhagic disease of infancy can be ascribed to the immaturity of the liver, which forms too little of the

coagulation factors, but it is caused primarily by a vitamin K deficiency. Breastfed infants are more susceptible than those fed otherwise⁸⁷.

Diagnostic inclusion criteria include visible bleeding, such as that from the nose, umbilicus, skin, urinary tract, and gastrointestinal tract, as well as iatrogenic bleeding resulting from needle pricks or operations. Intracranial, pulmonary and gastrointestinal bleeding is often diagnosed only retrospectively, as central nervous system disturbances (convulsions, impairment of consciousness, vomiting) breathing difficulties, and anemia can also be due to conditions other than bleeding.

In a bleeding infant, a prolonged one-stage prothrombin time in association with reduced activity of coagulation factors II, VII, IX, X, normal vitamin K-independent coagulation factors, a normal (or increased) fibrinogen level and platelet count are strong indications for VKDB. The diagnosis is proven if administration of vitamin K is followed by a shortening of the prothrombin time, within 30 minutes, or by cessation of bleeding⁸⁸. Several reasons for a low vitamin K status are summarized in Table 2. The placental barrier for maternal vitamin K leads to a ratio of vitamin K levels in cord blood and maternal blood of about 1:30⁸⁹. After birth breastfed children have an increased risk of developing classical VKDB^{90,91}, since human milk has a low content of vitamin K⁹². Another reason may be of various gastrointestinal etiology and the intake of medications, which interfere with vitamin K metabolism^{93,94} (Table 2).

Table II: Classification of VKDB by etiology:

ETIOLOGY OF VKDB	CAUSES
NUTRITIONAL VKDB (Reduced vitamin K intake)	<ul style="list-style-type: none"> • abnormally low VK intake (breastfed babies) • poor feeding • parenteral nutrition (no vegetables)
ABSORPTION VKDB (Reduced VK absorption)	<p>Cholestasis</p> <ul style="list-style-type: none"> • α_1 antitrypsin deficiency • biliary atresia • cystic fibrosis • viral diseases • celiac disease • ascaris infection • blockage of the bile ducts <p>Gastrointestinal disease</p> <ul style="list-style-type: none"> • short bowel syndrome • inflammation • prolonged diarrhea <p>Medications</p> <ul style="list-style-type: none"> • antibiotics, sulfonamides antiepileptics, salicylates, coumarins

Introduction

HDN was first thought that it was limited to the first five days of life. It was later reported that breastfed infants 3 to 4 months of age suffered intracranial hemorrhage caused by vitamin K deficiency. The disease is strongly correlated with low socioeconomic status, and it occurs mainly in breast-fed infants⁹⁵, boys being twice more affected than girls⁹⁶. Further classification into early, classical and late forms is important because of pathogenic and clinical differences (Table 3).

Table III: Forms of vitamin K deficiency bleeding (VKDB) in infancy.

FORM OF VKDB	AGE AT ONSET	CAUSES AND RISK FACTORS	LOCALIZATION IN ORDER OF FREQUENCY	FREQUENCY WITHOUT VK PROPHYLAXIS
EARLY	Younger than 24 hours	Medications taken during pregnancy: anticonvulsivants, oral anticoagulants, tuberculostatics.	Cephalhematoma, umbilicus, intracranial, intraabdominal, gastrointestinal	very rare ⁹⁴
CLASSICAL	Days 2-7	Late onset of feeding, inadequate milk intake, marginal VK content in breast milk.	Gastrointestinal tract, umbilicus, ENT region, needle-prick sites, circumcision, intracranial	0 - 4.4 per 10 ³ births, ^{97,98}
LATE	Week 2-8	Marginal VK content in breastmilk in association with low VK intake and absorption.	Intracranial (>50%), skin. ENT region, gastrointestinal tract, needle-prick sites, umbilicus, urogenital tract, intrathoracic	4-10 per 10 ⁵ births (more common in South East Asia)

1.2.3. VKDB prophylaxis in newborns

In the 1950s it became widespread practice to give vitamin K prophylactically to protect neonates against VKDB⁹⁹. The finding of an association between the synthetic form of vitamin K used (K₃; Synkavit) and blood hemolysis causing subsequent kernicterus¹⁰⁰ in the 1960s was a setback. Administration of vitamin K₁ was safe in this respect, but the rarity of classical VKDB, the trauma of intramuscular injections, and the cost involved by the treating of all babies have been the cause of a continuing debate¹⁰¹. Some pediatricians maintain that the only safe practice is to

give intramuscular vitamin K to all babies, others that oral vitamin K suffices in those at low risk for haemorrhagic disease of the newborn, and a minority that this second group needs no prophylaxis at all¹⁰². In 1992 suspicions were raised that parenteral VK prophylaxis doubled the risk of cancer^{103,104}. However, these studies have not been confirmed by others¹⁰⁵.

In the 1980s numerous cases of the late form of VKDB appeared after the neonatal period, because single oral VK prophylaxis was not very protective^{106,107}. With intramuscular VK prophylaxis the problem seemed to be solved again, but intramuscular VK prophylaxis was replaced in many countries by triple oral VK prophylaxis^{97,108}. The change to oral prophylaxis may have improved vitamin K acceptance. In Switzerland the recommendation is to give 2 mg vitamin K₁ on postnatal days 1 and 4, whereas in other European countries three doses are recommended. To facilitate resorption, particularly in infants with cholestatic liver disorders, a new mycellar form of oral vitamin K (Konaktion MM paediatric, Roche, Switzerland) was introduced in January 1995¹⁰⁹. Cornelissen et al. showed that one single oral dose of 1 mg vitamin K at birth followed by repeated maintenance doses of 25 µg/day or 1 mg/ week of phyloquinone during three months provides optimal protection against VKDB^{110,111}.

1.2.4. Hypervitaminosis

Vitamins K₁ and K₂ are not toxic. In mice, menadione (K₃) administered in high doses determines a prolongation of blood coagulation time, haemorrhages, effects contrary to those induced by the administration of low doses of this vitamin. The effect of high doses of vitamin K₃ (10 to 50 mg/kg/day, administered intramuscularly) show that overdoses of vitamin K₃ in rats alter lipid metabolism, causes jaundice (due to mainly unconjugated bilirubin as result of hemolysis), anemia becoming evident at doses of 40 and 50 mg/kg/day¹¹². In humans, vitamin K₃ administration in parenteral doses of 5-25 µg/day of water-soluble menadione have induced hemolytic anemia, hyperbilirubinemia and kernicterus in newborns¹¹³. The toxic effects of high doses of menadione may not be the result of its vitamin K activity, but are probably side effects of the unphysiological form of the preparation. Menadione reduces the glutathione level in blood, interacts with the redox system of the erythrocytes, causes the formation of methemoglobin, destabilizes the erythrocytes and causes hemolysis. Among the vitamins K the best tolerated by adults and children is vitamin K₁. No cases of hypervitaminosis K₁ have been reported in humans or animals. However, headache, chest pain, urticaria, hot flush after intravenous phytomenadione injections (K₁) have been occasionally reported. Various local reactions seen at the site of intramuscular injections of many different drugs have been reported¹¹⁴, but

morphea-like changes have only been reported after phytonadione administration (AquaMEPHYTON, Konakion, Mephyton)¹¹⁵. These effects should not be attributed to the action of vitamin K₁, but to the emulsifying substances used for solubilization. This is why vitamins K should be administered intravenously only in severe cases, when the rapid action of vitamins is essential.

1.3. GLA PROTEINS INVOLVED IN BONE METABOLISM

1.3.1. About bone metabolism

Bone consists of cells and extracellular matrix, the latter comprising organic (35%) and inorganic (65%) components. The organic component includes type I collagen (90%) and non-collagenous proteins such as osteocalcin (OC), osteonectin, matrix Gla protein (MGP), protein S, proteoglycans, glycosaminoglycans and lipids. The inorganic component mainly consists of calcium and phosphate in hydroxyapatite crystals. The skeleton stores 99% of the body's calcium, and is also a substantial store of sodium and magnesium¹¹⁶.

The process of *modeling* is an active bone formation and resorption process, which is responsible for the determination of the size and shape of the bone, a process which is active in all growing vertebrates. This process ends at adulthood. Another important process is *remodeling*, which is essential for the balance of bone formation and resorption and the maintenance of normal bone structure. The process starts with bone resorption, followed by bone formation, the entire remodeling process lasting about 120-200 days. The equilibrium between bone formation and bone resorption is realised by two types of bone cells: osteoclasts and osteoblasts. The osteoclasts are multinucleated cells involved in bone resorption. When osteoclasts are activated, they will arrange in a specialized ruffled border at the site of the bone resorption area. At the contact site of the ruffled border of the osteoclasts with the bone area to be resorbed, proton pumps are activated and lysosomal enzymes such as phosphatases, collagenases are secreted by osteoclasts. The resulting acid environment will dissolve the bone, developing an empty space, which will be filled in with new bone, during the process of bone formation. The process of bone resorption followed by an equal amount of formation has been termed *coupling* (Frost, 1964), and one of the most intriguing issues of bone cell biology is to learn how osteoblasts are programmed to replace the exact amount of bone which has been resorbed. This may be due to some local chemical signals. In Figure 5 the coupling process is illustrated. Bone formation process follows the resorption and is realised by the osteoblasts, which are young bone cells involved in the production of collagen type I,

osteocalcin and alkaline phosphatase. Osteoblasts lay down in the resorbed spaces an organic matrix called osteoid, which by mineralisation will form the new bone. The mineralisation process is dependent on local calcium and phosphate concentrations, in order to form hydroxyapatite.

Figure 5: The remodeling cycle starts with the activation of osteoclasts by the growth hormone, parathyroid hormone (PTH), interleukins (IL-1, IL-6, IL-11), estrogen. The network of cytokines and growth factors is essential for the regulation of both osteoclastogenesis and osteoblast formation: these factors play a pivotal role in the paracrine regulatory control of bone turnover under physiological conditions. (AOBL= Activated Osteoblast; OCN= Osteocalcin "de novo"; BAP= Bone Alkaline Phosphatase; COL I= Collagen type I; OCL= Osteoclast; HOP= Hydroxyproline; DPD= Deoxypyridinoline; OC= Osteocalcin; Ca= Calcium)

Osteoblastic synthesis of proteins can be detected in serum and permits indirect measurement of bone turnover. In older subjects the uncoupling of remodeling results in a net loss of bone, and the gradual decrease in bone mass is seen with ageing.

1.3.2. Bone turnover rate is influenced by several factors

Bone turnover represents the rate at which the existing bone is replaced by an equal amount of newly formed bone. Formation and resorption are closely coupled processes that are in balance in healthy people.

Three major influences on the equilibrium of bone tissue are: a) calcium and phosphate levels in the extracellular fluid, b) hormones and local factors (cytokines), and c) the mechanical stress, which helps stimulate osteoblastic activity and formation of the organic matrix.

Receptors for PTH, vitamin D and estrogens have been described in osteoblasts, while osteoclasts have receptors for vitamin D, calcitonin¹³⁶ and nuclear receptors for estrogens only¹¹⁷. PTH is supposed to act on osteoclasts indirectly, via the osteoblasts, that produce a soluble factor with affinity for the osteoclasts^{118,119}. In this context, increases in PTH will raise bone formation as well as bone resorption, equally, by increasing the rate of bone turnover. As compared to the adult population, bone turnover is high in young and old subjects. The age dependent manner of bone turnover rate variation^{120,121} is based on the relation between vitamin D metabolism, calcium absorption and parathyroid function¹²². Aging decreases the responsiveness of the renal 25(OH)₂D-1-hydroxylase to PTH, resulting small decreases in circulating levels of 1,25(OH)₂D, which may contribute to decreased calcium absorption in the elderly. Also, it seems that the bone turnover markers may vary substantially from country to country, suggesting systematic geographic and genetic differences that may potentially relate to osteoporotic fracture rates¹²³⁻¹²⁵. The quality of bone and the peak bone mass are closely related to nutritional aspects, such as calcium intake¹²⁵⁻¹²⁷, phosphate¹²⁸, vitamin D¹²⁹⁻¹³², vitamin K status¹³³, vitamin C, magnesium^{134,135}, to which risk factors including inheritance, parity, body weight, alcohol, tobacco and caffeine consumption are added.

Except PTH, other hormones influencing the balance of bone turnover, and for which bone cells present receptors, are calcitonin^{136,137} and the sex hormones (estrogens and testosterone)¹³⁸⁻¹⁴¹. Estrogens appear to stimulate bone formation, but this is rather indirectly, due to an inhibitory effect on the osteoclasts¹¹⁷. Oral contraception is associated with a moderate, but significant decrease in bone turnover, that may have a beneficial influence on bone mass only after prolonged use¹⁴². At menopause there is a change in biochemical markers and ultrasound densitometry¹⁴³. Except the sex hormones, glucocorticoids act as direct inhibitors on the proliferation and function of osteoblasts. They don't have an influence on bone turnover directly, but act by changing the production of hormones (testosterone¹⁴⁴) and local humoral factors (cytokines), that regulate bone and calcium metabolism^{145,146}. Glucocorticoids directly inhibit vitamin D-mediated intestinal calcium absorption, bone mineral mobilization and enhance the sensitivity of bone cells to 1,25(OH)₂D by stabilizing the 1,25(OH)₂D receptor or by increasing the affinity or number of receptors. Chronic anticonvulsant therapy can cause the development of osteomalacia, having multiple

effects on calcium metabolism, by altering the kinetics of the vitamin D-25-hydroxylase (Phenobarbital).

Also local factors may contribute to the modulation of bone metabolism, but their role needs further investigation. Cytokines that are stimulating osteoblasts are: tissular growth factor (TGF β), gamma interferon (IFN γ), while other factors such as TGF α , interleukin-1 (IL-1) activate osteoclasts, a process which is evident in some bone tumors by "malignant hypercalcemias"¹⁴⁷.

Overweight¹⁴⁸ and regular sport¹⁴⁹ are beneficial for the bone structure, while low weight and physical stress such as competitive sports¹⁵⁰ may influence bone turnover, inducing a change in bone turnover rate. The mechanisms are originating at various levels of the brain-hypothalamic-pituitary-gonadal axis¹⁵¹.

1.3.3. Biological markers for bone turnover

Bone formation and resorption markers are useful tools especially when they allow the clinician to predict the risk of a fracture or to evaluate a therapeutic effect. The bone markers are usually divided into two groups: the bone resorption markers which are detected mainly in urine, and the bone formation markers occurring in serum. Several assays for bone resorption are already available¹⁵², and the main assays may be classified in several groups: pyridinoline cross-link assays (hydroxylsyl pyridinoline, deoxy pyridinoline) and type I collagen telopeptide assays (for both carboxy - CTX and amino telopeptides - NTX). These two groups have been shown to better reflect bone resorption than hydroxyproline and calcium excretion assays, which in spite of their long-time use as the most frequently performed tests for the measurement of bone resorption, present the inconvenience of dietary interferences. Tartrate-resistant acid phosphatase (TRAP) also reflects osteoclast activity, but it is not used frequently because it is difficult to measure. Recently, a new immunoassay method was developed for tartrate-resistant acid phosphatase in serum¹⁵³.

The three main osteoblastic products used as markers for bone formation are: type I procollagen propeptides (PICP, PINP), bone alkaline phosphatase, and osteocalcin. Since the formation of bone takes about 120 days, it is expected that after the proliferation of the matrix, well represented by the production of collagen I, differentiation represented by matrix maturation and mineralisation will take place. These three analytes reflect different phases of osteoblast activity - proliferation and differentiation of the matrix. Figure 6 illustrates the main phases of bone formation and its corresponding bone markers.

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Figure 6: Total sequence of remodeling. The main stages of bone formation, reflecting the osteoblast proliferation and differentiation. OCL= Osteoclast, MN= mononucleated cells; PRE-OBL= Pre-Osteoblast; OBL= Osteoblast; OC= Osteocyte)

1.3.4. Gla proteins occurring in bone

Three Gla proteins have been identified in bone tissue: osteocalcin (bone Gla protein, BGP, OC)⁷⁸, matrix Gla protein (MGP)¹⁵⁴, and protein S¹⁵⁵. All three Gla proteins are synthesized by osteoblasts. Osteocalcin is exclusively synthesized by osteoblasts and odontoblasts¹⁵⁶, while MGP is expressed in many soft tissues¹⁵⁷ and protein S is produced in hepatocytes, megakaryocytes, endothelial cells^{158,159} as well. Together the three Gla proteins represent approximately 80% of the Gla store in the human body. In humans, at least two of the bone Gla proteins occur in an undercarboxylated form, osteocalcin at position 17, MGP at position 2^{80,81,160}.

Osteocalcin is a small (5.7 kDa) Ca^{2+} binding protein, containing 49 amino acids. The molecule presents two α -helical structures, stabilized by a disulphide bond between positions 23 and 29. The helical conformation is stabilized by Gla residues, that provide the optimum conformation for its adsorption to hydroxyapatite¹⁶¹. In bone, osteocalcin occurs in close association with the mineralized matrix, to which it is bound via the α -helical domain^{156,161}. The carboxy-terminal part of osteocalcin possesses chemo-attractant activity for osteoclasts¹⁶². These properties suggest that osteocalcin plays a regulatory role in the mineralization and remodeling of bone tissue^{156,163}. Indeed, osteocalcin-deficient transgenic mice developed a phenotype marked by longer bones, higher bone mass, and irregular orientation of the

hydroxyapatite crystals¹⁶⁴. The precise function of osteocalcin has remained unclear, however.

Osteocalcin synthesis is regulated by vitamins D and K. Vitamin D stimulates mRNA transcription^{165,166}, whereas vitamin K plays a role in the posttranslational carboxylation of Glu residues into Gla residues. After synthesis 80% of osteocalcin is incorporated in the bone matrix, whereas 20% is released in the blood stream. Circulating OC is metabolized in the liver and kidney^{167,168}. Serum osteocalcin is influenced by age¹⁶⁹, gender¹⁷⁰, renal function¹⁷¹, medication^{34,172,173}, menstrual cycle^{174,175}, season and time of the day^{176,177}.

Initially, osteocalcin detection was assessed by competitive radioimmunoassays (RIA), using bovine osteocalcin as a tracer¹⁷⁸. Recent developments are the use of nonradioactive tracers¹⁷⁹ or sandwich based assays^{180,181}. The difference in the populations studied, assay type, source of immunoglobulins (various antisera) and standards may be responsible for the difference between various kits¹⁸²⁻¹⁸⁴.

Matrix Gla Protein (MGP) is a 9.6 kDa Gla-protein containing 5 Gla residues. Insoluble in aqueous solutions¹⁵⁴, it interacts with organic bone constituents, such as collagen, proteoglycans or glycosaminoglycans. MGP is abundant in bone and cartilage¹⁸⁵, although these tissues contain lower levels of mRNA coding for MGP, as compared to the kidney, lung, liver, spleen, and vessel wall¹⁸⁶. In bone tissue, MGP is mainly expressed early in bone development, before the onset of mineralization¹⁸⁷, appearing earlier than osteocalcin. MGP mRNA transcription is regulated by vitamin D¹⁸⁸ and retinoic acid^{185,189}.

The function of MGP in the mineralization process was clearly demonstrated by Luo et. al.¹⁹⁰, who reported that MGP deficient mice underwent spontaneous calcification in the arteries and cartilage. Absence of MGP also results in the calcification of the growth plate cartilage, causing short stature and osteopenia. About 15% of the babies delivered by women who were treated with vitamin K antagonists during their pregnancy developed warfarin embryopathy syndrome or Fetal Warfarin Syndrome (FWS), characterized by nasal hypoplasia, stippled epiphyses, extremity shortening and vertebral abnormalities^{191,192}. Price et al. recently demonstrated that malformations appeared in FWS are similar to those observed in the MGP deficient mice. Specifically blocking the extrahepatic carboxylases by using a combination of warfarin and vitamin K, these authors showed that rats developed calcifications of the elastic lamellae in arteries and heart valves within 3-5 weeks¹⁹³, thus demonstrating that Gla residues are required for MGP function.

Protein S is a single polypeptide chain molecule with a molecular mass of 69 kDa, containing 11 Gla residues, playing a role in blood coagulation by acting as a cofactor for activated protein C¹⁹⁴. 40% of circulating protein S occurs in a free form,

the rest is bound in the complex C4b-binding protein, involved in the regulation of the complement system¹⁹⁵. In 1990 Pan et al. reported that two children with well-established protein S deficiency suffered from severe osteopenia and had reduced bone mineral density and vertebral body compression fractures¹⁹⁶. This finding proposes an additional role of protein S in bone metabolism, and is sustained by the discovery that protein S is also synthesized by osteoblasts¹⁹⁷.

1.4. INTRODUCTION TO THIS THESIS

After the discovery of the unusual aminoacid γ -carboxyglutamate in 1974, the function of vitamin K has been extensively studied, and vitamin K-dependent carboxylase has been found in liver and in a large number of extrahepatic tissues. The number of Gla-containing proteins that have been identified and characterized has remained surprisingly low thus far. Osteocalcin and MGP are two examples of Gla-proteins. The molecular structure is precisely known, but their mode of action in regulating bone formation and tissue mineralization has remained obscure. It is generally accepted, however, that their biological activity depends on the presence of three (osteocalcin) and five (MGP) Gla-residues per molecule.

Once it has been formed, Gla cannot be metabolized or re-used so that after protein degradation Gla is excreted in the urine, mostly as the free aminoacid¹⁹⁸, a minor part as short peptides. In a steady state situation, the urinary Gla-concentration may therefore reflect the turnover of the total body pool of Gla-proteins. In chapters 2 and 3 we have compared two animal models for vitamin K-deficiency: rats receiving a diet deficient in vitamin K and rats treated with coumarins (vitamin K antagonists). The major difference between both models is that in the former one the vitamin K cycle is operational, so that supplemented K-vitamins are recycled and utilized 500 to 1,000 fold more efficiently than in the coumarin-treated model. In these models we have compared the ability of various K-vitamins to function as a cofactor in the blood clotting factor synthesis. Moreover, we have tested whether urinary Gla-excretion may be used as a sensitive overall marker for vitamin K status.

In chapter 4 we address the question of how to define and measure human vitamin K status, and it appeared that the hydroxyapatite binding capacity (HBC) of serum osteocalcin is the most sensitive marker for human vitamin K status presently available. Accurate measurement of serum osteocalcin concentrations is a critical step in this technique. Therefore, we have developed an automated enzymatic assay for the quantitative determination of osteocalcin in human serum (chapter 5).

Chapter 6 is based on a review article which forms the problem statement for this thesis. Based on the concept of tissue-specific vitamin K requirement we hypothesize that for optimal carboxylation of Gla-proteins in bone tissue a higher nutritional vitamin K intake is needed than for the carboxylation of the clotting factors in the liver. This implies that in the apparently healthy population sub-groups may be at risk for developing subclinical vitamin K-deficiency, possibly leading to poor bone development and accelerated bone loss. If so, these sub-groups should be identified to find out the effect of vitamin K-supplementation on bone markers, bone mass, and bone strength. At the start of this project one such group (postmenopausal women) had been identified already, and clinical trials were already in progress.

In the subsequent chapters we describe an inventory of other potential candidates for further evaluation. The studies presented are relatively small, cover widely different subjects, and were ment as exploratory studies before placebo-controlled clinical trials might be initiated. In *chapter 7* we have investigated institutionalized children living in Romanian children's homes. Since prophylactic vitamin K administration is not common in these institutions, they form one of the last places in Europe where vitamin deficiencies can be studied in a non-supplemented population. In *chapter 8* we report the effect of vitamin K-administration to female elite athletes. The training and work load of these women is high and frequently leads to long lasting amenorrhea and rapid loss of bone mass. In *chapter 9* we describe a unique experiment onboard the Russian space station MIR. It is well known that during microgravity conditions astronauts undergo rapid loss of bone mass, which may be as high as several percent per month. During the 6-month flight we performed a 3-month intervention study with 10 mg/day of vitamin K and regular onboard venipunctuations and urine collections to monitor the biochemical bone markers.

In *chapter 10* we have summarized the conclusions from this thesis.

References

1. Dam H. Cholesterinstoffwechsel in Hühnereiern und Hünchen. *Biochem. Zeitschr.* 1929; 215: 475-492.
2. McFarlane, W.D., Graham, W.R., & Richardson, F. The fat-soluble vitamin requirements of the chick. *Biochem. J.*; 1931; 25: 358-366.
3. Dam H., *Nature* 1935; 133:652-662.
4. International symposium on recent advances in research on vitamins K and related quinones (vitamin K, ubiquinones or coenzymes Q, plastoquinones) in honor of

- Professor Henrik Dam. Historical survey and introduction. *Vitam. Horm.* 1966; 24: 295-306.
5. Dirckx, J.H. Armand J. Quick: pioneer and prophet of coagulation research. *Ann. Intern. Med.* 1980; 92(4): 553-558.
6. Raju, T.N. The Nobel chronicles. 1943: Henrik Carl Peter Dam (1895-1976); and Edward Adelbert Doisy (1893-1986). *Lancet* 1999; 353: 761.
7. McKee, R.W., Binkley, S.B., MacCorquodale, D.W., Thayer, S.A. & Doisy, E.A. J. *Am. Chem. Soc.* 1939; 61:1295-1299.
8. Shofield, F.W. A brief account of a disease in cattle simulating hemorrhagic septicemia due to feeding sweet clover. *Can. Vet. Rec.* 1922; 3: 74-79.
9. Campbell, M.A., Link, K.P. Haemorrhagic disease in cattle. *J. Biol. Chem.* 1941; 18: 21-33.
10. Campbell, M.A., Link, K.P. Studies on the hemorrhagic sweet clover disease. The isolation and crystallization of the hemorrhagic agent. *J. Biol. Chem.* 1941; 138: 21-33.
11. Hemker, H.C., Veltkamp, J.J., Hensen A., Loeliger, E.A. Nature of prothrombin biosynthesis: preprothrombinemia in vitamin K deficiency. *Nature* 1963; 200: 589-590.
12. Ganrot, P.O., Nilehn, J.E. Plasma prothrombin during treatment with dicumarol. Demonstration of an abnormal prothrombin fraction. *Scand. J. Clin. Lab. Invest.* 1968; 22: 23-28.
13. Stenflo J. Dicumarol-induced prothrombin in bovine plasma. *Acta Chem. Scand.* 1970; 24: 3762-3763.
14. Hemker, H.C., Veltcamp, J.J., Loeliger, E.A. Kinetic aspects of the interaction of blood clotting enzymes. *Thromb. Diathes. Haemorrh.* 1968; 19: 346-363.
15. Helgeland, L. The submicrosomal site for conversion of prothrombin precursor to biologically active prothrombin in rat liver. *Biochim. Biophys. Acta* 1977; 499: 181-193.
16. Suttie, J.W. & Preusch, P.C., Studies of vitamin K-dependent carboxylase and vitamin K epoxide reductase in rat liver. *Haemostasis* 1986; 16: 193-215.
17. Fasco, M.G., Hildebrandt, E.F., Suttie, J.W., Evidence that warfarin anticoagulant action involves two distinct reductase activities. *J. Biol. Chem.* 1982; 257: 11210-11212.
18. Hildebrandt, E.F., Suttie, J.W.. Mechanism of coumarin action: sensitivity of vitamin K metabolizing enzymes of normal and warfarin-resistant rat liver. *Biochemistry* 1982; 21: 2406-2411.
19. Ulrich, M.M.W., Knapen, M.H.J., Herremann-Erlee, M.P.M., Vermeer, C. Vitamin K is no antagonist for the action of warfarin in rat osteosarcoma UMR 106. *Thromb. Res.* 1988; 50: 27-32.

20. Price, P.A., Kaneda, Y. Vitamin K counteracts the effect of warfarin in liver but not in bone. *Throm. Res.* 1987; 46:121-131.
21. Price, P.A., Williamson, M.K. Effects of warfarin on bone: studies on the vitamin K-dependent bone protein of rat bone. *J. Biol. Chem.* 1981; 256: 12754-12759.
22. Suttie, J.W., Preusch, P.C. & Mc Tighe, J.J. In: *Posttranslational Covalent modifications of Proteins* (ed. Johnson, B.C.) Academic Press, New York 1983; 253-279.
23. Lian, J.B., Friedman, P.A. The vitamin K-dependent synthesis of gamma-carboxyglutamic acid by bone microsomes. *J. Biol. Chem.* 1978; 253: 6623-3326.
24. Hauschka, P.V., Friedman, P.A., Traverso, H.P., Gallop, P.M. Vitamin K-dependent gamma-carboxyglutamic acid formation by kidney microsomes in vitro *Biochem. Biophys. Res. Commun.* 1976; 71: 1207-1213.
25. de Boer-van den Berg, M.A.G., Verstijnen, C.P.M.J., Vermeer, C. Vitamin K-dependent carboxylase in skin. *J. Invest. Dermatol.* 1986; 87:377-380.
26. Buchthal, S.D., Bell, R.G., Vitamin K dependent carboxylation of the glutamic residues to gamma-carboxyglutamate in microsomes from spleen and testes: comparison with liver, lung, and kidney. *Biochemistry* 1983; 22: 1077-1082.
27. van Haarlem, L.J.M., Ulrich, M.M., Hemker, H.C., Soute, B.A.M., Vermeer, C. Isolation and partial characterization of a vitamin K-dependent carboxylase from bovine aortae. *Biochem. J.* 1987; 245: 251-255.
28. Friedrich, W. *Vitamins*. Berlin; New York: de Gruyter 1988. p. 285-338.
29. Ramotar, K., Conly, J.M., Chub, H., & Louie, T.J., J. Production of menaquinones by intestinal anaerobes. *Infectious Dis.* 1984; 150: 213-218.
30. Bentley, R. & Meganathan, R. Biosynthesis of vitamin K (menaquinone) in bacteria. *Microbiol. Rev.* 1982; 46: 241-280.
31. Dialameh, G.H., Taggart, W.V., Matschiner, J.T.&Olson, R.E. Isolation and characterisation of menaquinone-4 as a product of menadione metabolism in chicks and rats. *Int.J.Vitam.Nutr.Res.*; 1971; 41:391-400.
32. Richardson, L.R., Wilkes, S. & Ritchey, S.J. Comparative vitamin K activity of frozen, irradiated and heat-processed food. *J.Nutr.* 1962; 73:369-374.
33. Sorano, G.G., Biondi, G., Conti, M.Mameli, G., Licheri, D., Marongiu, F. Controlled vitamin K content diet for improving the management of poorly controlled anticoagulated patients: a clinical practice proposal. *Haemostasis* 1993; 23:77-82.
34. Knapen, M.H., Hamulyak, K. & Vermeer, C. The effect of vitamin K supplementation on circulating osteocalcin (bone Gla protein) and urinary calcium excretion. *Ann. Intern. Med.* 1989; 111:1001-1005.

Introduction

35. Shearer, M.J., Bach, A. & Kohlmeier, M. Chemistry, nutritional sources, tissue distribution and metabolism of vitamin K with special reference to bone health. *J. Nutr.* 1996; 126: 1181s-1186s.
36. Langenberg, J.P., Tjaden, U.R., DeVogel, E.M., & Langerak, D.I. Determination of phyloquinone (vitamin K1) in raw and processed vegetables using reversed phase HPLC with electrofluorometric detection. *Acta Alimentaria* 1986; 15: 187-198.
37. Hirauchi, K., Sakano, T., Notsumoto, S., Nagaoka, T., Morimoto, A., Fujimoto, K., Masuda, S. & Suzuki, Y. Measurement of K vitamins in foods by high-performance liquid chromatography with fluorometric detection. *Vitamins (Japan)* 1989; 63: 147-151.
38. Sumi, H., Yanagisawa, Y. and Yatagai, C. Vitamin K (Menaquinone-7) in Plasma Increased After Oral Administration of Natto Bacilli (*B. Subtilis* Natto) Abstracts for the International Society on Thrombosis and Haemostasis 1999 Annual Meeting, Washington.
39. Barnes, R.H., Fiala, G., McGehee, B. & Brown, A. Prevention of coprophagy in the rat. *J. Nutr.* 1957; 63: 489-498.
40. Mameesh, M.S. & Johnson, B.C. Dietary vitamin K requirement of the rat. *Proc. Soc. Exp. Biol. Med.* 1960; 103: 378-380.
41. Matschiner, J.T. & Bell, R.G. Effect of sex and sex hormones on plasma prothrombin and vitamin K deficiency. *Proc. Soc. Exp. Biol. Med.* 1973; 144:316-320.
42. Olson, J.A. Recommended dietary intakes (RDI) of vitamin K in humans. *Am. J. Clin. Nutr.* 1987; 45:687-692.
43. Food and Nutrition Board. Recommended Dietary Allowances. National Academy Press, Washington D.C., 10th ed. 1989.
44. Suttie, J.W., Mumma-Schendel, L.L., Shah, D.V., Lyle, B.J., Greger, J.L. Vitamin K deficiency from dietary vitamin K restriction in humans. *Am.J.Clin.Nutr.* 1988; 47: 475-480.
45. Ferland, G., Sadowski, J.A. & O'Brien, M.E. Dietary induced subclinical vitamin K deficiency in normal human subjects. *J. Clin. Invest.* 1993; 91: 1761-1768.
46. Sadowski, J.A., Hood, S.J., Dallal, G.E., Garry, P.J., Phyloquinone in plasma from elderly and young adults: factors influencing its concentration. *Am. J. Clin. Nutr.* 1989; 50: 100-108.
47. Saupe, J., Shearer, M.J., Kohlmeier, M. Phyloquinone transport and its influence on γ -carboxyglutamate residues of osteocalcin in patients on maintenance hemodialysis. *Am. J. Clin. Nutr.* 1993; 58: 204-208.
48. Booth, S.L., Tucker, K.L., McKeown, N.M., Davidson, K.W., Dallal, G.E & Sadowski, J.A. Relationships between dietary intakes and fasting plasma concentrations of fat-soluble vitamins in humans. *J. Nutr.* 1997; 127: 587-592.

49. Booth, S.L., Sokoll, L.J., O'Brien, M.E., Tucker, K., Dawson-Hughes, B. & Sadowski, J.A. Assessment of dietary phyloquinone intake and vitamin K status in postmenopausal women. *Eur. J. Clin. Nutr.* 1995; 49: 832-841.
50. Shearer, M.J., McBurney, A. & Barkhan, P. Studies on the absorption and metabolism of phyloquinone (vitamin K1) in man. *Vitam. Horm.* 1974; 32: 513-542.
51. Savage, D., Lindenbaum, J. Clinical and experimental human vitamin K deficiency. *Nutrition in Haematology*. Churchill Livingstone, New York 1983, pp. 435-447.
52. Akiyama, Y., Hara, K., Matsumoto, A., Takahashi, S., & Tajima, T. Comparison of intestinal absorption of vitamin K2 (menaquinone) homologues and their effects on blood coagulation in rats with hypoprothrombinaemia. *Biochem. Pharmacol.* 1995; 49: 1801-1807.
53. Gijssbers, B.L.M.G., Jie, K-S. G. & Vermeer, C. Effect of food composition on vitamin K absorption in human volunteers. *J. Nutr.* 1996; 76: 223-229.
54. Shearer, M.J., Barkhan, P., & Webster, G.R. Absorption and excretion of an oral dose of tritiated vitamin K1 in man. *Br. J. Haematol.* 1970; 18: 297-308.
55. Hagstrom, J.N., Bovill, E.G., Soll, R.F., Davidson, K.W., & Sadowski, J.A. The pharmacokinetics and lipoprotein fraction distribution of intramuscular vs. oral vitamin K1 supplementation in women of childbearing age: effect on hemostasis. *Thromb. Haemost.* 1995; 74:1486-1490.
56. Siest, G., Pillot, T., Régis-Bailly, A., Leininger-Muller, B., Steinmetz, J., Galteau, M.-M. & Sophia Visvikis Apolipoprotein E: An important gene and protein to follow in laboratory medicine, *Clin. Chem.*, 1995; 41(8): 1068-1086.
57. Davignon, J. Apolipoprotein E polymorphism and atherosclerosis. In: *New horizons in coronary heart disease*. Current Science 1993; 5: 1-21.
58. Shiraki, M., Akiguchi, I., Itoh, H., Kaneko, T., Matsuo, M. Vitamin K in the aged with special reference to the clinical significance of the urinary gamma-carboxy glutamic acid content as a new indicator for vitamin K status. *Nippon Ronen Igakkai Zasshi*, 1983; 20: 128-132.
59. Kohlmeier, M., Saupe, J., Drossel, H.J. & Shearer, M.J. Variation of phyloquinone (vitamin K1) concentrations in hemodialysis patients. *Thromb. Haemost.* 1995; 74(5): 1252-1254.
60. Konishi, T., Baba, S. & Sone, H. Whole-body autoradiographic study of vitamin K distribution in rat. *Chem. Pharm. Bull. Tokyo* 1973; 21: 220-224.
61. Uchida, K., Komero, T. Relationship between dietary and intestinal vitamin K, clotting factors levels, plasma vitamin K, and urinary Gla. In: *Current advances in vitamin K research*. Suttie J.W., Ed. Elsevier, New York 1988; 477-492.

Introduction

62. Shearer, M.J., McCarthy, P.T., Crampton, O.E., Mattock, M.B. The assessment of human vitamin K-status from tissue measurements. In: *Current advances in vitamin K research*. Suttie J.W., Ed. Elsevier, New York 1988; 437-452.
63. Usui, Y., Tanimura, H., Nishimura, N., Kobayashi, N., Okanoue, T. & Ozawa, K. Vitamin K concentrations in the plasma and liver of surgical patients. *Am. J. Clin. Nutr.* 1990; 51: 846-852.
64. Hodges, S.J., Pilkington, M.J., Shearer, M.J., Bitenski, L. & Chayen, J. Age-related changes in the circulating levels of congeners of vitamin K2, menaquinone-7 and menaquinone-8. *Clin. Sci. Colch.* 1990; 78: 63-66.
65. Thijssen, H.H.W. & Drittij Reijnders, M.J. Vitamin K distribution in rat tissues: dietary phylloquinone is a source of tissue menaquinone-4. *Br. J. Nutr.* 1994; 72: 415-425.
66. Thijssen, H.H.W., Drittij-Reijnders, M.J. & Fischer, M.A. Phylloquinone and menaquinone-4 distribution in rats: synthesis rather than uptake determines menaquinone-4 organ concentrations. *J. Nutr.* 1996; 126: 537-543.
67. Suttie, J.W. In: *Handbook of Vitamins*. Dekker, New York; Machlin, p. 147.
68. Shearer, M.J., Barkhan, P. Studies of the metabolites of phylloquinone (vitamin K1) in the urine of man. *Biochim. Biophys. Acta* 1973; 297:300-312.
69. Deutsche Gesellschaft für Ernährung. Empfehlungen für die Nährstoffzufuhr 4. Überarbeitung (Umschau Verlag, Frankfurt/Main).
70. Friedman, P.A. Vitamin K dependent proteins. *N. Engl. J. Med.* 1984; 310: 1458-1460.
71. Widdershoven, J., Munster van P., De Abreu, R., et. al. Four methods compared for measuring des-carboxy-prothrombin (PIVKA-II). *Clin. Chem* 1987; 33: 2074-2078.
72. von Kries, R., Greer, F.R., Suttie, J.W. Assessment of vitamin K status of the newborn infant. *J. Pediatr. Gastroen. Nutr.* 1993; 16:232-238.
73. Ferland, G., Sadowski, J.A., O'Brien, M.E. Dietary induced subclinical vitamin K deficiency in normal human subjects. *J. Clin. Invest.* 1993; 9: 1761-1768.
74. Blanchard, R.A., Furie, B.C., Jorgensen, M., Kruger, S.F., Furie, B. Acquired vitamin K-dependent carboxylation deficiency in liver disease. *New Eng. J. Med.* 1981; 305: 242-248.
75. Okuda, H., Nakanishi, T., Furukawa, M., Obata, H. Vitamin K-dependent proteins and hepatocellular carcinoma. *J. Gastroent. Hep.* 1991; 6: 392-399.
76. Schubiger, G., Gruter, J., Shearer, M.J. Plasma vitamin K1 and PIVKA-II after oral administration of mixed-micellar or cremophor EL-solubilized preparations of vitamin K1 to normal breast-fed newborns. *J. Pediatr. Gastroenterol. Nutr.* 1997 Mar; 24(3): 280-284.

77. Hauschka, P.V., Lian, J.B., Gallop, P.M. Direct identification of the calcium-binding amino acid, γ -carboxyglutamate, in mineralized tissue. *Proc. Nat. Acad. Sci* 1975; 72: 3925-3929.
78. Price, P.A., Otsuka, A.S., Poser, J.W., Kristaponis, J., Raman, N. Characterization of a γ -carboxyglutamic acid containing protein from bone. *Proc. Natl. Acad. Sci USA* 1976; 73: 1447-1451.
79. Szulc, P., Chapuy, M.C., Meunier, P.J., and Delmas, P. Serum undercarboxylated osteocalcin is a marker of the risk of hip fracture in elderly women. *J. Clin. Invest.* 1993; 91: 1769-1774.
80. Cairns, J.R., Price, P.A. Direct demonstration that the vitamin K dependent bone Gla protein is incompletely γ -carboxylated in humans. *J. Bone Miner. Research*, 1994; 9(12): 1989-1997.
81. Nakao, M., Nishiuchi, Y., Nakata, M., Kimura, T., Sakakibara, S. Synthesis of human osteocalcins: γ -carboxyglutamic acid at position 17 is essential for a calcium - dependent conformational transition. *Peptide Research*, 1994; 7(4): 171-174.
82. Plantalech, L.C., Chapuy, M.C., Guillaumont, M., Chapuy, P., Leclercq, M., Delmas, P. Impaired carboxylation of serum osteocalcin in elderly women: effect of vitamin K1 treatment. In *Osteoporosis 1990*, ed. Christiansen, K., Overgaard, Copenhagen: Aps. p. 345-347.
83. Plantalech, L., Guillaumont, M., Vergnaud, P., Leclercq, M., Delmas, P.D. Impairment of gamma carboxylation of circulating osteocalcin (bone Gla protein) in elderly women. *J. Bone Miner. Res.* 6: 1211-1216.
84. Jie, K.-S.G., Hamulyák, K., Gijssbers, B.L.M.G., Roumen, F.J.M.E., Vermeer, C. Serum osteocalcin as a marker for vitamin K-status in pregnant women and their newborn babies. *Thromb. Haemost.* 1992; 68: 388-391.
85. Price, P.A. Vitamin K nutrition and postmenopausal osteoporosis (editorial). *J. Clin. Invest.* 1993; 91: 1268-1273.
86. Townsend, C.W.: The haemorrhagic disease of the newborn. *Arch. Pediatr* 1894; 11: 559-565.
87. Nagao, T. & Iizuka, A. Idiopathic vitamin K deficiency in infancy: its entity and enigmas. *Acta Haematol. Jpn.* 1982; 45: 849-859.
88. Sutor, A.H., Kunzer, W. Time interval between vitamin K administration and effective homeostasis. In: Suzuki S., Hataway, W.E, Bonnar, J., Sutor, A.H. (Eds). *Perinatal Thrombosis and Haemostasis* 1991. Springer. Berlin, pp 257-262.
89. Shearer, M.J., Rahim, S., Barkhan, P., Stimmier, L. Plasma vitamin K1 in mothers and their newborn babies. *Lancet.* 1988; ii: 460-463.
90. Lane, P.A. & Hathaway, W.E. Vitamin K in infancy. *J. Pediatr* 1985; 106:351-359.

Introduction

91. von Kries, R., Shearer, M.J. & Gobel, U. Vitamin K in infancy. *Eur. J. Pediatr.* 1988; 147: 106-112.
92. Greer, F.R., Marshall, S., Cherry, J. & Suttie, J.W. Vitamin K status of lactating mother, human milk, and breast-feeding infants. *Pediatrics* 1991; 88: 751-756.
93. Moslet, U. & Hansen, E.S. A review of vitamin K, epilepsy and pregnancy. *Acta Neurol. Scand.* 1992; 85: 39-43.
94. Cornelissen, M., Steegers-Theunissen, R., Kollee, L., Eskes, T., Vogels-Mentink, G., Motohara, K., De Abreu, R. & Monnens, L. Increased incidence of neonatal vitamin K deficiency resulting from maternal anticonvulsant therapy. *Am. J. Obstet. Gynecol.* 1993; 168: 923-928.
95. Benno, Y., Sawada, K. & Mitsuoka, T. The intestinal microflora of infants: fecal flora of infants with vitamin K deficiency. *Microbiol. Immunol.* 1985; 29: 243-250.
96. Sutor, A.H., Dagres, N., Niederhoff, H., Late form of vitamin K deficiency bleeding in Germany. *Klin. Pädiatrie* 1995; 207: 89-97.
97. von Kries, R., Wirtz, P., Göbel, U. Krebs durch Vitamin-K-Prophylaxe? *Dtsch Ärzteblatt*, 1993; 90: 36-40.
98. McNinch, A.W., Tripp, J.H. Haemorrhagic disease of the newborn in the British Isles: two-year prospective study. *BMJ* 1991; 303: 1105-1109.
99. Lehman, J. Vitamin K as prophylaxis in 13,000 infants. *Lancet* 1994; 493-494.
100. American Academy of Pediatrics: Committee on Nutrition. Vitamin K compounds and water soluble analogues: use in therapy and prophylaxis in pediatrics. *Pediatrics*, 1961; 28: 501-507.
101. Tripp, J.H., McNinch, A.W. Haemorrhagic disease and vitamin K. *Arch. Dis. Child.* 1987; 62: 436-437.
102. Handel, J., Tripp, J.H. Vitamin K prophylaxis against haemorrhagic disease of the newborn in the United Kingdom. *BMJ* 1991; 303: 1109-1112.
103. Golding, J.R., Greenwood, R., Birmingham, K., Mott, M. Childhood cancer, intramuscular vitamin K, and pethidine given during labour. *BMJ*, 1992; 305:341-346.
104. Golding, G., Paterson, M., Inlen, I.J. Factors associated with childhood cancer in a national cohort study. *Br. J. Cancer* 1990; 62: 304-308.
105. Ekelund, H., Finnström, O., Gunnarskog, J., Källén, B., Larson, Y. Administration of vitamin K to newborn infants and childhood cancer. *BMJ* 1993; 307: 89-91.
106. Ekelund, H.: Late haemorrhagic disease in Sweden 1987-1989. *Acta Paedr. Scand.* 1991; 80: 966-968.
107. von Kries, R., Göbel, U.: Vitamin K prophylaxis and late hemorrhagic disease of the newborn. *Acta Paediatr. Scand.* 1992; 81: 655-657.

108. Kurinezuk, J.J., Henderson-Smart, D.J., Stanley, F.J. Vitamin K and childhood cancer: A dilemma in practice. *J. Paediatr. Child. Health* 1993; 29: 169-170.
109. Schubiger, G., Tönz, O., Grüter, J., Shearer, M.J. Vitamin K concentration in breastfed neonates after oral or intramuscular administration of a single dose of a new mixed micellar preparation of phylloquinone. *J. Pediatr. Gastroenterol. Nutr.* 1993; 16: 435-439.
110. Cornelissen, E.A., Kollee, L.A., De Abreu, R.A., Motohara, K. & Monnens, L.A. Prevention of vitamin K deficiency in infancy by weekly administration of vitamin K. *Acta. Paedr.* 1993; 82: 656-659.
111. Cornelissen, E.A., Kolle, L.A., van Lith, T.G., Motohara, K. & Monnens, L.A. Evaluation of a daily dose of 25 micrograms vitamin K1 to prevent vitamin K deficiency in breast-fed infants. *J. Pediatr. Gastroenterol. Nutr.* 1993; 16: 301-305.
112. Alarcon, O.M., Reinosa, F.A., Silva, L., Padron, F. Changes of serum lipids in vitamin K3 (menadione) treated rats. *Arch. Latinoam. Nutr.* 1995; 45: 286-289.
113. Briggs, M., *Vitamins in Human Biology and Medicine*. CRC Press, Boca Raton, Florida.
114. Robinson, W., Odom, R.B. Delayed cutaneous reaction to phytomenadione. *Arch. Dermatol.* 1978; 114: 1790-1792.
115. Morell, A., Betlloch, I., Sevilla, A., Baáulus, J., Botella, R. Morphea-like reaction from vitamin K1. *Int. Journal of Dermatology*; 1995; 34 (3): 201-202.
116. Martin, T.J. Bailliere's Metabolic Bone Disease. In: *Clinical endocrinology and metabolism. International practice and research* 1988; 2 (1): 1-29.
117. Oursler, M.J., Pyfferoen, J., Osdoby, P. et. al: *J. Bone Miner. Res.* 1991; 6(Suppl): 203-207.
118. McSheehy, P.M., Chambers, T.J. Osteoblastic cells mediate osteoclastic responsiveness to parathyroid hormone. *Endocrinology* 1986; 118: 824-828.
119. Rodan, G.A., Martin, T.J. Role of osteoblasts in hormonal control of bone resorption - a hypothesis. *Calcif. Tissue Int.* 1981; 33: 349-351.
120. Epstein, S., Bryce, G., Hinman, J.W., Miller, O.N., Riggs, B.L., Hul, S.L., Johnston, C.C. The influence of age on bone mineral regulating hormones. *Bone* 1986; 7: 421-425.
121. Fatayerji, D., Eastell, R. Age-related changes in bone turnover in men. *J. Bone and Min. Res.* 1999; 14: 1203-1210.
122. Eastell, R., Yergey, A., Vieira, N.E., Cedel, S.L., Kumar, R., Riggs, B.L. Interrelationship among vitamin D metabolism, true calcium absorption, parathyroid function, and age in women: evidence of an age-related intestinal resistance to 1,25-dihydroxyvitamin D action. *J. Bone and Miner. Res.* 1991; 6: 125-132.

123. Cohen, F.J., Eckert, S., Mitlak, B.H. Geographic differences in bone turnover: data from a multinational study in healthy postmenopausal women. *Calcif. Tissue Int.* 1998; 63: 277-282.
124. Anderson, J.J.B., Tylavski, F.A., Lacey, J.M., Adeleke, V.M. Ethnicity, Nutrition, and Bone Mass, Challenges of Modern Medicine; Nutritional aspects of osteoporosis '94, Editors: P. Burkhardt, R.P. Heaney, Ares-Serono Symposia Publications 1994; 7: 27-44.
125. Lau, E.M.C. Osteoporosis in Asians - The role of calcium and other nutrients. In: Nutritional aspects of osteoporosis '94, Editors: P. Burkhardt, R.P. Heaney, Ares-Serono Symposia Publications 1994; 7:45-54.
126. Uusi-Rasi, K., Heinonen, A., Oja, P., Pasanen, M., Sievänen, H., Vuori, I. Calcium intake and bone mineral density in perimenopausal women. Nutritional aspects of osteoporosis '94, Editors: P. Burkhardt, R.P. Heaney, Ares-Serono Symposia Publications 1994; 7: 77-80.
127. Matkovic, V., Ilich, J.Z., Skugor, M. Calcium intake and skeletal formation. Nutritional aspects of osteoporosis '94, Editors: P. Burkhardt, R.P. Heaney, Ares-Serono Symposia Publications 1994; 7: 129-146.
128. Faisant, C., Lauque, S., Stebenet, Rouillon, M., Vellas, B., Albarède, J.L. Calcium and Phosphorus Intake in different groups of elderly persons with good or poor nutritional status. In: Nutritional aspects of osteoporosis '94, Editors: P. Burkhardt, R.P. Heaney, Ares-Serono Symposia Publications 1994; 7: 209-212.
129. Scharla, S.H., Scheidt-Nave, C., Leidig, G., Seibel, M., Ziegler, R. Association between serum 25-hydroxyvitamin D and bone mineral density in a normal population sample in Germany. In: Nutritional aspects of osteoporosis '94, Editors: P. Burkhardt, R.P. Heaney, Ares-Serono Symposia Publications 1994; 7: 325-328.
130. Nieves, J., Cosman, F., Herbert, J., Shen, V., Lindsay, R. High prevalence of vitamin D deficiency and reduced bone mass in multiple sclerosis. In: Nutritional aspects of osteoporosis '94, Editors: P. Burkhardt, R.P. Heaney, Ares-Serono Symposia Publications 1994; 7: 329-334.
131. Ooms, M.E., Lips, P., Roos, J.C., van-der-Vijgh, W.J., Popp-Snijders, C., Bezemer, P.D, Bouter, L.M. Vitamin D status and sex hormone binding globulin: determinants of bone turnover and bone mineral density in elderly women. *J. Bone Miner. Res.* 1995; 10(8): 1177-1784.
132. Lukert, B., Higgins, J., Stoskopf, M. Menopausal bone loss is partially regulated by dietary intake of vitamin D. *Calcif. Tissue Int.* 1992; 51(3): 173-179.
133. Feskanich, D., Weber, P., Willet, W.C., Rockett, H., Booth, S., Colditz, G.A. Vitamin K intake and hip fractures in women: a prospective study. *Am. J. Clin. Nutr.* 1999; 69: 74-79.

134. New, S.A., Robins, S.P., Garton, M.J., Martin, J.C., Bolton-Smith, C., Grubb, D.A., Reid, D.M. The influence of factors (dietary & non-dietary) upon bone mineral density and bone metabolism in premenopausal women. In: *Nutritional aspects of osteoporosis '94*, Editors: P. Burkhardt, R.P. Heaney, Ares-Serono Symposia Publications 1994; 7: 73-76.
135. Paunier, L. Effect of magnesium on phosphorus and calcium metabolism. *Monatsschr. Kinderheilkd.* 1992; 140: 17-20.
136. Holtrop, M.E., Raisz, L.G., Simmons, H.A. The effects of parathyroid hormone, colchicine, and calcitonin on the ultrastructure and the activity of osteoclasts in organ culture. *J. Cell Biol.* 1974; 60: 346-355.
137. Rodriguez, M., Felsenfeld, A.J., Torres, A., Pederson, L., Llach, F. Calcitonin, an important factor in the calcemic response to parathyroid hormone in the rat. *Kidney Int.* 1991; 40: 219-225.
138. Oursler, M.J., Landers, J.P., Riggs, B.L., Spelsberg, T.C. Oestrogen effects on osteoblasts and osteoclasts. *Ann. Med.* 1993; 25(4): 361-371.
139. Gay, C.V., Kief, N.L., Bekker, P.J. Effect of estrogen on acidification in osteoclasts. *Biochem. Biophys. Res. Commun.* 1993; 192(3): 1251-1259.
140. Liel, Y., Kraus, S., Levy, J., Shany, S. Evidence that estrogens modulate activity and increase the number of 1,25 dihydroxyvitamin D receptors in osteoblast-like cells. *Endocrinology* 1992; 130 (5): 2597-2601.
141. Ohta, H., Ikeda, T., Masuzawa, T., Makita, K., Suda, Y., Nozawa, S. Differences in axial bone mineral density, serum levels of sex steroids, and bone metabolism between postmenopausal and age-and body size-matched premenopausal subjects. *Bone* 1993; 14(2): 111-116.
142. Garnero, P., Sornay-Rendu, E., Delmas, D. Decreased bone turnover in oral contraceptive users. *Bone* 1995; 16: 499-503.
143. Kawana, K., Kushida, K., Takahashi, M., Ohishi, T., Denda, M., Yamazaki, K., Inoue, T. The effect of menopause on biochemical markers and ultrasound densitometry in healthy females. *Calcif. Tissue Int.* 1994; 55: 420-425.
144. Reid, I.R., France, J.T., Pybus, J., Ibbertson, H.K. Low plasma testosterone levels in glucocorticoid treated male asthmatics. *BMJ* 1985; 291: 574-579.
145. Reid, I.R., Veale, A., France, J.T. Glucocorticoid osteoporosis, *Journal of Asthma* 1994; 31(1): 7-18.
146. Cosman, F., Nieves, J., Herbert, J., Shen, V., Lindsay, R. High-dose glucocorticoids in multiple sclerosis patients exert direct effects on the kidney and skeleton. *J. Bone and Miner. Res.* 1994; 9(7): 1097-1105.
147. Mundy, G.R. Calcium homeostasis; hypercalcemia and hypocalcemia. Martin Dunitz, London 1989; 64-91.

Introduction

148. Gossain VV, Rao DS, Carella MJ, Divine G, Rovner DR. Bone mineral density (BMD) in obesity effect of weight loss. *J. Med.* 1999; 30(5-6):367-376.
149. Lehtonen-Veromaa, M., Mottonen, T., Nuotio, I., Heinonen, O.J., Viikari, J. Influence of physical activity on ultrasound and dual-energy X-ray absorptiometry bone measurements in peripubertal girls: a cross-sectional study. *Calcif. Tissue Int.* 2000; 66(4):248-254.
150. Bennell, K.L., Malcom, S.A., Brukner, P.D., Green, R.M., Hopper, J.L., Wark, J.D., Ebeling, P.R. A 12-month prospective study of the relationship between stress fractures and bone turnover in athletes. *Calcif. Tissue Int.* 1998; 63: 80-85.
151. Keizer, H.A., Rogol, A.D. Physical exercise and menstrual cycle alterations. What are the mechanisms? *Sports Med.* 1990, 10 (4): 218-235.
152. Calvo, M.S., Eyre, D.R., Gundberg, C.M. Molecular basis and clinical application of biological markers of bone turnover. *Endocrine Reviews* 1996, 17 (4): 333-368.
153. Cheung, C.K., Panesar, N.S., Haines, C., Masarei, J., Swaminathan, R. Immunoassay of a tartrate-resistant acid phosphatase in serum. *Clin. Chem.* 1995; 41: 679-686.
154. Price, P.A., Urist, M.R., Otawara, Y. Matrix Gla protein, a new gammacarboxyglutamic acid-containing protein which is associated with the organic matrix of bone. *Biochem. Biophys. Res. Commun.* 1983; 117: 765-771.
155. Maillard, C., Berruyer, M., Serre, C.M., Dechavanne, M., Delmas, P.D. Protein S, a vitamin K dependent protein, is a bone matrix component synthesized and secreted by osteoblasts. *Endocrinology* 1992; 130: 1599-1604.
156. Hauschka, P.V., Lian, J.B., Cole, D.E.C., Gundberg, C.M. Osteocalcin and matrix Gla protein: vitamin K dependent proteins in bone. *Phys. Rev.* 1989; 69: 990-1047.
157. Furie, B., Furie, B.C. Molecular basis of vitamin K-dependent γ -carboxylation. *Blood* 1990; 75:1753-1762.
158. Fair, D.S., Marlar, R.A. Biosynthesis and secretion of factor VII, protein C, protein S and the protein inhibitor by a human hepatoma cell line. *Blood* 1986; 67: 64-70.
159. Fair, D.S., Marlar, R.A., Levin, E.G. Human endothelial cells synthesize protein S. *Blood* 1986; 67: 1168-1171.
160. Price, P.A., Williamson, M.K. Primary structure of bovine matrix Gla protein, a new vitamin K-dependent bone protein. *J. Biol. Chem.* 1985; 260: 14971-14975.
161. Hauschka, P.V., Carr, S.A. Calcium-dependent α -helical structure in osteocalcin. *Biochemistry* 1982; 21: 2538-2547.
162. Chenu, C., Colucci, S., Grano, M., Zigrino, P., Barattolo, R., Zamboni, G., Baldini, N., Vergnaud, P., Delmas, P.D., Zallone, A.Z. Osteocalcin induces chemotaxis, secretion of matrix proteins, and calcium mediated intracellular signaling in human osteoclast-like cells. *The J. of Cell Biol.* 1994; 127(4): 1149-1158.

163. Partridge, N.C., Bloch, S.R., Pearman, A.T. Signal transduction pathways mediating parathyroid hormone regulation of osteoblastic gene expression. *J. of Cell Biochem.* 1994; 55: 321-327.
164. Ducy, P., Desbois, C., Boyce, B., Pinero, G., Story, B., Dunstan, C., Smith, E., Bonadio, J., Goldstein, S., Gundberg, C., Bradley, A., Karsenty, G. Increased bone formation in osteocalcin-deficient mice. *Nature* 1996; 382: 448-452.
165. Price, P.A., Baukol, S.A. 1,25-dihydroxyvitamin D3 increases synthesis of the vitamin K-dependent bone protein by osteosarcoma cells. *J. Biol. Chem.* 1980; 255: 11660-11667.
166. Kruse, K., Kracht, U. Evaluation of serum osteocalcin as an index of altered bone metabolism. *Eur J Pediatr* 1986; 145: 27-33.
167. Price, P.A., Williamson, M.K., Lothringer, J.W. Origin of the vitamin K-dependent bone protein found in plasma and its clearance by kidney and bone. *J. Biol. Chem.* 1981; 256: 12760-12766.
168. Farrugia, W., Melik, R.A. Metabolism of osteocalcin. *Calcif. Tissue Int.* 1986; 39: 234-238.
169. Gundberg, C. Gamma carboxyglutamic acid and serum osteocalcin and their use in the clinical situation as markers of bone disease. In: *Vitamin K and vitamin K-dependent proteins*; CRC Press, Inc. 1993; 295-328.
170. Resch, H., Pietschmann, P., Kudlacek, S., Woloszczuk, Krexner, E., Bernecker, P., Willvonseder, R. Influence of sex and age on biochemical bone metabolism parameters. *Miner. Electrolyte Metab.* 1994; 20: 117-121.
171. Saupe, J., Shearer, M.J., Kohlmeier, M. Phylloquinone transport and its influence on gamma-carboxyglutamate residues of osteocalcin in patients on maintenance hemodialysis. *Am. J. Clin. Nutr.* 1993; 58(2): 204-208.
172. Baran, D. Osteoporosis: monitoring techniques and alternate therapies. *Calcitonin, fluoride, bisphosphonates, vitamin D. Primary Care of the Mature Women* 1994; 21(2): 321-334.
173. Menon, R.K., Gill, D.S., Thomas, M., Kernoff, P.B., Dandona, P. Impaired carboxylation of osteocalcin in warfarin-treated patients. *J. Clin. Endocrinol. Metab.* 1987; 64: 59-61.
174. Nielsen, H.K., Brixen, K., Bouillon, R. Changes in biochemical markers of osteoblastic activity during the menstrual cycle. *J. of Clin. Endocr. and Metab.* 1990; 70(5): 1431-1437.
175. Moreno, J.M.L., Gonzalez, G.V., Campino, C.J., Salvatierra, A.M., Croxatto, H.B. Serum osteocalcin in normal menstrual cycle. *Medicina* 1992; 52: 37-40.
176. Namgung, R., Tsang, R.C., Specker, B.L., Sierra, R.I., Ho, M.L. Low bone mineral content and high serum osteocalcin and 1,25-dihydroxyvitamin D in summer versus

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winter-born infants: an early fetal effect? *J. of Pediatric Gastroenterology and Nutrition* 1994; 19: 220-227.

177. Eastell, R., Simmons, P.S., Colwell, A., Assiri, A.M.A., Burritt, M.F., Russel, G.G., Riggs, B.L. Nyctohemeral changes in bone turnover assessed by serum bone Gla-protein concentration and urinary deoxypyridinoline excretion: effects of growth and ageing. *Clinical Science* 1992; 83: 375-382.
178. Price, P.A., Nishimoto, S.K. RIA for the vitamin K-dependent protein of bone and its discovery in plasma. *Proc. Natl. Acad. Sci USA* 1980; 77: 2234-2238.
179. Kao, P.C., Riggs, B.L., Schryver, P.G. Development and evaluation of an osteocalcin chemiluminoimmunoassay. *Clin. Chem.* 1993; 39: 1369-1374.
180. Gamaro, P., Grimeaux, M., Demiaux, B., Preaudat, C., Seguin, P., Delmas, P.D. Measurement of serum osteocalcin with a human specific two-site immunoradiometric assay. *J. Bone Min. Res.* 1992; 12: 1389-1398.
181. Hosoda, K., Egushi, H., Nakamoto, T., et. al. Sandwich immunoassay for intact human osteocalcin. *Clin. Chem.* 1992; 38: 2233-2238.
182. Power, M.J., Fottrell, P.F. Osteocalcin: diagnostic methods and clinical applications. *Crit. Rev. Clin. Lab. Sci* 1991; 2: 327-335.
183. Delmas, P.D., Christiansen, C., Mann, K.G., Price, P.A. Bone Gla Protein (Osteocalcin) assay standardization report. *J. Bone Miner. Res.* 1990; 5: 5-11.
184. Masters, P.W., Jones, R.G., Purves, D.A., Cooper, E.H., Cooney, J.M. Commercial assays for serum osteocalcin give clinically discordant results. *Clin. Chem.* 1994; 40: 358-363.
185. Hale, J.E., Fraser, J.D., Price, P.A. The identification of matrix Gla-protein in cartilage. *J. Biol. Chem.* 1988; 266: 5820-5824.
186. Fraser, J.D., Price, P.A. Lung, heart, and kidney express high levels of mRNA for the vitamin K-dependent matrix Gla-protein. Implications for the possible functions of matrix Gla-protein and for the possible distribution of the gamma carboxylase. *J. Biol. Chem.* 1988; 263: 11033-11036.
187. Otawara, Y., Price, P.A. Developmental appearance of matrix Gla protein during calcification in the rat. *J. Biol. Chem.* 1986; 261: 10828-10832.
188. Fraser, J.D., Otawara, Y., Price, P.A. 1,25-Dihydroxyvitamin D3 stimulates the synthesis of Matrix γ -carboxyglutamic acid Protein by osteosarcoma cells. *J. Biol. Chem.* 1988; 263: 911-916.
189. Cancela, M.L., Price, P.A. Retinoic acid induces matrix Gla protein gene expression in human cells. *Endocrinology* 1992; 130: 102-108.
190. Luo, G. et al. Spontaneous calcification of arteries and cartilage in mice lacking matrix Gla protein. *Nature* 1997; 385: 78-81.

191. Hall, J.G., Pauli, R.M., Wilson, K.M. Maternal and fetal sequelae of anticoagulation during pregnancy. *Am. J. Med.* 1980; 68: 122-140.
192. Harrold, M.G., Sherrod, P.S. Warfarin embryopathy in siblings. *Obstetric. Gynecol.*, 1981; 57: 673-676.
193. Price, P.A., Faus, S.A., Williamson, M.K. Warfarin causes rapid calcification of the elastic lamellae in rat arteries and heart valves. *Arterioscler. Thromb. Vasc. Biol.* 1998; 18: 1400-1407.
194. Dahlbäck, B., Lundwall, A., Stenflo, J. Localization of thrombin cleavage sites in the amino-terminal region of bovine protein S. *J.Biol.Chem.* 1986; 261: 5111-5115.
195. Hessing, M. The interaction between complement component C4b-binding protein and the vitamin K-dependent protein S forms a link between blood coagulation and the complement system. *Biochem. J.* 1991; 277: 581-592.
196. Pan, E.Y., Gomperts, E.D., Millen, R., Gilsanz, V. Bone mineral density and its association with inherited protein S deficiency. *Thromb. Res.* 1990; 58: 221-231.
197. Maillard, C., Berruyer, M., Serre, C.M., Dechavanne, M., Delmas, P.D. Protein S, a vitamin K-dependent protein, is a bone matrix component synthesized and secreted by osteoblasts. *Endocrinology* 1992; 130(3): 1599-1604.
198. Shah, D.V., Tews, J.K., Harper, A.E. and Suttie, J.W. Metabolism and transport of gamma-carboxyglutamic acid. *Biochem. Biophys. Acta* 1978; 539: 209-217.

CHAPTER 2

Bio-availability of orally ingested vitamins K in vitamin K-deficient rats as compared to brodifacoum treated rats

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Summary

Vitamin K is a group name for a number of prenylated 2-methyl-1,4-naphthoquinones, which may differ in their ability to function as a cofactor for prothrombin biosynthesis. To quantify the bioactivity of different forms of vitamin K, two experimental animal systems are frequently used: vitamin K-deficient rats and anticoagulated rats. In this paper both models are compared, and it is shown that the results obtained depend on the model used. The main reason for this discrepancy is the difference in recycling of vitamin K-epoxide, which results in a 500 times higher vitamin K requirement in anticoagulated rats. Absorption and hepatic accumulation of long chain menaquinones seem to be restricted to a maximum, whereas the lipophilic nature of long chain menaquinones may also hamper the quinone-quinol reduction in anticoagulated animals. If these data were extrapolated to patients, food items rich in K₁ and MK-4 could be expected to influence the stability of oral anticoagulation to a much larger extent than food items primarily containing higher menaquinones.

Introduction

Vitamin K functions as a cofactor for the posttranslational enzyme γ -glutamylcarboxylase, which is involved in the biosynthesis of a number of blood coagulation factors¹⁻³. The result of vitamin K action is the conversion of a glutamate into a γ -carboxyglutamate (Gla) residue. Normal prothrombin contains 10 Gla-residues, which are essential for its procoagulant activity⁴. In this paper "vitamin K-activity" is defined as the ability to promote prothrombin biosynthesis in the rat model system used. By the action of a second enzyme, vitamin K 2,3 epoxide reductase (KO-reductase), vitamin K is recycled and extensively re-utilized⁵. Various forms of vitamin K are known, which all have a naphthoquinone group in common, but which differ according to the length and degree of saturation of their aliphatic side chain. In phylloquinone (vitamin K₁) the side chain is invariably composed of 4 isoprenoid residues, one of which is unsaturated. Because in natural menaquinones (the K₂-vitamins) the length of this side chain may vary from 4 to 13 isoprene residues, their lipophilicity may vary as well. These large structural differences may result in differences regarding intestinal absorption, transport, hepatic metabolism and utilization as a cofactor for carboxylase⁶⁻⁸.

To quantify the bioactivity of different forms of vitamin K, and the effect of the food matrix on its intestinal absorption, two experimental animal models are generally used: vitamin K-deficient rats and anticoagulated rats (i.e. rats treated with vitamin K-antagonists like warfarin or brodifacoum). In the former case Gla-formation comes to

a halt because the endogenous supply of vitamin K is exhausted, which results in very low circulating prothrombin levels. After oral administration of a single dose of vitamin K, the latter is recycled maximally, and thus used optimally. Hence small dosages of vitamin K result in substantial effects on the level of circulating prothrombin^{9,10}. In the anticoagulated model, the recycling is blocked by the irreversible inhibition of KO-reductase. After absorption, each vitamin K molecule can be used only once so that the endogenous supply of vitamin K is rapidly exhausted, and very high doses are needed to ensure the normal carboxylation of coagulation factors¹¹. From a technical point of view, it is much more simple to anticoagulate rats than to make them vitamin K-deficient. Therefore, the efficacy and disposition of K-vitamins is often tested in the anticoagulated model^{7,9}. Sometimes the interpretation of the data is even extrapolated to human vitamin K utilization. In earlier studies we used the vitamin K-deficient model, and we observed that the stimulation of prothrombin synthesis was much higher for menaquinone-9 than for phyloquinone, and lowest for menaquinone-4^{9,10}. Using the anticoagulated model, contradictory results were published by Akiyama et al., who demonstrated that the potency of MK-4 to counteract hypoprothrombinemia was much higher than that of MK-9¹². Therefore, we compared both rat model systems, and we demonstrate that they differ according to vitamin K disposition.

Materials and Methods

Chemicals

Phylloquinone (K₁) and menaquinone-4 (MK-4) were obtained from Sigma (St.Louis,MO). MK-9 and 2',3'-dihydrophylloquinone were kind gifts from Hoffmann-La Roche (Basel, Switzerland). The non-ionic surfactant HCO-60 (polyoxyethylene hydrogenated castor oil derivatives) was purchased from Nikko-Chemicals (Tokyo, Japan). Brodifacoum was a gift from Sorex (Cheshire, United Kingdom) and it was dissolved in dimethyl sulfoxide (DMSO; J.T.Baker, Deventer, Holland) to a concentration of 1 mg/ml, which was mixed with 3 volumes of a buffer containing 150 mM NaCl, 20 mM Tris-HCl, pH 7.5 to give a final brodifacoum concentration of 0.25 mg/ml in 25% (v/v) of DMSO. All other chemicals were of analytical grade or better.

Animals and diets

All studies were performed in male rats of the Lewis strain. They were 12 weeks old when entering the experiment. The animals were housed in individual metabolism cages with a controlled temperature (20 ± 2 °C) and humidity ($50 \pm 10\%$) and a 12-h light-dark cycle, and they had free access to food and water. During all experiments

the food intake was verified for regular intake. Animals used for the vitamin K-deficient model had free access to a radiated (0.9 Mrad) vitamin K deficient diet (Hope Farms, Woerden, The Netherlands), which was mixed with cooked and dried rice in a 1:1 (w/w) ratio prior to use^{9,13}. The commercial vitamin K-deficient food contained (g/kg): vitamin-free milk proteins (300), synthetic methionine (2), cellulose (50), sugars (450), starch (70), sunflower oil (50), and the standard amounts of minerals, vitamins (excluding vitamin K) and trace elements; each lot number was carefully checked that it did not contain detectable amounts of vitamin K. On day 7 of this regimen, prothrombin concentrations were found to range between 5-10% of initial values, and at that time the animals were treated with vitamin K as indicated. Animals used for the anticoagulated model received a commercial rat diet (SRM-A, Hope Farms), the vitamin K content of which was 4-6 mg menadione and 2 mg phyloquinone per kg of food. The rats were anticoagulated by a single oral dose of 0.25 mg brodifacoum¹⁴. Three days later their prothrombin levels were less than 2 % of the starting values, and at that time the animals had been treated with vitamin K as indicated. The various solutions of K₁, MK-4 and MK-9 were prepared in 0.7% HCO-60 as described earlier¹⁰. In all cases the amount of vitamin K to be administered was prepared in a volume of 1 ml, and given orally via a syringe equipped with a plastic cannula, which was protruded into the esophagus. To quantify the efficacy of the various vitamers, we have calculated the Areas Under the Curve (AUC) for the circulating prothrombin concentration as a function of time.

Plasma prothrombin assay

Blood (0.15 ml) was collected in 0.015 ml of 0.14 M trisodium citrate after puncture of the tail vein. The samples were centrifuged for 15 min at 3,000 x g and the supernatant plasma was frozen at -80 °C until use. Prothrombin concentrations were determined in the one-stage assay using a coagulometer (KC-4, Amelung, Germany), a commercial thromboplastin preparation (Thromborel S) and clotting factor II deficient plasma (both from Behringwerke AG, Marburg, Germany). Prothrombin concentrations were calculated with the aid of a reference curve from pooled normal rat plasma (same strain, sex and age as in the experiment).

Vitamin K determination

Circulating vitamin K was determined in the serum. For this purpose blood was collected from the abdominal aorta at the end of the experiment. The samples were centrifuged as described above and serum was frozen at -80 °C until use. Concentrations of vitamin K₁, MK-4 and MK-9 were analyzed simultaneously after electrochemical reduction, HPLC separation and fluorometric detection as described

previously¹⁵. The hepatic contents of vitamin K quinone and epoxide were analyzed according to the method initially described by Thijssen et al.¹⁶. In short, livers were perfused with saline (0.15 M NaCl) via the portal vein and removed from the body, washed, and homogenized in three volumes of saline. At this stage 2',3'-dihydrophyloquinone was added to 1 ml of liver homogenate as an internal standard, with subsequent extraction and HPLC analysis.

Results

1. Effect of K-vitamins on plasma prothrombin in vitamin K-deficient and in brodifacoum-treated rats

The three vitamins tested below were: K₁, MK-4, and MK-9, and their biological effect was defined as the ability to increase the plasma prothrombin concentration. In the experiment, the results of which are given in fig. 1A, 24 rats were made vitamin K-deficient and subdivided into 4 groups of 6 animals each. One group received only solvent (controls), the other groups received a single oral dose of 10 nmol of either K₁, MK-4, or MK-9. The effect of each vitamin was measured by taking blood at regular time intervals, followed by prothrombin assessment. The time between ingestion and maximal effect (t_{max}) was found to be between 6 and 10 h for K₁ and MK-4; a more accurate assessment of the t_{max} could not be made because the number of sampling points was limited. The response to MK-9 was much slower, with a maximum at about 24 h. From these curves we calculated the AUC-values, which were 433, 1318, and 2806 h% for MK-4, K₁, and MK-9, respectively. Hence the efficacy of K₁ was about 3 times higher than that of MK-4, and that of MK-9 (expressed on a molar basis) was at least 6.5 times that of MK-4.

In a parallel experiment, brodifacoum-treated rats ($n = 6$ per group) were given a single oral dose of 5 μ mol of either K₁, MK-4, or MK-9. The quantity of the dose had been established in a pilot experiment, showing that in anticoagulated rats the dose of K₁ required to observe a significant effect was 500 times higher than in nutritionally deficient rats. As is shown in fig. 1B, there were two striking differences between the vitamin K-deficient and the anticoagulated model: (i) MK-9 had no effect in anticoagulated rats, and (ii) the effect of K₁ was equivalent to that of MK-4. AUC-values for 5 μ mol of MK-4, K₁, and MK-9 were calculated to be 963, 839, and 0 h%, respectively. At 1 μ mol the effect of MK-4 was even larger than that of K₁ (data not shown), but the poor response at this low dose did not allow us to make reliable calculations. By comparing figures 1A and 1B it can be seen that in both models the t_{max} for the effect of MK-4 seemed to be slightly shorter than that of K₁. In the

experiments described below we have tried to find an explanation for the differences between the vitamin K-deficient and the brodifacoum-treated models.

Fig. 1. Effect of a single dose of vitamin K on circulating prothrombin concentrations in vitamin K-deficient and in anticoagulated rats. Vitamin K was administered as a detergent-solubilized preparation. Each point represents the average of six different rats $\pm$ SEM. If no error bar is shown, the SEM falls within the symbol. The mean prothrombin concentrations in the control group were subtracted from those in the test groups at each time point. The Area Under the Curve (AUC) was calculated from the equation: $AUC = 1/2(x_2 - x_1)(y_1 + y_2)$. A: 10 nmol of each vitamer in vitamin K-deficient rats; B: 5 μ mol of each vitamer in brodifacoum-treated rats. Symbols: $\bullet$ K₁; $\square$ MK-4; $\circ$ MK-9.

2. Differential absorption at low and high intake of vitamers

Forty rats were made vitamin K-deficient and treated with a single oral dose of a mixture containing either 20 nmol, or 10 μ mol of each of the three K-vitamins. Blood and livers were taken from 2 different rats at each of the following time points: 0, 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 h after administration, and their vitamin K contents were analysed. The pharmacokinetic behaviour of the K vitamers in the high dose administration is shown in figs. 2A (serum) and B (liver). The complete data are summarized in table I. Both at low and high doses the t_{max} in serum was 1-2 h for K₁ and MK-4, and 3-4 h for MK-9; the highest AUC_{0-8} were observed for K₁. After the high dose intake the circulating levels of MK-9 remained low, suggesting that this vitamer was absorbed poorly. Maximal hepatic concentrations of K₁ and MK-4 were found between 2 and 3 h after ingestion, both at a low and high dose intake. The hepatic accumulation of MK-9 was extremely low and in the high dose group it was still far from completed at $t=8$ h (see fig. 2B).

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Fig. 2. Appearance and disappearance of K-vitamins in serum and in liver after oral ingestion. To reduce base-line levels all animals were made vitamin K-deficient before the start of the experiment. All animals received a single, oral dose containing 10 μmol of each vitamer, and at each time point indicated on the graph 2 animals were killed in order to obtain serum and liver. Each point represents the mean of two rats $\pm$ SD. Symbols are as in fig. 1.

Table 1: Absorption of K-vitamins from an orally administered vitamins mixture.

Composition of mixture		Serum		Liver		Ratio liver/serum
Vitamer	Dose (μmol)	AUC ₀₋₈ (nmol.h.ml ⁻¹)	t _{max} (h)	AUC ₀₋₈ (nmol.h.g ⁻¹)	t _{max} (h)	
K ₁	0.02	0.108	1.5	0.954	4	8.83
MK-4	0.02	0.021	1	0.091	3	4.33
MK-9	0.02	0.022	3	0.249	6	11.4
K ₁	10	17.5	2	127.2	3	7.3
MK-4	10	8.6	2	24.3	2.5	2.8
MK-9	10	0.56	4	11.3	>8	20.2

Mixtures of the three vitamers were administered orally to vitamin K-deficient rats in a single, equimolar dose. Between 0 and 8 h after administration 2 rats were killed at $t=0$, 2, 1 h and at each of the subsequent 1 h intervals to obtain blood and liver. The total recovery was quantitated as the Area Under the Curve (AUC), which was calculated on the basis of means of two animals per time point (see also fig. 1).

The hepatic specificity for the three vitamers was calculated from the ratio between the AUC_{0-8} values for liver and serum, showing that at low dose intake there was a slight preference for MK-9. At a 500 times higher dose, the AUC_{0-8} values for serum vitamin K increased 160 fold for K_1 and 400 fold for MK-4, but only 25 fold for MK-9. High serum levels resulted in high hepatic vitamin K concentrations with AUC_{0-8} values which were 135 and 275 times the respective low dose values for K_1 and MK-4, but in not more than a 45 times increased AUC_{0-8} value for MK-9.

To exclude the possibility that the relatively low circulating MK-9 concentrations, notably found in the high dose group, were caused by competition with the other K-vitamers we compared the appearance and disappearance of K_1 , MK-4 and MK-9 in the circulation after ingestion of the vitamins either as a mixture, or as homogenous solutions, using 4 groups of 4 animals each and blood samplings at 1 h intervals between 0 and 8 h. As is shown in table II, the AUC_{0-8} values for each of the vitamins were independent of the presence of other vitamers in the mixture.

Table II: Absorption of K-vitamers from mixtures and from homogenous solutions.

Vitamin	Recovery (AUC) in serum (nmol.h.ml ⁻¹)	
	from vitamin K mixture	from homogenous solution
K_1	18.1	19.2
MK-4	8.3	6.7
MK-9	0.6	0.5

The three K-vitamers were administered orally to vitamin K-deficient rats in a single dose. Animals in one group received a mixture containing 10 μ mol of each vitamin, animals in three other groups received a homogenous solution containing 10 μ mol of only one vitamin. Each group consisted of 4 animals. Two animals from each group were used for the collection of blood at $t = 0, 2, 4, 6$, and 8 h after vitamin K administration, the other two animals in each group donated blood at $t = 0, 1, 3, 5$, and 7 h. The total recovery was quantitated as the Area Under the Curve (AUC), which was calculated on the basis of means of two animals per time point (see also fig. 1).

3. Effect of brodifacoum on absorption of K vitamers

Regarding the important observation that in anticoagulated rats the effect of MK-4 was similar or even larger than that of K_1 , whereas it was substantially lower than that of K_1 in vitamin K-deficient animals (see fig.1), we checked whether brodifacoum treatment could change the absorption characteristics of MK-4. Five groups of 4 rats each were either made vitamin K-deficient or anticoagulated with brodifacoum (as indicated in table III). Ten μ mol of K_1 or MK-4 were administered either separately or as a mixture, and the serum concentration of both vitamers was assessed at 4 h after

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administration. As can be seen from table III, the serum concentrations of both K₁ and MK-4 were closely similar in the vitamin K-deficient and in the brodifacoum-treated rats.

Table III: Effect of brodifacoum on serum levels of K-vitamins after oral ingestion.

Pretreatment of rats	Type of vitamin K used	Recovery (pmol/ml) in	
		serum	liver
Brodifacoum	Mixture: K ₁ MK-4	9.7 ± 2.1	65.2 ± 12.3
		2.5 ± 1.0	12.6 ± 2.7
Brodifacoum	Separate K ₁	6.9 ± 1.8	51.1 ± 13.5
	Separate MK-4	2.2 ± 0.4	7.1 ± 1.8
Vitamin K-deficient	Separate K ₁	8.2 ± 2.5	56.2 ± 14.4
	Separate MK-4	2.5 ± 0.8	8.3 ± 1.9

The recovery of both vitamins is expressed as the sum of the K quinone and K epoxide concentrations in serum and liver at this time point. Data represent means of 4 rats ± SD.

This seems to exclude the possibility that brodifacoum affects the rate or extent of vitamin K adsorption. The fact that the values obtained for K₁ + MK-4 mixtures were comparable to those obtained for separate administrations is consistent with the earlier conclusion that there is no mutual influence of intestinal absorption.

Discussion

In this paper we have shown that the relative bioavailability of various K-vitamins depends on the model system used. A single dose of MK-9, for instance, had a relatively high "vitamin K activity" when tested in vitamin K-deficient rats, but no measurable activity in anticoagulated animals. Hence, conclusions concerning vitamin K utilization in anticoagulated rats may not be extrapolated to vitamin K-deficient or non-treated rats^{7,12}. If a similar mechanism was operational in anticoagulated patients, diets containing primarily K₁ (spinach, kale) and MK-4 (meat and liver-paste, chicken) would be expected to influence the stability of oral anticoagulation to a much larger extent than meals containing a comparable content of higher menaquinones (curd, cheese, yoghurt). Accordingly it is important to mention that the most abundant K-vitamin in the western diet is K₁¹⁷.

The poor effect of MK-9 in anticoagulated rats may be explained in part by its limited intestinal absorption or by poor hepatic uptake of long chain menaquinones.

Consequently, the higher concentrations required to overcome the effect of anticoagulants are not reached. The mechanism underlying the poor absorption of MK-9 is not clear, but the fact that at high dose intake the ratio between liver and serum MK-9 had increased rather than decreased does not suggest an upper limit for the hepatic uptake of long chain menaquinones. Moreover, the data in table II demonstrate that there is no competition between the various vitamers for a common intestinal or circulatory transport system. From table I it can also be seen that at an intake of 10 μmol the hepatic concentration of MK-9 is still about half that of MK-4, which should be sufficient for a detectable effect. We conclude, therefore, that there is a second factor contributing to the very poor effect of MK-9 in the livers of anticoagulated animals. This factor might be a different distribution of the various K-vitamers over the subcellular organelles. Reedstrom and Suttie have shown previously that MK-9 primarily accumulates in rat liver mitochondria, whereas higher concentrations of K_1 were found in the microsomes⁷. Additionally also the marked difference in hydrophobicity might play a role: in vitamin K-deficient rats all forms of vitamin K may be reduced into their hydroquinone form (i.e. the active cofactor for γ -glutamylcarboxylase) by the dithiol-dependent KO-reductase. The latter enzyme is an integral membrane protein located in the endoplasmic reticulum, which is also the site where the highly lipophilic MK-9 is expected to accumulate. In anticoagulated rats, however, the KO-reductase is blocked, and the conversion of vitamin K quinone to hydroquinone may be taken over by the much more water-soluble cytosolic DT-diaphorase¹⁸. It seems at least plausible that the short-chain K-vitamers like K_1 and MK-4, which have a far less lipophilic nature than MK-9, are less tightly bound to the microsomal membranes, and may be reduced preferentially by the DT-diaphorase. Another noteworthy observation was that the apparent vitamin K activity of MK-4 was half that of K_1 in vitamin K-deficient rats, whereas the effects of both vitamers were similar in anticoagulated animals. Brodifacoum treatment by itself affected the absorption of neither K_1 nor MK-4 (table III). In table I it can be seen that at low dose intake the hepatic accumulation of K_1 is about 10 times higher than that of MK-4. The observation that at a 10 fold higher K_1 concentration the efficacy of K_1 is not more than twice that of MK-4 suggests that in vitamin K-deficient rats MK-4 is used five times more efficiently than is K_1 . Earlier studies in which we employed an *in vitro* assay system, showed that the K_M^{app} of γ -glutamylcarboxylase for MK-4 is about 4 times lower than that for K_1 ¹⁹, which explains the observed larger *in vivo* efficacy. From table I it can also be seen that at high dose intake the hepatic accumulation of K_1 was not more than five times that of MK-4. Taking into account the lower K_M^{app} values for MK-4, this fully explains the fact that the biological effect of both vitamers were similar at the high doses used in anticoagulated animals.

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as compared to brodifacoum treated rats**

References

1. Suttie, J.W. (1985) Vitamin K-dependent carboxylase. *Annu. Rev. Biochem.* 54, 459-477.
2. Vermeer, C. (1990) Gamma-carboxyglutamate containing proteins and the vitamin K-dependent carboxylase. *Biochem. J.* 266, 625-636.
3. Furie, B. and Furie, B.C. (1990) Molecular basis of vitamin K-dependent gamma-carboxylation. *Blood* 75, 1753-1762.
4. Furie, B. and Furie, B.C. (1988) The molecular basis of blood coagulation. *Cell* 53, 505-518.
5. Suttie, J.W. and Preusch, P.C. (1986) Studies of the vitamin K-dependent carboxylase and vitamin K epoxide reductase in rat liver. *Haemostasis* 16, 193-215.
6. Suttie, J.W. (1995) The importance of menaquinones in human nutrition. *Annu. Rev. Nutr.* 15, 399-417.
7. Reedstrom, C.K. and Suttie, J.W. (1995) Comparative distribution, metabolism, and utilization of phyloquinone and menaquinone-9 in rat liver. *Proc. Soc. Exptl. Biol. Med.* 209, 403-409.
8. Will, B.H. and Suttie, J.W. (1992) Comparative metabolism of phyloquinone and menaquinone-9 in rat liver. *J. Nutr.* 122, 953-958.
9. Groenen-van Dooren, M.M., Soute, B.A., Jie, K.-S., Thijssen, H.H.W., and Vermeer, C. (1993) The relative effects of phyloquinone and menaquinone-4 on the blood coagulation factor synthesis in vitamin K- deficient rats. *Biochem. Pharmacol.* 46, 433-437.
10. Groenen-van Dooren, M.M., Ronden, J.E., Soute, B.A. and Vermeer, C. (1995) Bioavailability of phyloquinone and menaquinone after oral and colorectal administration in vitamin K-deficient rats. *Biochem. Pharmacol.* 50, 797-801.
11. Park, B.K., and Leck, J.B. (1982) A comparison of vitamin K antagonism by warfarin, difenacoum and brodifacoum in the rabbit. *Biochem. Pharmacol.* 31, 3635-3639.
12. Akiyama, Y., Hara, K., Matsumoto, A., Takahashi, S. and Tajima, T. (1995) Comparison of intestinal absorption of vitamin K2 (menaquinone) homologues and their effects on blood coagulation in rats with hypoprothrombinemia. *Biochem. Pharmacol.* 49, 1801-1807.
13. Mathers, J.C., Fernandez, F., Hill, M.J., McCarthy, P.T., Shearer, M.J. and Oxley, A. (1990) Dietary modification of potential vitamin K supply from enteric bacterial menaquinones in rats. *Brit. J. Nutr.* 63, 639-652.
14. Mosterd, J.J. and Thijssen, H.H.W. (1991) The long-term effects of the rodenticide, brodifacoum, on blood coagulation and vitamin K metabolism in rats. *Brit. J. Pharmacol.* 104, 531-535.

15. Gijsbers, B.L., Jie, K.-S. and Vermeer, C. (1996) Effect of food composition on vitamin K absorption in human volunteers. *Brit. J. Nutr.* 76, 223-229.
16. Thijssen, H.H.W. and Drittij-Reijnders, M.J. (1996) Vitamin K status in human tissues: tissue-specific accumulation of phyloquinone and menaquinones-4. *Brit. J. Nutr.* 75, 121-127.
17. Shearer, M.J., Bach, A., and Kohlmeier, M. (1996) Chemistry, nutritional sources, tissue distribution and metabolism of vitamin K with special reference to bone health. *J. Nutr.* 126, 1181S-1186S.
18. Wallin, R., and Suttie, J.W. (1981) Vitamin K-dependent carboxylation and vitamin K epoxidation. Evidence that the warfarin-sensitive microsomal NAD(P)H dehydrogenase reduces vitamin K₁ in these reactions. *Biochem. J.* 194, 983-988.
19. Buitenhuis, H.C., Soute, B.A.M. and Vermeer C. (1990) Comparison of the vitamin K₁, K₂ and K₃ as cofactors for the hepatic vitamin K-dependent carboxylase. *Biochim. Biophys. Acta* 1034, 170-175.

Nutritional vitamin K intake and urinary gamma-carboxyglutamate excretion in rats

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Summary

Using the rat as an experimental animal model we found that prothrombin synthesis reaches its maximal level at a relatively low dietary vitamin K intake. At even higher vitamin K intakes, however, the urinary Gla-excretion was substantially increased, showing a different vitamin K requirement for liver and extrahepatic tissues. The increased urinary Gla-excretion was found for both phyloquinone and menaquinone-4, but not for menaquinone-8, which questions the bioavailability of higher menaquinones for extrahepatic tissues. A discrepancy was found between effects of nutritional vitamin K-deficiency and treatment with a vitamin K-antagonist (brodifacoum). With both regimens plasma prothrombin rapidly decreased to well below 10% of the starting values, but in the case of K-deficiency urinary Gla had hardly decreased in 7 days, whereas after 3 days of brodifacoum treatment Gla-excretion had decreased to 17% of the starting values. An explanation for this observation is that prothrombin procoagulant activity does not decrease proportionally to the prothrombin Gla-content, but that a wide range of undercarboxylated prothrombins lost nearly all their activity. During vitamin K-deficiency the remaining low levels of vitamin K would mainly give rise to undercarboxylated prothrombin, whereas during brodifacoum treatment only non-carboxylated prothrombin is formed. It seems plausible that in the latter case the urinary Gla originates from proteins with long half-life times, such as bone Gla-proteins.

Introduction

Vitamin K is a group name for a number of related compounds which all have a naphthoquinone ring structure, but which differ from each other by the length and degree of saturation of their aliphatic side chain at the 3-position¹. The three K-vitamins which were studied in this paper are: phyloquinone, menaquinone-4, and menaquinone-8. Phyloquinone and menaquinone-4 are different with respect to their degree of saturation, whereas menaquinone-4 and menaquinone-8 differ in their aliphatic side chain length.

The only known function of vitamin K in mammals is to act as a coenzyme for the endoplasmic enzyme γ -glutamylcarboxylase during the posttranslational conversion of protein-bound glutamate residues into γ -carboxyglutamate (Gla)^{2,3}. The daily requirement of vitamin K is extremely low, because a second enzyme (KO-reductase) is capable of recycling the vitamin so that one molecule may catalyze the formation of several thousand Gla-residues^{2,4}. A number of coumarin derivatives (e.g. warfarin and brodifacoum) inhibit the recycling of vitamin K by blocking the KO-reductase, thus rapidly exhausting the pool of bioavailable vitamin K^{5,6}.

Once it has been formed Gla cannot be metabolized or re-used, so eventually all Gla is excreted stoichiometrically via the urine, most of it as a free amino acid and less than 5% in a peptide-bound form⁷⁻⁹. Hence urinary Gla levels may reflect the turnover of the total body pool of Gla proteins; in a steady state situation the urinary Gla excretion will even be a measure for the total Gla production. By comparing plasma prothrombin and urinary Gla under different conditions an impression may be obtained of the extent and vitamin K-requirement of extra-hepatic carboxylation events.

The enzymes of the vitamin K-cycle (i.e.: carboxylase and reductase) were discovered in the liver, but afterwards they were detected in most tissues and cell types¹⁰⁻¹³. The hepatic system is involved in the production of several blood coagulation factors, the most abundant of which is prothrombin. Osteoblasts in bone produce osteocalcin and matrix Gla-protein, two proteins which are structurally not related to the hepatic Gla-proteins¹⁴. Recent studies in humans have indicated that the nutritional vitamin K-requirement for complete carboxylation of osteocalcin may be higher than that for the carboxylation of prothrombin and related coagulation factors^{15,16}.

In this paper we have made use of the rat as an experimental model, and we have assessed the minimal dietary vitamin K intake at which the production of the hepatic Gla-protein prothrombin takes place at its maximal rate. To exclude small variations due to interindividual differences all animals were adapted for 3 weeks to a diet containing 3 times the minimal vitamin K requirement. In this model we have investigated the effect on plasma prothrombin concentration and urinary Gla excretion with a very high intake of various K vitamers, that of a nutritional vitamin K-deficiency, and finally the effect of administration of the vitamin K-antagonist brodifacoum.

Materials and Methods

Chemicals. Phylloquinone (vitamin K₁), D,L γ -carboxyglutamic acid (Gla), and ortho-phthaldialdehyde (OPA) were purchased from Sigma (St. Louis, MO), menaquinone-4 and menaquinone-8 were kind gifts from Hoffmann-La Roche (Basel, Switzerland). The non-ionic surfactant HCO-60 (polyoxyethylene hydrogenated castor oil derivatives) was purchased from Nikko Chemicals (Tokyo, Japan). Brodifacoum was a gift from Sorex (Cheshire, United Kingdom) and it was dissolved to a final concentration of 0.25 mg/mL in a buffer containing 150 mM NaCl, 20 mM Tris-HCl, pH 7.8 and 1% (w/v) HCO-60 shortly before use. The various K-vitamers were dissolved in the same buffer system to a final concentration of 10 μ mol/mL. All other chemicals were of analytical grade or better.

Animals and Diets. All studies were performed in male rats of the Lewis strain, which were 12 weeks old when entering in the experiment. The animals were housed in individual metabolic cages equipped for separate collection of urine and faeces (Tecniplast 170102 cages, B&E Industry, Utrecht, The Netherlands), with a 12-h light-dark cycle and controlled temperature (20 ± 2 °C) and humidity ($50 \pm 10\%$). They had free access to food and water. The following foods were used:

1. A commercial rat diet (SRM-A, Hope Farms, Woerden, The Netherlands), the vitamin K content of which was found to be 5 mg of menadione and 2 mg of phyloquinone per kg of food.
2. A radiated (0.9 Mrad) vitamin K-deficient diet (Hope Farms, Woerden, The Netherlands), which was mixed with cooked and dried white rice in a 1:1 (w/w) ratio prior to use¹⁷. This food was checked that it did not contain detectable amounts of vitamin K.
3. The same vitamin K-deficient diet, mixed with cooked and dried rice, to which 20 µg of menaquinone-4 per g of food were added.

All food was blended and mixed in a professional food processor before use. To ensure a controlled vitamin K intake the vitamins were administered orally, via a syringe equipped with a plastic cannula, which was protruded into the esophagus. All rats were weighed regularly, and checked that their body weights increased by about 1 gram per day, and that the various groups did not differ with respect to mean body weight gain. Blood (0.5 mL) was collected in 0.05 mL of 0.14 M trisodium citrate after puncture of the tail vein. The blood samples were centrifuged for 15 min at $3,000 \times g$ and the plasma was frozen at -80 °C until use. Urines were collected during the last 24 h of each measuring point, the volumes were recorded. Subsequently the samples were centrifuged for 10 min at $5,000 \times g$ and the supernatants were frozen at -80 °C until use.

Urinary Gla detection. The urine samples were diluted with distilled water between 4 and 160 times (in order to stay within the normal detection range), and the diluted samples were analyzed according to the procedure of Kuwada and Katayama¹⁸, using a Separations high precision HPLC pump (model 300), a Kratos Spectraflow 980 detector and a Nucleosil 100-5 SB anion exchange resin (5 µm particles, Macherey-Nagel, Düren, Germany) at a flow rate of 1 mL/min. Reference curves were prepared from samples with known concentrations of authentic Gla. The sensitivity of the assay was 0.3 µM. The intra- and interassay coefficients of variation were 2.2 and 6.4%, respectively.

Plasma prothrombin assay. Prothrombin concentrations were determined in the one-stage assay using a coagulometer (KC-4, Amelung, Germany), and a commercial

thromboplastin preparation (Thromborel S) and clotting factor II deficient plasma (both from Behringwerke AG, Marburg, Germany). Prothrombin concentrations were calculated with the aid of reference curve from pooled normal rat plasma.

Statistical analysis. The software package SPSS/PC+ version 5.0 (SPSS Inc., Chicago, Ill) was used for statistical analysis and the 0.05 level of significance was chosen. The data were analyzed using the paired Student's t-test for comparing groups of more than 20 animals, and the Wilcoxon test for experiments with less animals per group. All data are expressed as mean values $\pm$ S.E.M.

Results

1. Gla-excretion at the minimal vitamin K intake required for normal blood coagulation.

Sixty rats were made vitamin K-deficient by feeding them with a commercial diet devoid of vitamin K for a period of 1 week. At that time blood was collected, and it was found that the circulating prothrombin concentration had decreased to 10-20% of the starting values. Subsequently the rats were subdivided into 10 groups, and varying (known) amounts of menaquinone-4 were added to the diet. This regime was continued for 2 weeks before blood was taken for another prothrombin determination. It was found that at a menaquinone-4 content of 6 μ g (13.5 nmol) per g of food the prothrombin levels had reached their pre-treatment levels, and that they did not change at higher intakes (see fig. 1). Since the mean food consumption was 22 gram per day, it may be calculated that the minimal dietary menaquinone-4 requirement was around 300 nmol per day.

Figure 1: Menaquinone-4 requirement for normal prothrombin synthesis in the vitamin K-deficient rat. The food was administered ad libitum during a period of 2 weeks. Plasma prothrombin concentrations are expressed as a percentage of the starting values. Each point represents the mean value ($\pm$ SEM) for 6 rats.

On the basis of these data it was decided that for all experiments described below the reference rats (i.e.: vitamin K-sufficient) would receive a diet containing 20 µg (45 nmol) of menaquinone-4 per g. This diet will be referred to as the „standardized control diet”. Reference rats were defined further in an experiment in which we measured the plasma prothrombin concentration and the urinary Gla excretion in 25 animals before they entered the experiment and after they had been fed for 3 weeks with the standardized control diet. The data are given in Table I, and demonstrate that plasma prothrombin did not change during the switch from commercial food to the standardized control diet used for known vitamin K-intake. The urinary Gla-excretion had slightly decreased, however, which suggests that the Gla-excretion can be decreased without changing the plasma prothrombin concentration, and also that more vitamin K can be extracted from the commercial chow than from the standardized control diet.

Table I: Effect of diet on plasma prothrombin and urinary Gla excretion.

Marker	Before treatment	After treatment	Difference	p-value
Prothrombin (%)	80.2 ± 0.8	79.9 ± 1.8	- 0.28 ± 1.7	N.S.
Gla-excretion (nmol/day)	1470 ± 70	1340 ± 70	- 130 ± 40	< 0.005
Gla / prothrombin ratio	18.4 ± 1	17.0 ± 1	- 1.4 ± 0.5	< 0.02

The divergence between urinary Gla excretion and plasma prothrombin concentration is visualized most clearly from the ratio between both variables. As can be seen from table I this ratio had decreased significantly after the dietary change.

2. Effects of high doses of various K-vitamins on plasma prothrombin and on urinary Gla.

Thirty five rats were kept for 3 weeks on the standardized control diet, and subsequently they were subdivided into three groups of 10 and one of group of 5 animals. The first group (n=10) continued with the standardized control diet as its sole source of nutritional vitamin K; the other three groups received a high oral dose (20 µmoles) of vitamin K on each of three successive days. Three vitamins were studied: phyloquinone (n=10), menaquinone-4 (n=10), and menaquinone-8 (n=5). As is shown in Table II, these high doses of vitamin K did not change the circulating prothrombin concentrations. On the other hand, urinary Gla excretion was substantially and significantly increased by both phyloquinone and menaquinone-4, but not by menaquinone-8. Prolonged treatment with high doses of phyloquinone and menaquinone-4 until day 7 did not change these data

further (data not shown). Unfortunately our supply was insufficient to continue the treatment with menaquinone-8.

Table II: Effect of high vitamin K intake on plasma prothrombin and urinary Gla.

	Control n = 10	Excess K ₁ n = 10	Excess MK-4 n = 10	Excess MK-8 n = 6
Gla-excretion (nmol/day)				
Before treatment	1,464 ± 178	1,501 ± 204	1,372 ± 280	1,330 ± 75
After treatment	1,451 ± 165	1,853 ± 232	1,875 ± 406	1,486 ± 86
Difference	-13 ± 50	352 ± 110	503 ± 210	156 ± 70
Statistical significance (p)	N.S.	0.02	0.01	N.S.
Prothrombin (%)				
Before treatment	77.2 ± 1.8	84.4 ± 3.7	85.9 ± 4.5	81.6 ± 2.1
After treatment	83.4 ± 3.4	89.9 ± 5.1	90.6 ± 6.4	89.2 ± 3.8
Difference	6.2 ± 7.5	5.5 ± 7.1	4.7 ± 8.3	7.6 ± 3.5
Statistical significance (p)	N.S.	N.S.	N.S.	N.S.

K₁ stands for phyloquinone, MK for menaquinone. Statistical significance of differences was calculated by the Wilcoxon test.

3. Effects of dietary vitamin K-deficiency and oral anticoagulants on plasma prothrombin and on urinary Gla.

Thirty rats were kept for 3 weeks on the standardized control diet, and subsequently they were subdivided into three groups of 10 animals each. In the first group, which served as a control, the dietary regimen was continued, the second group received a vitamin K-deficient diet, whereas the third group received the standardized control diet + one oral dose of 1 mg of brodifacoum per kg body weight. Blood and urine samples were taken at day 3 (controls and brodifacoum group) and at day 7 (controls and vitamin K-deficient group). The data are given in Table III, and show that both after dietary vitamin K-deficiency as well as after brodifacoum treatment the circulating prothrombin concentrations fell to below 10% of the starting values. The urinary Gla-excretion, however, declined from 1.63 to 1.07 µmol/day (= 66%) in the vitamin K-deficient animals and from 1.59 to 0.30 µmol/day (= 19%) in the brodifacoum-treated animals. From these data it appears that nutritional vitamin K-deficiency gave rise to a substantial decrease of prothrombin activity, which is only partly accompanied by a decrease in Gla-excretion. A complete blockade of the vitamin K cycle by coumarin derivatives resulted in very low

prothrombin levels (below the level of detection, i.e. < 2%), and in a more pronounced reduction of Gla-excretion than in the case of vitamin K-deficiency. From the data in Table III it may be calculated that the ratio between urinary Gla-excretion and plasma prothrombin concentration was not constant, but increased 4.5-fold during 7 days of vitamin K-deficiency and even > 8.2-fold after 3 days of brodifacoum treatment. This suggests that a substantial fraction of the urinary Gla originates from Gla-proteins with a much longer half-life time than prothrombin.

4. Correlation between the plasma prothrombin concentration and urinary Gla-excretion.

Although all experiments were performed using an inbred strain of rats, there was substantial interindividual variation in the plasma prothrombin concentration, which ranged from 70-110% (mean value: 79%) of the pooled reference plasma. Urinary Gla-excretion ranged from 0.8-2.2 $\mu\text{mol/day}$ (mean value: 1.5 $\mu\text{mol/day}$, see fig. 2).

Figure 2. Histogram for Gla-excretion in untreated rats. Rats were housed in individual metabolic cages, and all urine was collected during a period of 24 h. Both the means and the median for excretion were 1.5 $\mu\text{mol}/24\text{ h}$.

Using the data from 30 animals which had been kept for 3 weeks on the standardized control diet no correlation was found between the plasma prothrombin level and the urinary Gla excretion ($r = -0.17$; $p > 0.4$). It was concluded that the interindividual differences in plasma prothrombin concentrations are not reflected in the urinary Gla excretion.

Table III: Effect of vitamin K-deficiency and vitamin K-antagonists on plasma prothrombin and urinary Gla.

	Control 1 n = 10	Brodifacoum-treatment n = 10	Control 2 n = 10	Vitamin K-deficiency n = 10
Gla-excretion (nmol/day)				
Before treatment	1,308 (± 88)	1,587 (± 141)	1,308 (± 88)	1,625 (± 59)
After treatment	1,299 (± 93)	301 (± 18)	1,284 (± 131)	1,065 (± 74)
Difference	- 9 (± 34)	-1,286 (± 151)	- 24 (± 18)	- 560 (± 70)
Statistical significance (p)	N.S.	0.005	N.S.	0.007
Prothrombin (%)				
Before treatment	77.8 (± 1.0)	86.3 (± 3.9)	77.8 (± 1.1)	72.3 (± 1.5)
After treatment	81.5 (± 2.2)	< 2	80.0 (± 1.7)	10.5 (± 1.2)
Difference	+ 3.7 (± 2.3)	< - 84.3	+ 2.2 (± 2.4)	- 61.8 (± 2.0)
Statistical significance (p)	N.S.	0.005	N.S.	0.005

Statistical significance of differences was established by the Wilcoxon test. Further details are as in the legend to Table I.

Discussion

The experiments presented in this paper were designed to bring rats in a steady state situation of nutritional vitamin K intake; it was assumed that in this situation the amount of urinary Gla-excretion equalled the total body Gla synthesis. Furthermore it was assumed that the plasma prothrombin concentration was a reliable marker for hepatic Gla synthesis. By comparing these two variables at different nutritional vitamin K intake we were able to investigate the vitamin K-requirement for hepatic and extra-hepatic Gla formation.

The data in this paper showed an almost linear relation between the dietary vitamin K concentration and the plasma prothrombin between 0 and 6 μg of menaquinone-4 per g of food. Vitamin K-sufficiency is generally defined as the vitamin K intake at which no further increase of plasma prothrombin takes place. For our studies we have taken rats fed with 20 μg (45 nmol) of menaquinone-4 per g of food as vitamin K-sufficient control animals, and it was shown that between 45 and 2,000 nmol of K-vitamins per g of food there was no change in the prothrombin concentration. On the other hand, the excess of phyloquinone and menaquinone-4 induced a significant increase in total Gla-excretion, suggesting that the vitamin K-requirement of a number of extrahepatic γ -glutamylcarboxylases is higher than that of the hepatic enzyme. This is consistent with earlier results from human studies showing that a major fraction of the bone Gla-protein osteocalcin occurs in an undercarboxylated form¹⁵, and that supplementation of pharmacological doses of phyloquinone increases both the synthesis of osteocalcin, as well as its degree of carboxylation¹⁹⁻²¹. High doses of menaquinone-8, on the other hand, did not affect the urinary Gla-excretion. Groenen-van Dooren et al. showed that the intestinal absorption of menaquinone-9 is slower than that of menaquinone-4 and phyloquinone, but these authors reported a maximal effect of menaquinone-9 at 24 h after ingestion of the vitamin. It must be assumed, therefore, that after 3 days of menaquinone-8 treatment the animals were in a steady state situation. This suggests that higher menaquinones do not have cofactor activity for extrahepatic carboxylases *in vivo*.

Remarkably, the Gla-excretion had only slightly decreased in the vitamin K-deficient state. The most plausible explanation for this phenomenon is that during nutritional vitamin K-deficiency one or more sources of vitamin K remain available (e.g.: liver or fat tissue stores, intestinal menaquinone production, coprophagy). The fact that the animals generally started bleeding after 7-10 days demonstrates that these sources are insufficient to maintain normal haemostasis. On the other hand this residual vitamin K might be sufficient to produce coagulation factors (or precursors thereof) with a reduced amount of Gla-residues. The occurrence of these undercarboxylated coagulation factors

in the circulation has been demonstrated in coumarin-treated humans and cows^{22,23}. Also if the undercarboxylated precursor proteins would be degraded intracellularly²⁴, the Gla-residues cannot be metabolized further, and are bound to reach the circulation to be excreted via the urine. It is well known that undercarboxylated coagulation factors have little (if any) procoagulant activity^{25,26}, but they might still substantially contribute to the urinary Gla-excretion.

In contrast to vitamin K-deficiency, brodifacoum efficiently and irreversibly blocks the vitamin K-cycle, thus preventing any further carboxylation events. Residual urinary Gla must originate from Gla-proteins formed before brodifacoum administration. After 3 days prothrombin (which is the coagulation factor with the longest half-life time, i.e. 9 h²⁷) had decreased below the level of detection, followed by serious bleeding during the fourth day. At that time the urinary Gla excretion had decreased to 17% of the starting value. Obviously the residual Gla originated from proteins with longer half-life times than prothrombin has, for instance those adsorbed to the hydroxyapatite matrix of bone.

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References

1. Shearer, M.J. (1995) Vitamin K. *Lancet* 345, 229-234.
2. Vermeer, C. (1990) Gamma-carboxyglutamate – containing proteins and the vitamin K – dependent carboxylase. *Biochem. J.* 266, 625-636.
3. Suttie, J.W. (1987) Recent advances in hepatic vitamin K metabolism and function. *Hepatology* 7, 367-376.
4. Suttie, J.W. and Preusch, P.C. (1986) Studies of the vitamin K-dependent carboxylase and vitamin K epoxide reductase in rat liver. *Haemostasis* 16, 193-215.
5. Fasco, M.J., Hildebrandt, E.F. and Suttie, J.W. (1982) Evidence that warfarin anticoagulant action involves two distinct reductase activities. *J. Biol. Chem.* 257, 11210-11212.
6. Mosterd, J.J. and Thijssen, H.H.W. (1991) The long term-effects of the rodenticide, brodifacoum, on blood coagulation and vitamin K metabolism in rats. *Brit. J. Pharmacol.* 104, 531-535.
7. Fernlund, P. (1976) Gamma-carboxyglutamic acid in human urine. *Clin. Chim. Acta* 72, 147-155.
8. Lian, J.B., Glimacher, M.J. and Gallop, P.M. (1971) in *Calcium binding proteins and calcium function* (Wasserman R.H. et al., eds.), pp 379-381, Elsevier North Holland, Amsterdam.
9. Shah, D.V., Tews, J.K., Harper, A.E. and Suttie, J.W. (1978) Metabolism and transport of gamma-carboxyglutamic acid. *Biochem. Biophys. Acta* 539, 209-217.

10. Buchthal, S.D. and Bell, R.G. (1983) Vitamin K dependent carboxylation of glutamate residues to gamma-carboxyglutamate in microsomes from spleen and testis: comparison with liver, lung, and kidney. *Biochemistry* 22, 1077-1082.
11. Vermeer, C., Hendrix, H. and Daemen, M. (1982) Vitamin K-dependent carboxylases from non-hepatic tissues. *FEBS Lett.* 148, 317-320.
12. De Boer-van den Berg, M.A.G., Uitendaal, M.P. and Vermeer, C. (1987) Direct measurement of vitamin K-dependent enzymes in various isolated and cultured tumor and non-tumor cells. *Mol. Cell. Biochem.* 75, 71-76.
13. Hauschka, P.V., Friedman, P.A., Traverso, H.P. and Gallop, P.M. (1976) Vitamin K-dependent gamma-carboxyglutamic acid formation by kidney microsomes in vitro. *Biophys. Res. Commun.* 71, 1207-1213.
14. Hauschka, P.V., Lian, J.B., Cole, D.E.C. and Gundberg, C.M. (1989) Osteocalcin and matrix Gla protein: vitamin K dependent proteins in bone. *Phys. Rev.* 69, 990-1047.
15. Price, P.A. and Cairns, J.R. (1994) Direct demonstration that the vitamin K-dependent bone Gla protein is incompletely gamma-carboxylated in humans. *J. Bone Miner. Res.* 9, 1989-1997.
16. Knapen, M.H.J., Jie, K.-S.G., Hamulyák, K. and Vermeer, C. (1991) *Thromb. Haemostas.* 65, 671.
17. Groenen-van Dooren, M.M.C.L., Soute, B.A.M., Jie, K.-S.G., Thijssen, H.H.W. and Vermeer, C. (1993) The relative effects of phyloquinone and menaquinone-4 on the blood coagulation factor synthesis in vitamin K-deficient rats. *Biochem. Pharmacol.* 433-437.
18. Kuwada, M. and Katayama, K. (1983) An improved method for the determination of gamma-carboxyglutamic acid in proteins, bone and urine. *Analyt. Biochem.* 131, 173-179.
19. Knapen, M.H.J., Hamulyák, K. and Vermeer, C. (1989) The effect of vitamin K supplementation on circulating osteocalcin (bone Gla protein) and urinary calcium excretion. *Ann. Int. Med.* 111, 1001-1005.
20. Knapen, M.H.J., Jie, K.-S.G., Hamulyák, K. and Vermeer, C. (1993) Vitamin K-induced changes in markers for osteoblast activity and urinary calcium loss. *Calcif. Tissue Int.* 53, 81-85.
21. Plantalech, L.C., Chapuy, M.C., Guillaumont, M., Chapuy, P., Leclercq, M. and Delmas, P.D. (1990) in *Osteoporosis 1990* (Christiansen, C. and Overgaard, K., eds.), pp 345-347, Osteopress, Copenhagen.
22. Friedman, P.A. (1984) Vitamin K-dependent proteins [editorial]. *N. Engl. J. Med.* 310, 1458-1460.
23. Malhotra, O.P. (1987) Electroimmunoassay of prothrombin. *Thromb. Res.* 47, 493-500.
24. Stanton, C. and Wallin, R. (1992) Processing and trafficking of clotting factor X in the secretory pathway. Effects of warfarin. *Biochem. J.* 284, 25-31.

25. Ratcliffe, J.V., Furie, B. and Furie, B.C. (1993) The importance of specific gamma-carboxyglutamic acid residues in prothrombin. Evaluation by site-specific mutagenesis. *J. Biol. Chem.* 268, 24339-24345.
26. Zhang, L. and Castellino, F.J. (1993) The contributions of individual gamma-carboxyglutamic acid residues in the calcium-dependent binding of recombinant human protein C to acidic phospholipid vesicles. *J. Biol. Chem.* 268, 12040-12045.
27. Vainieri, H. and Wingard, L.B. (1977) J. Effect of warfarin on the kinetics of the vitamin K-dependent clotting factors in rats. *Pharmacol. Exp. Ther.* 201, 507-517.

Circulating osteocalcin and urinary Gla as markers for human vitamin K status

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Introduction

The result of vitamin K action is the synthesis of γ -carboxyglutamate (Gla) residues in proteins¹. In humans Gla-containing proteins are synthesized by a wide range of tissues, in this paper it is our aim to compare circulating prothrombin and osteocalcin as well as the urinary excretion of free Gla for their capability to serve as markers for vitamin K status. Prothrombin is uniquely formed by hepatocytes and contains 10 Gla-residues per molecule², osteocalcin possesses 3 Gla-residues and is the product of osteoblasts³. After Gla-proteins have been metabolized, the free Gla residues cannot be re-used and are secreted in the urine.

During episodes of vitamin K-deficiency abnormal Gla-proteins (also known as undercarboxylated or descarboxy proteins) enter the circulation. Since the discovery of the chemical structure of Gla⁴ the name Proteins Induced by Vitamin K Absence (PIVKA's) has become obsolete. Assessment of circulating vitamin K is a time-consuming technique, only available in a small number of specialized laboratories⁵. Moreover, the relation between circulating and tissue vitamin K, as well as the level of plasma vitamin K at which a subject must be regarded as vitamin K-deficient have not yet been established. Clinical vitamin K-deficiency is generally assessed, therefore, by the occurrence of circulating descarboxy prothrombin. Neither the coagulation assays commonly used, nor gel immuno-electrophoretic techniques allow the detection of descarboxy prothrombin at levels below 3-5% of the normal prothrombin concentration. If measured in this way, vitamin K-deficiency is rarely seen, even among subjects who are known to have a low dietary vitamin K intake like newborns⁶ or elderly women⁷. It is well known, however, that due to the poor placental transport all newborns have very low circulating vitamin K levels, and are at risk for developing clinical vitamin K deficiency. The most sophisticated tests for descarboxy prothrombin presently available are based on monospecific or monoclonal antibodies, but even with these tests the occurrence of trace amounts of descarboxy prothrombin could be demonstrated in not more than 20% of the untreated newborns^{8,9}. It seems, therefore, that at decreasing vitamin K supply undercarboxylated blood coagulation factors are only formed during the later (more severe) stages of vitamin K deficiency. For that reason it must be questioned seriously whether descarboxy prothrombin forms an ideal marker to record the generation of vitamin K-deficiency in an early stage, and to assess the risk of developing hemorrhagic disease of the newborn (HDN). Seen from the point of detectability, a marker showing a larger fraction of its descarboxy form in an earlier phase of vitamin K-deficiency would be preferable.

Several arguments indicate that a poor vitamin K status manifests itself sooner in bone than in liver. The question is relevant, therefore, whether the vitamin K requirement of various tissues is similar. Firstly, it has been reported that substantial fractions of the bone Gla-proteins osteocalcin and matrix Gla-protein may occur in an undercarboxylated form¹⁰. Secondly, it is well known that nutritional vitamin K intake decreases with age, and that - in contrast to prothrombin - circulating undercarboxylated osteocalcin is common in elderly women^{7,11-13}. It must be expected, therefore, that the effect of a poor vitamin K status will be observable sooner in bone proteins than in clotting factors.

In this chapter we wish to report on two tests which may potentially provide information about K status before deviations in routine blood coagulation tests are detectable: the hydroxyapatite binding capacity (HBC) of serum osteocalcin, and the urinary excretion of Gla. Since no enzymatic activity of osteocalcin is known, functional assays are not available and its Gla-content is generally deduced from its affinity for hydroxyapatite^{7,10-13}. Urinary Gla-excretion is an overall test for the metabolism of Gla-proteins, and is low during vitamin K-deficiency. Subjects at risk for developing K-deficiency are newborns and elderly women. Here we report to which extend both markers respond to vitamin K supplementation in elderly women, in newborns and their mothers.

Materials and Methods

Subjects

For experiment 1, pregnant women were asked to participate in our study starting at the 34th week of their pregnancy. The women were recruited via the Department of Obstetrics of a local hospital and had an uneventful pregnancy. Vitamin K was administered (1 mg/day) from week 34 until delivery. Blood was taken from the mothers (shortly before delivery) and from the umbilical cords of their newborn babies.

In *experiment 2*, 29 healthy newborns were investigated. 15 of them received 1 mg K₁ in their first day of life, after collecting their first urine sample. 14 newborns were exclusively breastfed and did not receive any form of vitamin K₁ supplementation during three days. In all 29 newborns urine samples were collected in day 1 and day 3 after birth. In these urines GLA, calcium and magnesium were measured, and the concentrations were expressed as ratio with creatinine.

In *experiment 3*, apparently healthy, physically active women were recruited via a local newspaper call. Inclusion criteria were normal liver and renal functions, as well as serum calcium levels within the normal range. None of the participants had a

history of recent fractures or metabolic bone diseases, and those using medication known to affect calcium metabolism or vitamin K status were excluded from this study. All participants received vitamin K (Konakion®, 1 mg/day) for 4 weeks, blood and fasting urine were collected before and after the treatment period. Fast losers of urinary calcium are defined as those with a calcium/creatinine (mol/mol) ratio > 0.5 in the 2-h fasting urine; normal losers are defined as those in whom this ratio is ≤ 0.5 .

Samples

Urine was collected during the last 2 hours of a 16-hour fasting period, samples were acidified and stored at -30°C until serial testing. Blood was taken by venipuncture to prepare serum; after a short centrifugation step the samples were stored at -80°C until serial testing.

Biochemical determinations

Serum osteocalcin was assessed by radioimmunoassay (Incstar, Stillwater, MI) before and after extraction with 100 mg/ml hydroxyapatite¹², and the fraction of bound osteocalcin was calculated from the difference before and after extraction. HBC was expressed as the percentage of osteocalcin bound to hydroxyapatite relative to the total osteocalcin concentration. Subjects in whom the HBC is $< 70\%$ will be regarded as *biochemically vitamin K-deficient*. Prothrombin was tested with the one-stage and the two-stage assay¹⁴, and the ratio between both values (1-stage/2-stage) was regarded as a measure for undercarboxylation of prothrombin. Subjects in whom the ratio is < 1 are regarded to be *clinically vitamin K-deficient*. Urinary calcium concentrations were determined with an atomic absorption spectrophotometer (Perkin Elmer, Norwalk, CN), and creatinine with a commercial kit (Roche). Urinary calcium excretion is expressed as the molar ratio between calcium and creatinine in the fasting urine samples. Gla concentrations were assessed according to the procedure of Kuwada and Katayama¹⁵, except that the urine was diluted 4, 8, and 16 fold before adding the orthophthalaldehyde reagent. Measurements in more concentrated urine resulted in underestimation of the concentration of Gla¹⁶. Duplicate measurements of both dilutions were made to ensure that the samples gave a linear dose/response relation. Purified Gla (Sigma, St. Louis, MO) was taken as a standard.

Results

In two separate placebo-controlled studies we have tried to assess the vitamin K status with the aid of biochemical markers. Subjects investigated were: newborns

and their mothers (experiment 1), as well as adult pre- and postmenopausal women (experiment 2). In all subjects we have measured the effect of vitamin K administration (1 mg/day) during 4-8 weeks.

Experiment 1. Neither in the newborns nor in any of the adult women descarboxy-prothrombin was detectable with the assays employed (data not shown). On the other hand, high levels of circulating descarboxy osteocalcin were found in all cord sera of the placebo group. Consequently the HBC values for these newborns amounted about 35% (range: 12-52%), which is extremely low and comparable with values observed in coumarin-treated patients (fig. 1). Vitamin K administration to the mothers during the weeks preceding the delivery resulted in a substantial and significant increase of the cord HBC values (mean: 53%, range: 35-78%).

Figure 1: Effect of maternal vitamin K administration on hydroxyapatite binding capacity (HBC) of fetal serum osteocalcin. Serum was prepared by venipuncture of the cord veins, and frozen within 2 h at -80 °C until use. Closed symbols: mothers treated with placebo; open symbols: mothers treated with vitamin K (1 mg/day). The difference between both groups is significant at $p < 0.0001$.

This demonstrates that placental vitamin K transport increases as a response to elevated maternal vitamin K intake, leading to a raised fetal vitamin K status. At delivery the HBC values of placebo-treated mothers were slightly but significantly lower than those in vitamin K-treated women (fig. 2), suggesting that at that time both mother and child in the placebo group had a poor vitamin K status.

Figure 2: Effect of maternal vitamin K administration on HBC of maternal serum osteocalcin. Details are as in the legend to fig. 1. The difference between both groups is significant at $p < 0.02$.

Experiment 2. In their first day of life, newborns excreted 68.0 ± 6.52 mg Ca/g creat., 12.1 ± 1.1 mg Mg/g creat. and 34.8 ± 2.5 mg Gla/g creat. Figure 3 shows the histogram for Gla excretion in the first newborn urines ($n=29$). All newborns had higher calcium and magnesium excretion in day 3 of life (Table I). Urinary Gla did not increase in the vitamin K supplemented group, two days after 1mg K_1 intake.

Table I: Calcium, magnesium and Gla excretion in the newborns with (K_1) or without vitamin K_1 supplementation (exclusively breastfed).

MARKER	GROUP	DAY 1	DAY 3	P-VALUE
Gla/creat [mg Gla/g cr.]	K_1	36.9 ± 3.6	42.4 ± 5.9	N.s.
	Breastfed	32.6 ± 5.7	39.7 ± 7.9	N.s.
Calcium/creat [mg Ca/g cr.]	K_1	69 ± 7.1	102.2 ± 13.6	0.001
	Breastfed	67 ± 5.9	98.6 ± 10.4	0.01
Magnesium/creat [mg Mg/g cr.]	K_1	12.7 ± 1.2	16.2 ± 2.3	0.03
	Breastfed	11.5 ± 0.9	18.8 ± 2.7	0.01

Figure 3: Urinary Gla ratio with creatinine in 29 healthy newborns. The mean of ratios was 34.8 mg Gla/g creat. and the median 31.4 mg Gla/ g creat.

Experiment 3: Adult premenopausal women (25–40 years of age) had serum HBC values which were comparable to those found in men, and the effect of vitamin K administration was negligible (see fig. 4). The postmenopausal women (55–70 years of age) were selected from a previously described group¹², and included 15 fast losers of urinary calcium and 15 age-matched normal losers. It turned out that the postmenopausal women had lower HBC values than the premenopausal women, but the postmenopausal values returned to the premenopausal range upon vitamin K treatment. From these data it is clear that undercarboxylated serum osteocalcin is a very sensitive marker for vitamin K status and that, based on this marker, all newborns and the majority of the postmenopausal women tested were found to be biochemically vitamin K-deficient.

Figure 4: Effect of vitamin K administration on serum HBC in postmenopausal women. Participants were selected on the basis of their urinary calcium excretion, 21 fast losers and 13 normal losers of calcium were included. All participants received vitamin K (1 mg/day) during 4 weeks, and blood samples were taken before and after this period. The difference between the HBC values before and after vitamin K treatment was significant at $p < 0.005$. Explanation of symbols: untreated premenopausal women ($\blacktriangle$); fast losers of urinary calcium before ($\bullet$) and after ($\circ$) vitamin K treatment; normal losers of urinary calcium before ($\blacklozenge$) and after ($\square$) vitamin K treatment.

We have also investigated the extend to which changes in the degree of osteocalcin carboxylation are reflected in the urinary Gla-excretion. This was performed in the postmenopausal women described above. As is shown in fig. 5 their urinary Gla excretion remained unchanged by vitamin K treatment, both in fast and normal losers of urinary calcium. This suggests that only a minor fraction of urinary Gla originates from the degradation of circulating osteocalcin, and that Gla excretion is not a sensitive marker for subclinical vitamin K-deficiency.

Figure 5: Effect of vitamin K administration on urinary γ -carboxyglutamate (Gla) excretion in postmenopausal women. Gla was recorded in the 2-h fasting urines of the same postmenopausal women as shown in fig. 3. Gla was expressed relative to creatinine. The difference between the Gla excretion before and after vitamin K treatment was not significant. Explanation of symbols: o, normal losers of urinary calcium; ●, fast losers of urinary calcium.

Discussion

Our data confirm that undercarboxylated bone Gla-proteins are readily detectable in all newborns and in most elderly women. In the newborns the fraction of descarboxy osteocalcin was substantial, and the wide range of the HBC values observed suggests that this variable may become a sensitive and discriminating test to select those at high risk for developing a clinical vitamin K-deficiency. In none of the cases descarboxy prothrombin was detectable with the routine coagulation tests (one-stage and two-stage prothrombin concentration). The conclusion that low HBC values are indicative for a poor vitamin K status is supported by the fact that in all cases the HBC values increased upon vitamin K administration. Because substantial serum concentrations of descarboxy osteocalcin are detected well before descarboxy prothrombin appears in the circulation, it seems that bone tissue experiences K-deficiency sooner than does liver. The mechanism underlying the difference between liver and bone is unclear at this time, but could be related to the lipoprotein receptor density on hepatocytes and osteoblasts, respectively. Since lipoproteins, notably those containing apolipoprotein E, were reported to play an important role in the

plasma transport of vitamin K¹⁷, a low receptor density on osteoblasts might contribute to the relatively poor vitamin K status of the bones.

Whereas it is well known that the administration of vitamin K-antagonists leads to a substantial decrease of urinary Gla¹⁸, it is striking that an increase of vitamin K intake by subjects with low HBC values did not result in an increased Gla excretion. This demonstrates that in normal, apparently healthy individuals the major part of the Gla-containing proteins occurs in a fully carboxylated form, and that the test for urinary Gla is not sufficiently sensitive to record minor changes in the degree of carboxylation of the bone Gla-proteins. Apparently the nutritional vitamin K intake is sufficient to support the carboxylation of nearly all Gla-proteins to completion. There are two reasons why this may be different for serum osteocalcin. Firstly, the bioavailability of circulating vitamin K for bone may be lower than for liver tissue. This conclusion is supported by the observation that, like osteocalcin, another bone Gla-protein (MGP) also occurs in a partly undercarboxylated form¹⁰. Secondly, it must be realized that fully carboxylated osteocalcin has a much higher affinity for the hydroxyapatite matrix in bone than has descarboxy-osteocalcin. It is to be expected, therefore, that the descarboxy-form is preferentially set free in the blood stream thus causing an enlargement of the effect of minor reductions in vitamin K intake. For that reason we believe that serum descarboxy osteocalcin is an ideal marker for vitamin K status. By its nature, this marker is more sensitive than is descarboxy prothrombin or other blood coagulation factors. By using descarboxy osteocalcin as a marker for vitamin K status, we have demonstrated that all members of a group of 20 untreated newborns were characterized as being biochemically vitamin K-deficient, and that an increased maternal vitamin K intake during pregnancy will increase the fetal vitamin K status. At this time it is impossible to speculate whether HBC can be used as a discriminating test to select those at risk for developing clinical vitamin K deficiency. Moreover, the apparent biochemical vitamin K deficiency in newborns and elderly women need not necessarily be a harmful state during certain periods of development. Contrarily a poor vitamin K status might have an as yet unknown physiological importance, and whether pharmacological doses of vitamin K should be administered to all subjects with low HBC values remains to be seen.

References

1. Vermeer C, Soute BAM, Groenen-van Dooren MMCL, Gijsbers BLMG. Role of vitamin K in the biosynthesis of Gla-proteins. In *"Vitamin K in infancy"* (Ed. A.H. Sutor). Schattauer Verlag, Stuttgart.
2. Furie B, Furie BC. The molecular basis of blood coagulation. *Cell* 1988; 53: 505-518.

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3. Hauschka PV, Lian JB, Cole DEC, Gundberg CM. Osteocalcin and matrix Gla protein: vitamin K dependent proteins in bone. *Phys Rev* 1989; 69: 990-1047.
4. Stenflo J, Fernlund P, Egan W, Roepstorff P. Vitamin K dependent modifications of glutamic acid residues in prothrombin. *Proc Natl Acad Sci USA* 1974; 71: 2730-2733.
5. Haroon Y, Shearer MJ, Rahim S, Gunn WC, McEnery G, Barkhan P. The content of phylloquinone (vitamin K₁) in human milk, cow's milk and infant formula foods determined by high-performance liquid chromatography. *J Nutr* 1982; 112: 1105-1117.
6. Von Kries R, Shearer MJ, Göbel U. Vitamin K in infancy. *Eur J Pediatr* 1988; 147: 106-112.
7. Knapen MHJ, Hamulyák K, Vermeer C. The effect of vitamin K supplementation on circulating osteocalcin (bone Gla-protein) and urinary calcium excretion. *Ann Int Med* 1989; 111: 1001-1005.
8. Motohara K, Kuroki Y, Kan H, Endo F, Matsuda I. Detection of vitamin K deficiency by use of an enzyme-linked immunosorbent assay for circulating abnormal prothrombin. *Pediatr Res* 1985; 19: 354-357.
9. Widdershoven J, van Munster P, De Abreu R, Bosman H, van Lith T, van der Putten-van Meyel M, Motohara K, Matsuda I. Four methods compared for measuring des-carboxy-prothrombin (PIVKA-II). *Clin Chem* 1987; 33: 2074-2078.
10. Price PA. Role of vitamin K-dependent proteins in bone metabolism. *Annu Rev Nutr* 1988; 8: 565-583.
11. Plantalech L, Guillaumont M, Vergnaud P, Leclercq M, Delmas PD. Impairment of gamma carboxylation of circulating osteocalcin (bone Gla protein) in elderly women. *J Bone Miner Res* 1991; 6: 1211-1216.
12. Knapen MHJ, Jie K-SG, Hamulyák K, Vermeer C. Vitamin K-induced changes in markers for osteoblast activity and urinary calcium loss. *Calcif Tissue Int* 1993; 53: 81-85.
13. Szulc P, Chapuy M-C, Meunier PJ, Delmas PD. Serum undercarboxylated osteocalcin is a marker of the risk of hip fracture in elderly women. *J Clin Invest* 1993; 91: 1769-1774.
14. Vermeer C, Soute BAM, Hemker HC. A new method for the preparation of artificial factor II reagents from normal human and bovine plasma. *Thromb Res* 1977; 10: 495-507.
15. Kuwada M, Katayama K. An improved method for determination of gammacarboxyglutamic acid in proteins, bone and urine. *Analyt Biochem* 1983; 131: 173-179.
16. Goto K, Kato S, Sumiya S, Uehara S, Hirayama A, Sakamoto W, Tokumitsu Y, Takada M. Urinary levels of γ -carboxyglutamic acid and its clinical significance. *Biol. Pharm. Bull.* 1994; 17: 142-145.
17. Saupe J, Shearer MJ, Kohlmeier M. Phylloquinone transport and its influence on γ -carboxyglutamate residues of osteocalcin in patients on maintenance hemodialysis. *Am J Clin Nutr* 1993; 58: 204-208.
18. Levy RJ, Lian JB. γ -Carboxyglutamate excretion and warfarin therapy. *Clin Pharmacol Ther* 1979; 25: 562-570.

Development of a new enzymatic assay for quantitative determination of osteocalcin

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Summary

Circulating osteocalcin is a well known marker for bone formation, but none of the commercial kits presently available can be used in automated systems. Here we present the first semi-automated assay for human serum osteocalcin.

Polystyrene beads were coated with antibodies against the C-terminus of osteocalcin and used in the Cobas® EIA system. Osteocalcin was detected with peroxidase-conjugated antibodies against the osteocalcin N-terminus. The time required to analyse an unknown sample was 60 min, with a lower detection limit of 4.5 µg/L and a linear dose-response curve between 4.5 and 100 µg/L. The intra-assay imprecision (CV) was 5-8% (n=21), the inter-assay variation was 6-9% (n=14). In a number of samples from human volunteers and patients data generated with the newly developed assay were comparable to those obtained with standard microtiter plate-based assays. The coated beads assay may be implemented on fully automated analyzers, which may not only further reduce imprecision, but will also substantially increase the applicability of osteocalcin as a marker for bone metabolism in the routine clinical setting.

Introduction

Osteocalcin, also known as Bone Gla-Protein (BGP) is the most abundant non-collagenous protein in mature bone^{1,2}. Osteoblasts are the exclusive site of osteocalcin biosynthesis, which is regulated at the level of transcription by vitamin D^{3,4}, whereas vitamin K is required for the posttranslational formation of its three γ -carboxyglutamate (Gla) residues. Although its function on a molecular level has remained unclear thus far, increased bone formation including higher bone mass and improved bone strength were observed in osteocalcin-deficient (knock-out) mice⁵. These experiments have demonstrated that osteocalcin has an important role in the regulation of bone growth, and in the correct deposition of the mineral matrix in bone. Because 20-30% of the de novo synthesized osteocalcin is not accumulated in the bone tissue, but secreted into the blood stream, circulating osteocalcin is widely used as a biomarker for bone formation⁶⁻⁸.

During episodes of vitamin K-deficiency or during treatment with vitamin K-antagonists, Gla-containing proteins are synthesized in an undercarboxylated (Gla-deficient) form. Normal and undercarboxylated osteocalcin may be quantified separately on the basis of their different affinity for hydroxyapatite⁹. It has been reported by various groups that in the normal population a substantial fraction of circulating osteocalcin occurs in its undercarboxylated form⁹⁻¹¹, and that the level of

undercarboxylated osteocalcin may have an independent diagnostic value for the assessment of bone mass and bone fracture risk¹²⁻¹⁴. During recent years an increasing number of test kits for serum osteocalcin has become commercially available all of which are either radio immuno assays, or enzyme-based immuno assays for microtiter plates. Drawbacks of such tests are that they are laborious and prone to personal errors, and that they generally require long incubation steps (either overday or overnight) before the data will become available. Also, the various osteocalcin kits may give widely different values when the same serum sample is tested^{6,15}. This may be related to different specificities of the antibodies used for normal and undercarboxylated osteocalcin, and to the question whether or not osteocalcin degradation products are recognized.

To improve the accuracy and reproducibility of osteocalcin quantitation, we have used commercial antibodies which were coated onto polystyrene beads and used in the Roche Cobas EIA System. The performance of this semi-automated enzyme immunoassay is compared with commercial radiometric and microtiter plate-based assays for various markers of bone metabolism.

Materials and Methods

Subjects

The normal ranges for the concentrations of osteocalcin (OC) and bone-specific alkaline phosphatase (BAP) were established in serum samples obtained from 151 apparently healthy subjects (60 men, 91 women) between 19 and 86 years of age who were recruited via a local newspaper call. The within-day and day-to-day variations of OC and several other markers were examined in 12 apparently healthy volunteers (6 men and 6 women) aging between 19 and 27 years recruited among the students of the Maastricht University.

During the first 24 h of the experiment urine and blood were collected at 09.00 a.m. (start) and at 11.00, 14.00, 17.00, 21.00, 23.00, 03.00, 07.00. Subsequent samples were collected at 09.00 a.m. of days 2, 8, 15, 22, 29, and 57. All samples taken at 09.00 a.m. were obtained after an overnight fast (only water allowed after 19.00 of the preceding night). Changes of bone markers during the follow-up of treatment were recorded in serum and urine samples from 30 osteoporotic women (>65 years of age), before and after treatment with either bisphosphonate (Alendronate, Merck, New Jersey, 5-10 mg/day for 15 months), estrogen (Livial, Organon, The Netherlands, 2.5 mg/day, 6 months), or calcitonin (Miacalcic nasal application, Sandoz, Switzerland, 100 IU/day, 12 months). All studies have been approved by the University Hospital Medical Ethics Committee.

Sample collection and storage

Blood (10 mL) was taken by venipuncture to prepare serum, 0.5 mL aliquots of which were frozen at -80 °C until use within 2 h after sample collection. All urine samples collected at the 09:00 h time points were obtained after an overnight fasting period (14 h) and represent the 2 h second morning void. Other urine samples collected during the first day were non-fasting samples. All urine samples were stored in 0.5 mL aliquots at -80 °C until use.

Procedure for the semi-automated osteocalcin immuno assay

The assay is based on commercial monoclonal antibodies against synthetic peptides homologous to OC residues 1-19 and 20-43 (Osteometer, Copenhagen, Denmark), which will be designated here as mAb¹⁻¹⁹ and mAb²⁰⁻⁴³. Polystyrene beads (Ø 2 mm) were coated with mAb²⁰⁻⁴³ by Osteometer, other components for the test were those in the microtiter-based N-midTM Osteocalcin ELISA (Osteometer, Copenhagen, Denmark), and are described by Rosenquist et al.¹⁶. The test procedure, for which we used the Cobas EIA system from Hoffmann-La Roche (Basel, Switzerland), was as follows: 25 µL of sample (either calibrator, or control, or serum) were pipetted into polycarbonate tubes, together with 225 µL of buffer A (0.14 M NaCl, 0.01 M Na-phosphate, pH 7.4) and 25 µL of peroxidase-conjugated mAb¹⁻¹⁹. Subsequently one coated bead was added to each tube and incubated for 30 min at 37 °C while shaking in the Cobas EIA Incubator. Next, the beads were washed with distilled water in the Cobas EIA Washer, and 250 µL of substrate solution (tetramethylbenzidine) was added, after which the tubes were incubated for another 15 min at 37 °C with constant shaking. The reaction was stopped by adding 1 mL of 0.1 M H₂SO₄ and within 1 h after termination of the reaction the optical density at 450 nm was recorded using a 25-channel COBAS EIA Photometer. Serum osteocalcin concentrations were calculated using a 4-parameter logistic curve fit based on the calibrators of the assay (0, 6.25, 12.5, 25, 50, and 100 µg/L).

Other tests used

The data obtained with the experimental OC assay were compared with commercial kits for OC (ELSA-Osteo, CIS Bio-international, Gif-sur-Yvette, France), and bone alkaline phosphatase (BAP: Tandem-R Ostase, Hybritech Inc., San Diego, CA). Both tests are two-site immunoradiometric assays (IRMA). Intact parathyroid hormone (PTH) was quantified in serum with the N-tact PTH radioimmuno assay from Incstar (Stillwater, MN). Markers tested in urine were: hydroxyproline (OHPro: hypronosticon, Organon Teknika, Oss, The Netherlands), deoxypyridinoline (DPD: pyrilinks-D, Metra Biosystems, Mountain View, CA), type I collagen C-terminal telopeptides (CTX:

crosslaps, Osteometer BioTech, Copenhagen, Denmark). Creatinine was assessed in urine by standard enzymatic techniques (Boehringer Mannheim, Germany) on a Beckman Synchron CX7-2 autoanalyser (Fullerton, CA). Urinary calcium was determined by atomic absorption spectrophotometry (Perkin Elmer, Norwalk, CN). Urinary markers are expressed as the ratio between these markers and creatinine throughout this report.

Data analysis.

Statistical analysis was performed with the software package SPSSWin, version 7.5 (SPSS, Chicago, IL). All results are given as the mean value $\pm$ SD. Differences between the groups were investigated with the unpaired Student's t-test. The Wilcoxon test was used for the evaluation of differences within groups. Differences were considered to be significant at $p < 0.05$. Correlations between the data obtained with different test procedures were evaluated using linear regression.

Results

Calibration curve and test characteristics

Calibration curves were constructed on 12 different days using six calibrator solutions with OC concentrations in the range of 0-100 $\mu\text{g/L}$. Each calibrator was measured in triplicate, and the mean absorbance at 450 nm ($\pm$ SD) was expressed as a function of the OC concentration (figure 1). The lower limit to detection, defined as the mean absorbance $\pm$ 3 SD for calibrator A (0 $\mu\text{g/L}$), was 4.45 $\mu\text{g/L}$ (absorbance = $0.05 + 3 \times 0.02 = 0.11$). The intra- and inter-assay variation of the test were determined using three serum pools of known OC concentrations. The intra-assay variation was calculated by expressing the standard deviation as a percentage of the mean concentration as calculated from 21 replicates of each serum pool, which was repeated on three different days. Mean values obtained were: 7.6, 6.0, and 4.8% for serum pools containing 10, 22, and 83 $\mu\text{g/L}$ of OC, respectively. The same serum pools were determined in duplicate on 14 consecutive days and inter-assay variations of the means of duplicates were calculated by expressing the standard deviations as a percentage of the means: 6.0, 9.1, and 5.9%, respectively.

Figure 1. Calibration curve for osteocalcin by Coated Beads Assay. Calibrators used were from the commercial N-midTM Osteocalcin assay (Osteometer, Denmark). Error bars indicate standard deviation.

To determine the linearity on dilution we used three human serum samples containing pathologically high OC concentrations (from patients with renal failure). Samples with OC concentrations well above the highest calibrator (F: 100 $\mu\text{g/L}$) were pre-diluted with calibrator A (0 $\mu\text{g/L}$) before use to give the following OC concentrations: sample A (undiluted) 97.4 ± 2.2 $\mu\text{g/L}$, sample B (pre-diluted two-fold) 94.3 ± 2.6 $\mu\text{g/L}$, and sample C (pre-diluted 5-fold): 75.3 ± 0.7 $\mu\text{g/L}$. On three different days each sample was diluted serially and tested in duplicate. Recoveries were calculated and expressed as a percentage of the starting values (table I), and it is obvious that sample A can be diluted two fold without significant loss of recovery. As was the case with all ($n=6$) microtiter plate-based kits and radioimmuno assays checked by us thus far (data not shown), the recovery declined at higher dilutions. Both pre-diluted samples (B and C) showed a strong decrease of recovery after further dilutions, which shows that more than 2-fold dilution of serum samples will lead to substantial underestimation of the OC concentration in these samples if the dilutions are prepared with the calibrator A (0 $\mu\text{g/L}$) having not a sufficiently similar matrix to serum.

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Table 1: Recovery of osteocalcin in pathological serum samples after serial dilution.

	Sample A undiluted serum	Sample B 2-fold pre-diluted	Sample C 5-fold pre-diluted
OC concentration (µg/L)	117.4	114.3	75.3

Dilution factor	Recovery of osteocalcin (%) after dilution		
1.00	100.0 ± 1.2	100.0 ± 2.3	100.0 ± 2.9
1.25	102.7 ± 1.8	91.9 ± 3.4	93.6 ± 4.2
1.67	105.0 ± 4.7	88.4 ± 3.9	88.7 ± 7.4
2.00	99.7 ± 4.7	90.0 ± 8.9	85.1 ± 5.4
2.50	96.6 ± 2.4	84.0 ± 6.2	79.9 ± 4.5
5.00	80.0 ± 5.1	77.5 ± 3.5	69.5 ± 6.0

The samples were diluted with calibrator A, and the observed OC concentrations were multiplied by the dilution factor. All samples were tested in triplicate, and the experiment was repeated on three different days. Mean values are given ± SD.

To investigate whether the antibodies used in our assay discriminate between osteocalcins containing different numbers of Gla-residues, full length synthetic osteocalcins¹⁷ containing either 0 or 3 Gla-residues were dissolved in calibrator A and tested in various dilutions. Both synthetic peptides were recognized well, but the antibodies did not differentiate between fully carboxylated and non-carboxylated osteocalcin (data not shown).

Variations related to age and gender

In this experiment we have assembled serum samples from apparently healthy men (n=60) and women (n=91) of different ages, and we have compared the new assay with commercially available test kits for OC and for BAP. The two OC assays are closely similar, with all three bone formation markers slightly elevated in the young men, possibly because in this group the peak bone mass was not yet reached (table II). Only in the case of the commercial OC kit was this difference statistically significant, however. Also in the higher age group in women the bone formation markers had a tendency to increase, which may be related to the increased bone turnover frequently seen during postmenopausal bone loss. Only in the case of BAP was this increase statistically significant, however.

Day-to-day and within-day variations

Twelve subjects (6 men and 6 women) were enrolled in an experiment at which blood and urine samples were collected at various time points during the first 24 hours, and at weekly intervals during the first month with a final sample collection after 2 months. In the serum we measured OC by two assays, BAP, and PTH, and in the urine DPD, CTX, OHPPro, total Ca^{2+} , and creatinine. For each subject and each variable separately the individual mean values of the 7 fasting morning samples (taken at 09.00 a.m.) were calculated and used for calculating the group mean values (table III, lanes 1, 3, and 5). Individual day-to-day variation coefficients were expressed as a percentage of the corresponding individual mean values and used for calculating the mean intra-individual variation in the group (table III, lanes 2, 4, and 6). It turned out that the serum markers (except PTH) were relatively stable in time, and that both the inter- and intra-individual variation of the Coated Bead Assay were comparable with commercial kits for bone markers. The urinary markers, notably OHPPro and CTX had large intra- and inter-individual variation coefficients.

The within-day variation of the various markers was assessed from the samples obtained during the first 24 hours of the experiment (figure 2). The individual variation of the bone formation markers OC (determined with both assays) and BAP are given in the different plots. No distinct diurnal variation was found for any of the three assays. A more pronounced diurnal pattern was observed for the bone resorption markers, but due to the large interindividual variation, the difference between zenith and nadir did not reach statistical significance (data not shown).

Table II: Bone formation markers in different age groups of men and women.

Age range	mean age	n	Osteocalcin (µg/L)		Bone-specific alkaline phosphatase (µg/L)
			Beads	IRMA	
Men:					
19-40	27.5 ± 6.3	30	21.7 ± 10.1	25.2 ± 10.4	16.7 ± 5.3
59-86	74.4 ± 6.5	30	16.8 ± 11.9	16.6 ± 9.6*	13.9 ± 8.1
Women:					
19-40	30.5 ± 6.1	31	18.8 ± 6.0	16.6 ± 6.1	11.5 ± 4.7
41-58	51.1 ± 4.9	28	18.4 ± 6.6	16.5 ± 6.8	9.5 ± 5.0
59-86	75.1 ± 6.3	32	22.0 ± 10.6	17.5 ± 9.4	15.1 ± 6.4 [#]

*All samples were tested in triplicate, and mean values are given ± SD. Statistics were performed with the unpaired Student t-test. Explanation of symbols: *, significantly different from men in the young age group; [#], significantly different from women in the middle-aged group.*

Table III: Day to day variation of bone markers during two months.

Test	All (n = 12)		Men (n = 6)		Women (n = 6)	
	Mean $\pm$ SD	Day to day variation (%)	Mean $\pm$ SD	Day to day variation (%)	Mean $\pm$ SD	Day to day variation (%)
<i>In serum</i>						
OC (μ g/L, coated beads)	23.7 $\pm$ 6.0	10.8	23.1 $\pm$ 6.5	8.3	24.3 $\pm$ 2.7	15.5
OC (μ g/L, ELSA-Osteo)	26.0 $\pm$ 6.1	9.1	25.0 $\pm$ 6.4	9.6	27.2 $\pm$ 6.1	8.4
BAP (μ g/L)	15.8 $\pm$ 7.7	11.7	11.9 $\pm$ 3.2	13.3	19.6 $\pm$ 9.2	9.9
PTH (ng/L)	31.1 $\pm$ 5.6	29	28.9 $\pm$ 6.1	27.5	33.2 $\pm$ 4.5	30.5
<i>In urine</i>						
DPD/creat (μ mol/mol)	6.9 $\pm$ 2.8	24.1	5.2 $\pm$ 2.2	20.7	8.6 $\pm$ 2.5	27.6
OHP/creat (g/mol)	2.2 $\pm$ 0.9	42.3	1.7 $\pm$ 0.5	44.5	2.7 $\pm$ 0.9	40
CTX/creat (mg/mol)	160.8 $\pm$ 104.2	52.4	109.3 $\pm$ 56.6	58.7	212.3 $\pm$ 119.7	46.1
Calcium/creat (mol/mol)	0.25 $\pm$ 0.09	36.3	0.2 $\pm$ 0.06	37	0.3 $\pm$ 0.1	35.5

Abbreviations used: OC, osteocalcin; BAP, bone-specific alkaline phosphatase; PTH, parathyroid hormone; DPD, deoxypyridinoline; OHP, hydroxyproline; CTX, type I collagen C-terminal telopeptides; creat, creatinine.

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Figure 2. Within-day variation of bone formation markers. Fig. 2A: Individual plots of osteocalcin (OC) determined with the Coated Beads Assay during consecutive time points within 24 hours, fig. 2B: Individual plots of osteocalcin determined with the Elsa-Osteo assay, fig. 2C: Individual plots of bone alkaline phosphatase (BAP). All curves are presented as the concentration of each marker ($\mu\text{g/L}$). The mean values per time point are given by the bold line. Error bars indicate the standard deviation for each time point.

Follow-up during osteoporosis treatment

To test the ability of the Coated Beads Assay to detect changes in serum OC concentration during therapy, three groups of 10 postmenopausal women were followed during their treatment with either bisphosphonates, estrogen, or calcitonin (see table IV). Changes during therapy were more pronounced for OC than for BAP, with similar relative changes for the two OC assays. Therapy-induced changes were also large in the bone resorption markers, but because of the large standard deviation of urinary markers, the changes were not statistically significant in all cases.

Correlation between bone formation markers

To investigate the correlation between the various tests for bone formation, the data from the former experiments were pooled. In total the data of 181 subjects (60 men, 91 women and the baseline measurements of 30 postmenopausal women) were used to perform linear regression. The correlation between both OC assays was $R=0.879$ ($p<0.0001$) and the standard deviation of the residual was $4.66 \mu\text{g/L}$. Regression analysis between BAP and both OC assays resulted in rather poor regression coefficients and higher standard deviations of the residual (see table V).

Table IV: Bone markers in postmenopausal women before and after therapy.

Treatment	OC (beads) (µg/L)	OC (ELSA-Osteo) (µg/L)	BAP (µg/L)	Ca/creat (mol/mol)	DPD/creat (mol/mol)	CTX/creat (mg/mol)
Biphosphonates:						
before	28.2 ± 13.1	29.8 ± 12.5	12.9 ± 4.0	0.61 ± 0.40	29.8 ± 54.0	532 ± 1698
after	10.3 ± 3.07**	8.9 ± 3.91**	7.0 ± 2.06**	0.63 ± 1.14	17.6 ± 26.2	173 ± 332*
Hormone replacement:						
before	23.6 ± 7.7	28.0 ± 8.7	6.6 ± 2.3	0.49 ± 0.33	19.8 ± 26.4	578 ± 307
after	17.6 ± 4.2*	18.5 ± 5.0**	5.6 ± 2.9*	0.31 ± 0.29*	10.1 ± 4.8	210 ± 144*
Calcitonin:						
before	21.1 ± 8.0	23.8 ± 8.7	14.1 ± 2.7	0.4 ± 0.3	13.0 ± 6.6	801 ± 459
after	16.0 ± 5.1*	19.1 ± 9.7*	11.9 ± 1.7*	0.4 ± 0.3	11.7 ± 5.2	609 ± 338*

Changes within each treatment group were analyzed with the Wilcoxon test. Explanation of symbols: *, $p < 0.05$; **, $p < 0.005$. For abbreviations see legend to table III.

Table V: Linear regression analysis of bone formation markers.

Dependent variable	Independent variable	Slope	Intercept	R	P	S_{yx} $\mu\text{g/L}$
OC (Elsa-Osteo)	OC (coated beads)	0.89 (0.036)	1.86 (0.81)	0.89	<0.0001	4.66
BAP	OC (coated beads)	0.32 (0.059)	7.21 (1.32)	0.378	<0.0001	7.58
BAP	OC (Elsa-Osteo)	0.22 (0.061)	9.41 (1.35)	0.259	<0.0001	7.91

Linear regression analysis was performed according the equation: $y = a_0 (SD) + a_1 (SD) \times X$, in which y is the dependent variable and X is the independent variable. a_0 is the intercept and a_1 is the slope of the equation, both with corresponding standard deviation (SD). For each equation the regression coefficient (R) and level of significance (p-value) are given. S_{yx} is the standard deviation of the residual.

Discussion

Osteocalcin is the most abundant non-collagenous protein in bone. It is synthesized by the osteoblasts and after its cellular secretion about 80% is bound to the hydroxyapatite matrix. The remaining part is set free in the blood stream, where it is available for detection². Circulating osteocalcin is used as a marker for bone formation, and high levels have been observed in children² and in patients with high bone turnover, as is found in Paget's disease and postmenopausal osteoporosis^{18,19}. Commercial test kits for osteocalcin detection are based on either of two principles: radio immuno assay or enzyme-linked immuno assays. The use of osteocalcin as a marker for bone metabolism in routine clinical chemistry is mainly restricted because of two reasons: the large differences between the various kits, and the fact that no automated test is available thus far. The former problem may be related to the fact that serum osteocalcin occurs in at least two conformations: normal osteocalcin containing three residues of the unusual aminoacid Gla, and undercarboxylated osteocalcin with 0-2 Gla-residues^{20,21}. Antibodies (either polyclonal or monoclonal) used in different kits differ from each other in their relative affinity for normal and undercarboxylated osteocalcin¹⁵, and thus in the amounts which are detected in the same serum sample. An additional problem is that some kits detect only intact osteocalcin, whereas others recognize both intact and fragmented osteocalcin. Hence standardization of reference samples and antibodies used is an absolute requirement before data obtained with various kits may be compared. From a technical point of view a serious drawback of existing kits is that they are test tube or microtiter plate based assays, which have to be pipetted by hand. This is laborious, and forms a potential source of imprecision and mistakes. Therefore, we have used and evaluated a semi-automated method for osteocalcin detection using the antibodies and standards present in a commercial enzyme-linked immuno sorbent assay. The test is based on the sandwich principle with antibodies directed against epitopes at the N- and C-terminus of osteocalcin outside the Gla-domain, thus ensuring that only intact osteocalcin (both normal and undercarboxylated) is detected. It turned out that the newly developed assay (provisionally designated as Coated Beads Assay) has a high precision, and compares well with the kit from CIS Biointernational both in within-day and day-to-day variation, as well as in patient follow-up studies. Like other test for bone formation, the standard deviation of the Coated Beads Assay was much lower than that of bone resorption markers, and a good correlation was observed with osteocalcin values determined with the CIS kit. In conclusion, we have demonstrated that with the semi-automated assay osteocalcin may be determined within 1 hour, and that the assay is at least comparably accurate

as existing kits. The new test still requires a small number of pipettations, but the coated beads may be used equally well in fully automated auto-analyzers.

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References

1. Price PA. Role of vitamin K-dependent proteins in bone metabolism [Review]. *Annu Rev Nutr* 1988;8:565-83.
2. Hauschka PV, Lian JB, Cole DEC, Gundberg CM. Osteocalcin and matrix Gla protein: vitamin K dependent proteins in bone [Review]. *Phys Rev* 1989;69:990-1047.
3. Kesterson RA, Stanley L, DeMayo F, Finegold M, Pike JW. The human osteocalcin promoter directs bone-specific vitamin D-regulatable gene expression in transgenic mice. *Mol Endocrinol* 1993;7:462-7.
4. Ozono K, Liao J, Kerner SA, Scott RA, Pike JW. The vitamin D-responsive element in the human osteocalcin gene. Association with a nuclear proto-oncogene enhancer. *J Biol Chem* 1990;265:21881-8.
5. Luo G, Ducy P, McKee MD, Pinero GJ, Loyer E, Behringer RR, Karsenty G. Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. *Nature* 1997;386:78-81.
6. Power MJ, Fottrell PF. Osteocalcin: diagnostic methods and clinical applications [Review]. *Crit Rev Clin Lab Sci* 1991;28:287-335.
7. Garnero P, Grimaux M, Demiaux B, Preaudat C, Seguin P, Delmas PD. Measurement of serum osteocalcin with a human-specific two-site immunoradiometric assay. *J Bone Miner Res* 1992;7:1389-98.
8. Eastell R, Robins SP, Colwell T, Assiri AMA, Riggs BL, Russell RGG. Evaluation of bone turnover in type I osteoporosis using biochemical markers specific for both bone formation and bone resorption. *Osteoporosis International* 1993;3:255-60.
9. Knapen MHJ, Hamulyák K, Vermeer C. The effect of vitamin K supplementation on circulating osteocalcin (bone Gla-protein) and urinary calcium excretion. *Ann Int Med* 1989;111:1001-5.
10. Knapen MHJ, Jie K-SG, Hamulyák K, Vermeer C. Vitamin K-induced changes in markers for osteoblast activity and urinary calcium loss. *Calcif Tissue Int* 1993;53:81-5.

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11. Plantalech L, Guillaumont M, Vergnaud P, Leclercq M, Delmas PD. Impairment of gamma carboxylation of circulating osteocalcin (bone Gla protein) in elderly women. *J Bone Miner Res* 1991;6:1211-6.
12. Szulc P, Chapuy M-C, Meunier PJ, Delmas P.D. Serum undercarboxylated osteocalcin is a marker of the risk of hip fracture in elderly women. *J Clin Invest* 1993;91:1769-74.
13. Szulc P, Arlot M, Chapuy M-C, Duboeuf F, Meunier PJ, Delmas PD. Serum undercarboxylated osteocalcin correlates with hip bone mineral density in elderly women. *J Bone Miner Res* 1994;9:1591-5.
14. Knapen MHJ, Nieuwenhuijzen Kruseman AC, Wouters RSME, Vermeer C. Correlation of serum osteocalcin with bone mineral density in women during the first 10 years after menopause. *J Bone Miner Res* 1998;63:375-9.
15. Knapen MHJ, Eisenwiener H-G, Vermeer C. Osteocalcin detection in aging serum and whole blood: stability of different osteocalcin fractions. *Clin Chim Acta* 1996;256:151-64.
16. Rosenquist C, Qvist P, Bjarnason N, Christiansen C. Measurement of a more stable region of osteocalcin in serum by ELISA with two monoclonal antibodies. *Clin Chem* 1995;41:1439-45.
17. Houben RJTJ, Jin D, Stafford DW, Proost P, Ebberink RHM, Vermeer C, Soute BAM. Osteocalcin binds tightly to the γ -glutamyl carboxylase at a site distinct from that of the other known vitamin K-dependent proteins. *Biochem J* 1999;341:265-9.
18. Gundberg CM, Lian JB, Gallop PM, Steinberg JJ. Urinary gammacarboxyglutamic acid and serum osteocalcin as bone markers: studies in osteoporosis and Paget's disease. *J Clin Endocrinol Metab* 1983;57:1221-5.
19. Kaddam IMS, Iqbal SJ, Holland S, Wong M, Manning D. Comparison of serum osteocalcin with total and bone specific alkaline phosphatase and urinary hydroxyproline:creatinine ratio in patients with Paget's disease of bone. *Ann Clin Biochem* 1994;31:327-30.
20. Cairns JR, Price PA. Direct demonstration that the vitamin K-dependent bone Gla protein is incompletely γ -carboxylated in humans. *J Bone Miner Res* 1994;9:1989-97.
21. Szulc P, Chapuy M-C, Meunier PJ, Delmas PD. Serum undercarboxylated osteocalcin is a marker of the risk of hip fracture in elderly women. *J Clin Invest* 1993;91:1769-74.

Potential effects of vitamin K on bone mass and bone metabolism

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Summary

Vitamin K is involved in blood coagulation and in bone metabolism via the carboxylation of glutamate residues in (hepatic) blood coagulation factors and (osteoblastic) bone proteins. The bioavailability of nutritional vitamin K depends on the type of food, the dietary fat content, the length of the aliphatic side chain in the K-vitamer, and probably also the genetically determined polymorphism of apolipoprotein E. Whereas undercarboxylation of blood coagulation factors is very rare, undercarboxylated osteocalcin (bone Gla-protein) is frequently found in postmenopausal women. Supplementation of these women with extra vitamin K causes the markers for bone formation to increase. In parallel a decrease of the markers for bone resorption is frequently seen. Insufficient data are available to conclude that the regular administration of vitamin K concentrates will reduce the loss of bone mass in Caucasian women at risk for developing postmenopausal osteoporosis.

Introduction

Vitamin K is involved in the synthesis of a number of well-defined proteins, the vitamin K-dependent step being the addition of an extra carboxyl group at the γ -position of glutamate residues¹. The product formed is an unusual amino acid called γ -carboxyglutamate (Gla). There are two physiological processes in which Gla-proteins are known to play a role: blood coagulation and bone metabolism^{2,3}. Those participating in blood coagulation are of hepatic origin, whereas the bone Gla-proteins are synthesized by the bone-forming cells (osteoblasts). Recent publications indicate that Gla-proteins may also be involved in the regulation of cell growth^{4,5}.

The human vitamin K supply may originate from 2 sources: the intestinal flora and the diet. Vitamin K₁ (phyloquinone) originates from green vegetables, whereas vitamin K₂ (menaquinones) is present in meat, fish, and fermented foods^{6,7}. Also the bacteria in the gut produce menaquinones, but the question whether these products substantially contribute to human vitamin K supply has remained a matter of dispute⁸. On the other hand the vitamin K stores in human liver consist for more than 90% of menaquinones and for less than 10% of phyloquinone⁹. Whether these menaquinones can be recruited for the formation of Gla-residues in proteins is unclear.

It is generally accepted that a dietary vitamin K intake of about 1 $\mu\text{g/kg}$ body weight/day is required to ascertain the regular carboxylation of the various blood coagulation factors. Hence vitamin K-deficiency is defined as a state in which

undercarboxylated blood coagulation factors appear in the circulation¹⁰. During recent years increasing evidence has shown that complete carboxylation of the bone Gla-proteins may require a higher vitamin K intake. Based on the Gla-content of the bone protein osteocalcin a substantial part of the population must be characterized as biochemically vitamin K-deficient. In this paper we will review the available data.

Factors determining the bioavailability of nutritional vitamin K

All K vitamers are fat-soluble compounds requiring bile salts for their intestinal absorption. In the small intestine vitamin K is extracted from the food and incorporated into micelles consisting of bile salts, free fatty acids and monoglycerides. These micelles are subsequently taken up by the intestinal mucosa, from which the vitamin - bound to chylomicrons - is set free in the lymphatic system and in the circulation.

Phylloquinone is a component of the photosynthetic electron transport system in plants, and it occurs exclusively in the thylakoid membranes of the chloroplasts¹¹. This binding is very tight, which may be the reason for the fact that the intestinal absorption of phylloquinone from vegetables is poor. In a recent experiment among human volunteers it was shown by Gijsbers et al.¹² that, even after induction of bile secretion by the simultaneous ingestion of fat, less than 10% of the vitamin was absorbed. Nutritional menaquinones are generally dissolved in fats, which may facilitate their intestinal absorption. In Fig. 1 we have compared the cumulative amounts of vitamin K found in serum during the first 10 hours after ingestion of 1 mg of the vitamin, either in the form of spinach, spinach + fat, butter enriched with vitamin K, or a detergent-solubilized pharmaceutical preparation. From this figure it appears that the absorption of vitamin K is strongly dependent on the source from which it is obtained.

Figure 1: Effect of diet composition on circulating vitamin K. Five volunteers received 1 mg of phylloquinone after an overnight fast. The vitamin was either given in the form of Konakion® (week 1, bar A), butter enriched with vitamin K (week 2, bar B), spinach (227 g) + 25 g butter (week 3) or 227 g of spinach without additives (bar C). Serum vitamin K was measured at 1-h intervals during the first 10 h after consumption of these preparations, and corrected for the starting value in each subject. For each individual the cumulative serum vitamin K concentration was calculated from the sum of these 10 measurements. All data are expressed as the means of five volunteers $\pm$ SEM. Circulating phylloquinone was determined as described earlier [12].

A second factor which may influence the absorption of vitamin K is the length of the aliphatic side chain in the menaquinones. This was recently found by Groenen-van Dooren et al. and Craciun et al., who compared the extent to which the oral administration of various forms of vitamin were capable of restoring plasma prothrombin concentrations in vitamin K-deficient and in warfarin-treated rats^{13,14}. In the vitamin K-deficient animals the vitamin K cycle is fully operational and relatively low amounts of vitamin K were required to normalize plasma prothrombin. In this model system the long chain menaquinone-9 had a much higher biological activity than had menaquinone-4¹³. In warfarin-treated rats, on the other hand, the vitamin K cycle is blocked resulting in a more than 1,000 fold higher vitamin K requirement for maintaining normal prothrombin synthesis. In this model menaquinone-4 was perfectly capable of counteracting the effect of warfarin, whereas menaquinone-9 had no detectable effect¹⁴. We explain these results by assuming that - in contrast to menaquinone-4 - the absorption of menaquinone-9 is bound to a certain maximum, which is too low to overcome the effects of vitamin K-antagonists.

Potential effects of vitamin K on bone mass and bone metabolism

The chylomicrons in which vitamin K is set free in the circulation are composed of a hydrophobic core of triglycerides and cholesterylesters, surrounded by an amphipathic shell of apolipoproteins, phospholipids, and free cholesterol. Many kinds of apolipoproteins are known. They are generally classified in apo A, B, C, and E, whereas each class consists of various sub-types. It has recently been claimed that the genetically determined sub-types of apolipoprotein E play a crucial role in the transport of chylomicrons - and thus of vitamin K - to the liver and other target tissues^{15,16}. The mechanism underlying this hypothesis is that the various apolipoprotein subtypes have different affinities for the tissue apolipoprotein receptors. As a consequence they differ with respect to their plasma clearance rate. Since vitamin K is associated with these apolipoproteins, the serum vitamin K levels are low in subjects with apolipoprotein E4/4 or E3/4 (high clearance rate), and they are high in subjects with a low clearance rate (apolipoprotein E2/2 or E2/3). Obviously this hypothesis raises serious questions concerning the value of circulating vitamin K as a marker for tissue vitamin K status.

Undercarboxylated Gla-proteins

In normal, healthy adults undercarboxylated clotting factors are rarely seen. Even with most sensitive tests presently available descarboxyprothrombin was found in only a limited number of newborns¹⁷, a group well known to be prone of developing vitamin K-deficiency¹⁸. It may be concluded, therefore, that the liver is capable of efficiently extracting the required amount of vitamin K from the blood stream, even at very low circulating vitamin K concentrations. This is probably less so for extrahepatic tissues, notably bone. Since direct tests for undercarboxylated osteocalcin (ucOC) are not available, an indirect test has been developed, which is based on the differential affinity of normal and undercarboxylated osteocalcin for hydroxyapatite^{19,20}. In this test the serum immunoreactive osteocalcin (irOC) is fractionated into irOC_{bound} (with high affinity for HA) and irOC_{free} with low affinity. Both fractions may be established using an immunoradiometric assay, preferably one with which no osteocalcin degradation products are detected. It is generally assumed that irOC_{bound} consists of the fully carboxylated protein, whereas irOC_{free} is thought to be an undercarboxylated form of osteocalcin. Indeed the fraction of irOC_{bound} is strongly reduced during oral anticoagulant treatment^{21,22}. It was recently found that at low anticoagulant dosages the synthesis and carboxylation of osteocalcin were much stronger affected than was prothrombin (M.H.J. Knapen, unpublished data). It must be concluded, therefore, that in the same subject bone tissue may be vitamin K-deficient, whereas at the same time the liver is vitamin K-sufficient. Hence circulating

osteocalcin, specifically its carboxylated fraction is a far more sensitive marker for vitamin K status than are the Gla-containing blood coagulation factors²³.

It is at least surprising that $\text{irOC}_{\text{free}}$ has been detectable in all subjects tested. In two separate trials postmenopausal women were supplemented with pharmacological doses of vitamin K, which invariably caused an increase of $\text{irOC}_{\text{bound}}$ and a decrease of $\text{irOC}_{\text{free}}$, but in all cases the latter variable remained detectable even after 3 months of vitamin K treatment (1 mg/day). On the other hand it was found that in coumarin-treated patients the level of serum $\text{irOC}_{\text{bound}}$ decreased by 60-70% (M.H.J. Knapen, unpublished results), but that in the same patients the level of $\text{irOC}_{\text{free}}$ remained nearly constant. This raises the question whether ucOC has a physiological role and whether its secretion is regulated by the osteoblast. Two domains have been described for osteocalcin: the Gla domain, which is required for the very tight binding of the protein to the hydroxyapatite crystals in the bone²⁴, and the C-terminal domain, which has chemotactic properties for osteoclast-like cells^{25,26}. A general phenomenon in chemotaxis is that a gradient of the stimulus is formed against which the attracted cells move to their target, and it is clear that ucOC, with its low affinity for hydroxyapatite, is a much more plausible candidate for building up this concentration gradient than is normal osteocalcin. By regulating the cellular secretion of ucOC osteoblasts would thus be able to regulate the capture of osteoclasts and thus the rate of bone degradation.

Vitamin K status and bone metabolism

There are several indications that postmenopausal loss of bone mass is associated with a long lasting poor vitamin K status. Firstly, nutritional vitamin K intake decreases substantially with age²⁷. Secondly, patients with hip fractures or spinal crush-fractures were reported to have very low levels of circulating phylloquinone²⁸⁻³⁰ and menaquinones³⁰. Thirdly, circulating ucOC was reported to be inversely correlated with hip bone mineral density³¹ and subjects with increased levels of ucOC had a 6-fold increased risk of hip fracture³². Finally it has been shown that vitamin K supplementation increases the serum markers for bone formation (including osteocalcin and bone alkaline phosphatase) and may reduce urinary calcium and hydroxyproline excretion (a well-known marker for bone resorption)^{19,20,33}. It is not surprising, therefore, that clinical trials have been initiated in which the effect of long-term vitamin K supplementation on bone mass is investigated. The results of the first trial (in Japan) have been published and show a marked reduction of postmenopausal bone loss by vitamin K treatment³⁴. At this time the data have not yet been confirmed in the western population, however.

If vitamin K has a positive effect on net bone formation, it might be expected that vitamin K-antagonists (coumarin-derivatives, also known as oral anticoagulants) have an opposite effect. Indeed serious bone deformations were reported to develop in human fetuses during oral anticoagulant treatment of pregnant women³⁵. Animal models were developed in which this so-called fetal warfarin syndrome could be mimicked. It was shown that treatment of young rats with sublethal doses of warfarin induced excessive calcification with growth plate closure and reduced growth of the long bones^{36,37}, maxillonasal hypoplasia, reduced length of the nasal bones and massive calcification of the cartilage of the nasal septum³⁷. The reported effects of warfarin on bone in lambs were even more prominent and included osteopenia with 30% lower bone mass in 3 months (relative to control animals), mildly decreased bone resorption, strongly decreased bone formation, irregular calcium deposition, and remodeling abnormalities³⁸. It is striking that both in humans and in animals substantial effects of warfarin on young rapidly growing bone have been found, whereas in adults the effects are less clear. No placebo-controlled prospective trials have been published in which the potential effects of coumarins on bone were investigated. Conflicting data were obtained from cross-sectional studies among patients on long term oral anticoagulant therapy, with two studies showing a reduced bone mass^{39,40}, whereas in two others no significant differences were found between the bone mass in patients and that in age- and sex-matched controls^{41,42}. The fact that no profound effects of oral anticoagulant therapy on bone metabolism were observed suggests that the mode of action of vitamin K on bone cells may be different from that in the liver.

Without doubt vitamin K acts as a coenzyme for bone carboxylase in the formation of bone Gla-proteins. In this respect it is understandable that during periods of vitamin K-deficiency, or during coumarin treatment, the production of bone Gla-proteins is decreased. It is less clear why also vitamin K-independent bone markers, such as serum alkaline phosphatase or urinary hydroxyproline are affected by nutritional vitamin K intake. Recently it was reported by Hara et al. that menaquinone-4 inhibited the calcium release from mouse calvaria, as well as the formation of osteoclast-like multinucleated cell formation in a co-culture of spleen cells and stromal cells⁴³. A similar effect was not found for phyloquinone, and neither were the effects counteracted by warfarin. In a second series of experiments these investigators used only the aliphatic side chains in stead of the complete vitamers, and it turned out that only geranylgeraniol, the side chain of menaquinone-4, was capable of inducing the inhibitory effects described above. These data indicate that at least part of the effects of menaquinone-4 on bone resorption are not due to γ -carboxylation and that the side

chain of menaquinone-4 may regulate bone cell activity via an as yet unidentified mechanism.

References

1. Vermeer, C. (1990) Gamma-carboxyglutamate-containing proteins and the vitamin K-dependent carboxylase. *Biochem. J.* 266: 625-636.
2. Shearer, M.J. (1995) Vitamin K. *Lancet* 345: 229-234.
3. Hauschka, P.V., Lian, J.B., Cole, D.E.C. & Gundberg, C.M. (1989) Osteocalcin and matrix Gla protein: vitamin K dependent proteins in bone. *Phys. Rev.* 69: 990-1047.
4. Manfioletti G., Brancolini, C., Avanzi, G. & Schneider, C. (1993) The protein encoded by a growth arrest-specific gene (gas6) is a new member of the vitamin K-dependent proteins related to protein S, a negative coregulator in the blood coagulation cascade. *Mol. Cell. Biol.* 13: 4976-4985.
5. Varnum, B.C., Young, C., Elliot, G., Garcia, A., Bartley, T.D., Fridell, Y.W., Hunt, R.W., Trail, G., Clogston, C., Toso, R.J., Yanagihara, D., Bennett, L., Sylber, M., Merewether, L.A., Tseng, A., Escobar, E., Liu, E.T. & Yamane, H.K. (1995) Axl receptor tyrosine kinase stimulated by the vitamin K-dependent protein encoded by growth-arrest-specific gene 6. *Nature* 373: 623-626.
6. Hirauchi, K., Sakano, T., Notsumoto, S., Nagaoka, T., Morimoto, A., Fujimoto, K., Masuda, S. & Suzuki, Y. (1989) Measurement of K vitamins in food by high-performance liquid chromatography with fluorometric detection. *Vitamins (Japan)* 63: 147-151.
7. Sakano, T., Notsumoto, S., Nagaoka, T., Morimoto, A., Fujimoto, K., Masuda, S., Suzuki, Y. & Hirauchi, K. (1988) Measurement of K vitamins in food by high-performance liquid chromatography with fluorometric detection. *Vitamins (Japan)* 62: 393-398.
8. Lipsky, J.J. (1994) Nutritional sources of vitamin K. *Mayo Clin. Proc.* 69: 462-466.
9. Shearer, M.J. (1992) Vitamin K metabolism and nutrition. *Blood Rev.* 6: 92-104.
10. Vermeer, C. & Hamulyák, K. (1991) Pathophysiology of vitamin K deficiency and oral anticoagulants. *Thromb. Haemostas.* 66: 153-159.
11. Lichtenthaler, H.K. (1993) The plant prenyllipids, including carotenoids, chlorophylls and prenylquinones. In: *Plant Lipids* (Moore, T., ed.) pp 421-464. CRC Press, Boca Raton, USA.
12. Gijsbers, B.L.M.G., Jie, K.-S.G. & Vermeer, C. Effect of food composition on vitamin K absorption in human volunteers, submitted for publication.

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on bone mass and bone metabolism**

13. Groenen-van Dooren, M.M.C.L., Ronden, J.E., Soute, B.A.M. & Vermeer, C. (1995) Bioavailability of phyloquinone and menaquinones after oral and colorectal administration in the vitamin K-deficient rat. *Biochem. Pharmacol.*, in press.
14. Craciun, A.M., Groenen-van Dooren, M.M.C.L., Vermeer, C. Induction of prothrombin synthesis by K vitamins: a comparative study between vitamin K-deficient and brodifacoum-treated rats. *Biochim Biophys Acta* 1998; 1380: 75-81.
15. Saupe, J., Shearer, M.J. & Kohlmeier, M. (1993) Phylloquinone transport and its influence on γ -carboxyglutamate residues of osteocalcin in patients on maintenance hemodialysis. *Am. J. Clin. Nutr.* 58: 204-208.
16. Kohlmeier, M., Saupe, J. & Shearer, M.J. (1993) Lipoprotein metabolism determines the disposition of vitamin K1 in hemodialysis patients. *Int. J. Vit. Nutr. Res.* 63: 329.
17. Widdershoven, J., van Munster, P., De Abreu, R., Bosman, H., van Lith, Th., van der Putten-van Meyel, M., Motohara, K. & Matsuda, I. (1987) Four methods compared for measuring des-carboxy-prothrombin (PIVKA-II). *Clin Chem.* 33: 2074-2078.
18. Von Kries, R., Greer, F.R. & Suttie, J.W. (1993) Assessment of vitamin K status of the newborn infant. *J. Pediatr. Gastroenterol. Nutr.* 16 231-238.
19. Knapen, M.H.J., Hamulyák, K. & Vermeer, C. (1989) The effect of vitamin K supplementation on circulating osteocalcin (bone Gla-protein) and urinary calcium excretion. *Ann Int Med* 111: 1001-1005.
20. Knapen, M.H.J., Jie, K.-S.G., Hamulyák, K. & Vermeer C. (1993) Vitamin K-induced changes in markers for osteoblast activity and urinary calcium loss. *Calcif. Tissue Int.* 53: 81-85.
21. Menon, R.K., Gill, D.S., Thomas, M., Kernoff, P.B.A. & Dandona, P. (1987) Impaired carboxylation of osteocalcin in warfarin-treated patients. *J. Clin. Endocrinol. Metab.* 64: 59-61.
22. Jie, K.-S.G., Gijsbers, B.L.M.G., Knapen, M.H.J., Hamulyák, K., Frank, H.L. & Vermeer, C. (1993). Effects of vitamin K and oral anticoagulants on urinary calcium excretion. *Brit. J. Haematol.* 83: 100-104.
23. Knapen, M.H.J., Jie, K.-S.G., Hamulyák, K. & Vermeer, C. (1991) Vitamin K-deficiency redefined. *Thromb. Haemostas.* 65: 671.
24. Hauschka, P.V. & Carr, S.A. (1982) Calcium-dependent alpha-helical structure in osteocalcin. *Biochemistry* 21: 2538-2547.
25. Glowacki, J., Rey, C., Glimcher, M.J., Cox, K.A. & Lian, J.B. (1991) A role for osteocalcin in osteoclast differentiation. *J. Cell. Biochem.* 45: 292-302.

26. Liggett, W.H., Lian, J.B., Greenberger, J.S. & Glowacki, J. (1994) Osteocalcin promotes differentiation of osteoclast progenitors from murine long-term bone marrow cultures. *J. Cell. Biochem.* 55: 190-199.
27. Jie, K.-S.G., Bots, M.L., Vermeer, C., Witteman, J.C.M. & Grobbee, D.E. (1995). Vitamin K intake and osteocalcin levels in women with and without aortic atherosclerosis: a population-based study. *Atherosclerosis* 116: 117-123.
28. Hart, J.P., Catterall, A., Dodds, R.A., Klenerman, L., Shearer, M.J., Bitensky, L. & Chayen, J. (1984) Circulating vitamin K₁ levels in fractured neck of femur. *Lancet* ii: 283.
29. Hart, J.P., Shearer, M.J., Klenerman, L., Catterall, A., Reeve, J., Sambrook, P.N., Dodds, R.A., Bitensky, L. & Chayen, J. (1985) Electrochemical detection of depressed circulating levels of vitamin K₁ in osteoporosis. *J. Clin. Endocrinol. Metab.* 60: 1268-1269.
30. Hodges, S.J., Akesson, K., Vergnaud, P., Obrant, K. & Delmas, P.D. (1993) Circulating levels of vitamins K₁ and K₂ decreased in elderly women with hip fracture. *J. Bone Miner. Res.* 8: 1241-1245.
31. Szulc, P., Arlot, M., Chapuy, M.-C., Duboeuf, F., Meunier, P.J. & Delmas, P.D. (1994) Serum undercarboxylated osteocalcin correlates with hip bone mineral density in elderly women. *J. Bone Miner. Res.* 9: 1591-1595.
32. Szulc, P., Chapuy, M.-C., Meunier, P.J. & Delmas, P.D. (1993) Serum undercarboxylated osteocalcin is a marker of the risk of hip fracture in elderly women. *J. Clin. Invest.* 91: 1769-1774.
33. Plantalech, L.C., Chapuy, M.C., Guillaumont, M., Chapuy, P., Leclercq, M. & Delmas, P.D. (1990) Impaired carboxylation of serum osteocalcin in elderly women: Effect of vitamin K₁ treatment. In: *Osteoporosis 1990* (Christiansen, C. & Overgaard, K., eds), pp. 345-347. Osteopress, Aps, Copenhagen, Denmark.
34. Orimo, H., Shiraki, M., Fujita, T., Onomura, T., Inoue, T. & Kushida, K. (1992) Clinical evaluation of menatetrenone in the treatment of involutional osteoporosis - a double-blind multicenter comparative study with 1 α hydroxy vitamin D₃. *J. Bone Mineral Res.* 7: S122.
35. Pettifor, J.M. & Benson, R. (1975) Congenital malformations associated with the administration of oral anticoagulants during pregnancy. *J. Pediatr.* 86: 459-462.
36. Price, P.A., Williamson, M.K., Haba, T., Dell, R.B. & Jee W.S. (1982) Excessive mineralization with growth plate closure in rats on chronic warfarin treatment. *Proc. Natl. Acad. Sci. USA* 79: 7734-7738.

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37. Howe, A.M. & Webster, W.S. (1992) The warfarin embryopathy: a rat model showing maxillofacial hypoplasia and other skeletal disturbances. *Teratology* 46: 379-390.
38. Pastoureau, P., Vergnaud, P., Meunier, P.J. & Delmas, P.D. (1993) Osteopenia and bone-remodeling abnormalities in warfarin-treated lambs. *J. Bone Miner. Res.* 8: 1417-1426.
39. Fiore, C.E., Tamburino, C., Foti, R. & Grimaldi, D. (1990) Reduced bone mineral content in patients taking an oral anticoagulant. *South. Med. J.* 83: 538-542.
40. Resch, H., Pietschmann, P., Krexner, E. & Willvonseder, R. (1991) Decreased peripheral bone mineral content in patients under anticoagulant therapy with phenprocoumon. *Eur. Heart J.* 12: 439-441.
41. Rosen, H.N., Maitland, L.A., Suttie, J.W., Manning, W.J., Glynn, R.J. & Greenspan, S.L. (1993) Vitamin K and maintenance of skeletal integrity in adults. *Am. J. Med.* 94: 62-68.
42. Piro, L.D., Whyte, M.P., Murphy, W.A. & Birge, S.J. (1982) Normal cortical bone mass in patients after long term coumadin therapy. *J. Clin. Endocrinol. Metab.* 54: 470-473.
43. Hara, K., Akiyama, Y., Nakamura, T., Murota, S. & Morita, I. (1995) The inhibitory effect of vitamin K₂ (menatetrenone) on bone resorption may be related to its side chain. *Bone* 16: 179-184.

Association of vitamin D and vitamin K deficiency in nonsupplemented children

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Summary

One of the objectives of this study was to investigate whether vitamin D and Vitamin K deficiencies are associated in young children, and whether the apolipoprotein E genotype is a genetic factor contributing to these deficiencies. Study design: Vitamin K-deficiency bleeding of the newborn (VKDBN) was analysed in a patient-control design, rickets patients were obtained from a mid-winter cross-sectional study among 127 inhabitants (aged 4–48 months) of one children's home. It came out that VKDBN was not correlated with apoE genotype, but in healthy controls the urinary Gla excretion was significantly lower in subjects bearing the $\epsilon 2$ allele than in others. In rickets the $\epsilon 4$ allele was slightly overrepresented as compared to the adult control population. In January, 36% of all children analysed had developed rickets; besides low circulating vitamin D levels ($8.7 \pm 1.1 \mu\text{g/L}$), also their urinary Gla-excretion ($37.1 \pm 4.4 \text{ mg/g creatinine}$) was about half that in age-matched controls 18.0 ± 4 and 72.6 ± 13 , respectively). Children diagnosed as healthy in January further decreased in serum vitamin D ($7.0 \pm 1.4 \mu\text{g/L}$) and urinary Gla excretion ($45.7 \pm 5 \text{ mg/g}$), whereas 13 more had developed rickets in April. In the population investigated, rickets is frequently associated with subclinical vitamin K deficiency. Supplementation of these children with both vitamins throughout the winter is strongly recommended.

Introduction

Vitamins D and K are two fat-soluble compounds that play different but essential roles in bone metabolism. The normal human diet is a poor source of vitamin D and as much as 80–90% of the body's stores of vitamin D may come from UV irradiation of the skin^{1,2}. The most important sites of vitamin D action are the intestine, the skeleton, and the kidneys; at all three sites its major function is to maintain calcium and phosphate homeostasis.

Although vitamin K is widely distributed in foods, its major dietary source are green vegetables and some plant oils, with only a limited contribution by cheese, fish and meat³. A second potential source of vitamin K is the flora in the distal colon, but the highly lipophilic menaquinones produced there are tightly bound to the bacterial membranes and it is hard to envision how these compounds may be absorbed in the absence of bile salts⁴. Therefore, the contribution of gut bacteria to the human vitamin K status is still a matter of debate⁵. Vitamin K is involved in the posttranslational carboxylation of glutamate residues into γ -carboxyglutamate (Gla). Proteins containing one or more Gla-residues are generally known as Gla-proteins, and the only known function of Gla in these proteins is the binding of calcium ions⁶.

Examples of Gla-proteins are the vitamin K-dependent blood coagulation factors, which are all synthesized in the liver. A well-characterized extra-hepatic Gla-protein is osteocalcin, a 5.8 kD protein containing three Gla residues. Osteocalcin is exclusively synthesized by osteoblasts and odontoblasts in bone and dentin. Vitamin D stimulates osteocalcin synthesis at the level of transcription⁷, whereas its affinity for hydroxyapatite is greatly enhanced by the vitamin K-dependent step during posttranslational modification⁸.

Unlike vitamin K, vitamin D is not stored in the liver but during summer it accumulates in adipose tissue^{9,10}, from where it is set free in winter. Vitamin D deficiency occurs in special population groups such as the elderly (causing osteomalacia) and children (causing rickets); deficiencies are mostly seen during the winter season in northern climates where the atmospheric attenuation of UV radiation in the 290-315 nm band limits vitamin D synthesis in the skin¹¹. Two evolutive stages in rickets may be distinguished¹². In the first stage intestinal calcium absorption is decreased due to poor vitamin D status; this leads to low serum calcium concentrations which is compensated for by osteoclast recruitment and subsequent bone resorption via increased high parathyroid hormone (PTH) secretion. Biochemically, the first phase is characterized by low serum calcium and normal alkaline phosphatase. In the second stage the increased bone resorption has triggered osteoblastic bone formation: the osteoblasts lay down large quantities of osteoid which is not calcified, mainly because of insufficient calcium supply. Consequently, the newly formed uncalcified and weak osteoid gradually takes the place of the older bone that is absorbed. During the latter phase serum calcium is in the normal range, but the increased bone turnover is reflected by the high serum alkaline phosphatase levels.

Classical vitamin K deficiency appears in newborns during their first week of life in the form of gastrointestinal and intracranial bleedings. Factors contributing to the low vitamin K status of newborns are the poor vitamin K transport through the placenta¹³, the absence of gut flora at birth, and the low vitamin K content of breast milk¹⁴. Therefore, prophylactic vitamin K supplementation is common practice in most countries at this time. Romania forms an exception in this respect: all births occur in obstetric clinics, and only cases of classical VKDBN are treated with vitamin K supplements. Remarkably, the incidences of vitamin K-deficiency bleeding of the newborn (VKDBN), rickets (in children) and femur neck fractures (in the elderly) have similar seasonal rhythms, suggesting similar patterns of vitamin K and vitamin D status¹⁵.

Whether derived from skin synthesis or from the diet, vitamin D requires two sequential hydroxylations before becoming biologically active. The first step takes place in the liver and results in 25-hydroxyvitamin D₃ (25(OH)D₃), whereas the

second hydroxylation to 1,25-dihydroxyvitamin D₃ occurs in the kidney. The various vitamin D metabolites are transported in the blood stream in tight association with lipoproteins and vitamin D-binding protein (DBP). Also vitamin K is transported in chylomicrons and other lipoproteins, but no specific vitamin K-binding protein is known. Apolipoprotein E (apoE) is a major constituent of lipoproteins, and has an important role in the clearance thereof. The human apoE gene is polymorphic, with three common alleles (ϵ 2, ϵ 3, and ϵ 4) coding for three isoforms E 2/2, E3/3, E4/4 and three heteroforms E2/3, E3/4, E 2/4. The isoforms differ from each other by a single amino acid substitution, causing substantial differences in their binding affinities for the apoE receptors¹⁶. This may have implications for the tissue transport of important nutrients including vitamins K and D. Previous studies have shown that the occurrence of certain apoE genotypes is associated with the risk of developing diseases such as Alzheimer's dementia, cardiovascular disease, and osteoporosis^{17,18}.

In the present study we have investigated whether one of the apoE genotypes is over represented in either VKDB or in rickets. Moreover, we have used the urinary concentration of free Gla and the serum concentration of 25(OH)D₃ as markers for overall vitamin K and D status to investigate the incidence of combined vitamin K and D deficiency in children with and without rickets.

Materials and Methods

Subjects

In this paper we describe investigations in the following population cohorts:

1. To establish the incidence of VKDBN in non-supplemented babies we have analysed the 74,000 children born in two obstetric clinics over a 12-year period.
2. The incidence of rickets was analysed from data obtained from the Romanian Ministry of Health over the year of 1995. We restricted ourselves to the district of Cluj, in which 6,301 births were registered. To further analyse the risk for rickets as a function of age we have focussed on the 159 cases reported during the year of 1999 in one paediatric clinic in Cluj.
3. For the biochemical characterization of children with rickets or prone to develop rickets we have screened 127 institutionalized children (all from one children's home) between 4 and 48 months old.
4. ApoE genotyping in the Romanian population was performed in 100 apparently healthy subjects (19.7 ± 1.8 years old) who were recruited among the students of the University of Cluj, and in 2 (former) patient groups as specified below.

Study design

In January 1998, 127 institutionalized children were screened for rickets by measuring serum calcium and total alkaline phosphatase (TAP). At inclusion their age and present weight and length, as well as their Apgar score, weight and length at birth were registered. Rickets was diagnosed on the basis of either total serum calcium < 90 mg/L (normal values in our laboratory: 90-110 mg/L) or TAP > 300 U/L (normal values in children: < 300 U/L), in combination with clinical symptoms such as: teeth disorders, delayed fontanel closure, restlessness, sweating, frequent respiratory diseases, and enteritis. Subjects with hepatic diseases, gastrointestinal diseases or diabetes were excluded from our study. No other pre-selection was performed. Among the 127 children investigated, 46 subjects with rickets (27 boys and 19 girls) were identified. The remaining 81 apparently healthy children were randomized in two control groups, one re-investigated in March, the 6th, (control 1, n = 39 subjects) and the second in April, the 6th, (control 2, n = 42 subjects). At the second analysis another 24 had developed rickets (n=13) or missed the second evaluation (n=11), and these were excluded from our study. Hence the remaining healthy control groups consisted of 27 and 30 subjects, respectively.

ApoE genotyping was performed in the 46 rickets patients described above and in 16 subjects who had a history of VKDBN. The number of former VKDBN patients analysed included all subjects who had not moved out of the district of Cluj since their birth. For logistic reasons it appeared impossible to trace former patients who had moved outside this area.

This study was approved by the Medical Ethics Committee of the University Hospital Cluj, Romania.

Blood and urine sampling

Blood (2 mL) was taken by venipuncture after an overnight fast, and serum was prepared by incubating the samples for 2 h at room temperature, followed by centrifugation for 15 min at 3,000 x g, at 4 °C. Urine was collected as the first morning void after an overnight fasting period. All samples were immediately frozen and kept at -80 °C until use.

Biochemical analyses

Serum osteocalcin (OC) and bone alkaline phosphatase (BAP), and urinary deoxypyridinoline (DPD) were measured by ELISA using kits from Metra Biosystems (Palo Alto, USA) and serum 25(OH)D with the kit from Biomedica (Vienna, Austria). Microtiter plates were read with an Organon 230S ELISA reader (Organon, Oss, The Netherlands). Total calcium and magnesium concentrations in serum and urine were

established by atomic absorptiometry (Perkin Elmer, Norwalk, CN). Total serum alkaline phosphatase (TAP), and urinary creatinine and phosphate concentrations were assessed using a Cobas Mira Plus autoanalyser (Roche, Basel, Switzerland) and commercial kits (Roche).

Urinary Gla-excretion was used as a marker for vitamin K status. For Gla detection urines were diluted 2-16 fold with distilled water (as required to stay within the detection range), and the diluted samples were analysed according to the procedure of Kuwada and Katayama¹⁹, using a Separations high precision HPLC pump (model 300), a Kratos Spectraflow 980 detector and a Nucleosil 100-5 SB anion exchange resin (5 µm particles, Macherey-Nagel, Düren, Germany) at a flow rate of 1 mL/min. Reference curves were prepared from samples with known concentrations of authentic Gla (Sigma, St.Louis, MO). The sensitivity of the assay was 0.3 µM. The intra- and interassay coefficients of variation were 2.2 and 6.4%, respectively. Urinary Gla concentrations are expressed as the Gla/creatinine ratio throughout this paper. For apoE genotyping DNA was extracted from 200 µL blood, and approximately 400 ng DNA was used to perform the PCR technique as described by Main²⁰. Taq polymerase, primers F4 and F6, restriction enzyme Hha I, and markers for 82bp, 66bp, 48 bp were used from GibcoBRL (Breda, The Netherlands). The separation of the DNA fragments was performed in 5% metaphor agarose (Sigma, St. Lois, MO) in 89 mM Tris base, 89 mM boric acid, 2 mM EDTA.

Statistical analysis.

The software package SPSS/PC+ version 5.0 (SPSS Inc., Chicago, Ill) was used for the statistical analysis and the 0.05 level of significance was chosen. The data were analysed using the paired Student's t-test for differences within groups and the unpaired t-test for differences between groups of more than 20 subjects. The Wilcoxon test was used for groups smaller than 20 subjects. Data are expressed as mean values ± S.E.M.

Results

Incidence of VKDBN and rickets

VKDBN: among the 74,000 children born during the 12-year observation period, 39 cases (20 boys and 19 girls) had been reported, i.e. an incidence of 1 case per 1,897 newborns. The incidence showed circadian fluctuations with the highest incidence between October and February, and a low incidence during the summer months (see fig. 1). Rickets: among the 6,301 children born during the 1-year observation period, 230 cases had been recorded in the 0-1 year age group in the same district,

corresponding to an incidence of 1 case per 27.4 children. As is shown in figure 1 also the incidence of rickets had a circadian pattern with the highest incidence between October and May and a lower incidence in summer. Among the 159 cases of rickets reported during the year of 1999 in one paediatric clinic in Cluj, 49% of the cases were found in the 0-1 year age group, 30% in the 1-3 year group, 17% at age 3-10 , and 4% at age 10-18 years. Boys were more often affected than girls (65 vs. 35%). Neither vitamin K nor vitamin D deficiency were related to Apgar score, weight, or body mass index at birth or present weight and body mass index.

Figure 1. Seasonal incidence of rickets and VKDBN cases. The incidence of VKDBN was monitored over a 12-year period, the incidence of rickets was established from an analysis of the cases from one paediatric clinic in 1999.

ApoE genotyping of healthy subjects and patients

In the Romanian population the distribution of apoE alleles was: $\epsilon 2$ in 5.6%, $\epsilon 3$ in 85.9%, and $\epsilon 4$ in 8.6 of the subjects (see table I). Although the number of former VKDBN patients is small, it seems that the distribution of alleles was not different from that in the general Romanian population. In the rickets cases analysed for this study, the $\epsilon 4$ allele seemed to be slightly over represented (22.5 vs. 15%). Due to the

low number of $\epsilon 4$ alleles it remains to be seen whether the difference with the general population is statistically significant, however.

Table I: ApoE genotype in vitamin K and vitamin D deficiency.

ApoE genotype	General population	VKDBN	Rickets
ApoE 2/2	0	0	0
ApoE 2/3	9	2	6
ApoE 2/4	2	0	0
ApoE 3/3	74	12	29
ApoE 3/4	15	2	10
ApoE 4/4	0	0	1
Total number of cases:	100	16	46

The apoE genotype of the Romanian population was assessed in 100 apparently healthy students. Former VKDBN patients and rickets patients were obtained as described in Materials and Methods.

Urinary Gla-excretion was assessed in a subset of the reference population described in table I. The group investigated contained all apoE 2/3 and 3/4 genotypes but only part of the more common apoE 3/3. The data are presented in table II.

Table II: ApoE genotype and urinary Gla excretion.

Genotype	Gla/creatinine (mg/g) in:		
	Men	Women	Mean
2/3	9.3 $\pm$ 0.4 (n = 5)	13.1 $\pm$ 1.4 (n = 4)	11.0 $\pm$ 0.8 (n = 9)
3/3	19.7 $\pm$ 0.4 (n = 6)	20.4 $\pm$ 1.4 (n = 7)	20.1 $\pm$ 1.1 (n = 13)
4/3	18.6 $\pm$ 1.2 (n = 5)	22.2 $\pm$ 2.1 (n = 5)	20.4 $\pm$ 1.4 (n = 10)

Urinary Gla was assessed in a subset (n=32, aged 19.7 $\pm$ 1.8 years) of the healthy individuals described in table I. Both Gla and creatinine concentrations were measured in the first morning void after an overnight fast.

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There was a non-significant tendency for lower Gla/creat values in men than in women, but this is probably related to the higher creatinine excretion by men. Subjects bearing the $\epsilon 2$ allele secreted significantly lower Gla than others ($p < 0.005$), suggesting that their vitamin K status is sub-optimal. The combined data for Gla-excretion in the healthy adults were plotted as a function of DPD, a well-known urinary marker for bone resorption. As is shown in figure 2 both variables were correlated ($r=0.82$, $p<0.0001$).

Figure 2. Urinary DPD as a function of Gla in 32 healthy volunteers. The correlation curve ($r=0.82$, $p<0.0001$) was calculated by linear regression and resulted in the equation $DPD = 3.28 \cdot Gla + 7.15$.

Biochemical markers in rickets patients and rickets-prone children

Next, we have investigated whether the low combined vitamin D and K status in children during the winter is associated with a decrease in the urinary Gla-excretion. Since the incidence of vitamin D deficiency is highest during the second part of the winter we have included 57 children between 4 and 48 months old, half of whom were analysed with a 2-month interval (January 6th - March 6th), the other half with a 3-month interval (January 6th - April 6th). As is shown in table III there was a gradual decline of circulating 25(OH)D₃ and urinary Gla-excretion in time, with values for the healthy group reaching in April the same range as found for the rickets patients, notably those with low serum calcium and normal TAP. Like in the healthy adults, urinary Gla and DPD excretion were correlated ($r = 0.45$, $p < 0.01$) in the healthy children (analysed in January).

Table III: Decline of markers for vitamins K and D status in children during winter.

	Serum 25(OH)D ₃ (µg/L)	Urinary Gla/creat (mg/g)
Control group 1 at baseline	19.7 ± 5.4	76.0 ± 13.3
Control group 1 after 2 months	14.0 ± 3.1*	59.2 ± 11.3
Control group 2 at baseline	16.3 ± 7.1	69.2 ± 5.6
Control group 2 after 3 months	7.0 ± 1.4*	45.7 ± 5.0**
Rickets: low serum Ca, normal TAP	7.7 ± 1.5 [#]	37.1 ± 4.4 [#]
Rickets: normal serum Ca, high TAP	9.6 ± 1.7 [#]	67.6 ± 11.6

Mean age (± SD) for control group 1: 25.4 months ± 14.2; for control group 2: 27 months ± 12.3, for rickets 23.7 ± 10.9. Symbols: significantly different from baseline (*= $p < 0.05$, **= $p < 0.005$), and significantly different from total control group ($n=57$) at baseline ([#]= $p < 0.05$). Differences within one group were tested with the paired student t-test, differences between groups with the unpaired t-test.

In the rickets group with low serum calcium and normal TAP markers for bone formation (BAP: 86.5 ± 6.6 U/L) and bone resorption (DPD: 29.5 ± 3.0 nmol/g creat) were not different from the baseline values in healthy children 86.2 ± 5.2 U/L and 33.5 ± 2.7 nmol/g, respectively). In the rickets group with normal serum calcium and high TAP, however, both markers were significantly higher than in the control group, suggesting increased bone turnover in this stage of rickets (BAP: 108.9 ± 9.3 U/L, $p < 0.05$; DPD/creat: 40.3 ± 3.9 nmol/g, $p < 0.02$). Osteocalcin was slightly increased in both evolutive stages of rickets (31.3 ± 2.3 µg/L vs. 27.0 ± 1.8 µg/L in the controls, $p < 0.05$).

Discussion

In this paper we confirm that clinically expressed deficiencies of both vitamins D and K have closely similar seasonal incidences, with a highest frequency for the late winter and early spring. Our data on Romanian children are in good agreement with those reported for other populations^{11,15}. For a more detailed biochemical analyses we have recruited 127 institutionalized children (4-48 months of age) living in one of the children's homes in Cluj. The first analyses took place in January, and it appeared that even in early winter 36.2% (46 out of 127 cases) of the children were

suffering from rickets. In subsequent months this number increased further to 59 cases, which is half of the total population investigated at that time. This high incidence may be caused by vitamin malnutrition, lack of exposure to sunlight (the children are kept indoor most of the time) or a combination of both. The seasonal incidence of rickets was confirmed by measuring 25(OH)D₃ in the serum of the remaining 57 apparently healthy children; lowest values were found at the beginning of April, when circulating vitamin D levels were comparable with those of rickets patients (measured in January). It must be concluded, therefore, that during winter vitamin D-deficiency is acute in the majority of the children living in children's homes. Remarkably, 90% of the rickets patients followed the regular prophylaxis for vitamin D deficiency with intramuscular vitamin D administration at 4, 8, and 12 months. Hence this schedule is insufficient to prevent rickets, and we recommend that daily oral vitamin D administration to institutionalized children should be made mandatory. The seasonal incidence of VKDBN was consistent with the low urinary Gla-excretion in a 4-48 months age group, observed when analyzing these children in March and April. Remarkably, also very low Gla-excretion was observed in rickets patients. This was most apparent in those with low serum calcium and normal TAP. It must be concluded, therefore, that vitamin D deficiency is often associated with poor vitamin K status, and we have addressed the question of whether there is a common risk factor for both deficiencies. Neither Apgar score nor somatic indices for development (length, body mass index) were associated with vitamin K or D deficiency, and the question remains why some children develop rickets and VKDBN, while others do not. Since apolipoprotein E is involved in the transport of both vitamins, we have tested whether the genotype of one of the three common apoE variants is overrepresented in former VKDBN patients or in rickets patients. No clear deviation from the general Romanian population was observed for either of both diseases, although the number of ϵ 4 alleles in rickets was slightly higher than expected. This is consistent with data in postmenopausal women and in hemodialysis patients, showing that the ϵ 4 allele is associated with low bone mineral density²¹. Malnutrition or general vitamin deficiency is probably the predominant factor in the apparent correlation between vitamin K and D deficiency in the 4-48 months age group. In the adult study population we observed that subjects bearing the ϵ 2 allele excrete significantly lower amounts of Gla than others. The difference remained evident after adjustment for BMI, and may be related to the poor binding of apoE 2 to its receptor and thus to low tissue uptake of vitamin K. Whether these subjects are subclinically vitamin K deficient should be tested by monitoring Gla-excretion during vitamin K-supplementation studies, which was beyond the scope of this paper. In all groups investigated we observed a positive correlation between urinary Gla and DPD

excretion. Collagen type I in bone is the largest source of DPD, but there are two major stores of Gla-proteins: blood (containing the vitamin K-dependent coagulation factors) and bone (containing osteocalcin and matrix Gla-protein). Degradation of the clotting factors will result in a constant generation of free Gla-residues, which cannot be re-used and are excreted via the urine. It is hard to envision how this process may be correlated to the degradation of type I collagen. Degradation of bone-proteins, on the other hand, might be the underlying factor for the association between urinary DPD and Gla. This implies that resorption of bone tissue contributes for a substantial part to the total urinary Gla excretion.

Both vitamins K and D are involved in the synthesis of proteins which play important roles in the regulation of bone formation and in mineral deposition in bone. A number of studies in adults have demonstrated that long term use of vitamin K-antagonists may lead to low bone mass and osteopenia, whereas poor vitamin K status as measured by undercarboxylated osteocalcin is associated with low bone mass and strongly increased fracture rate²². At this time nothing is known about the effect of poor vitamin K status on bone in children, but animal experiments in young rats and lambs have clearly shown that the effects of vitamin K antagonists in growing animals are manifold more deleterious than in adult ones²³. There are no reasons to expect that this is different in humans. Since it seems that the extremely high incidence of rickets in the Romanian children's homes is associated with a combined vitamin D and K deficiency, supplementation of these children with both vitamins throughout the winter months is strongly recommended.

References

1. DeLuca HF. *The vitamin D story: a collaborative effort of basic science and clinical medicine*. FASEB J 1988;2:224-236.
2. Friedrich W. *Vitamins*. Berlin, New York: Walter de Gruyter; 1988. p. 141-216.
3. Schurgers LJ, Geleijnse JM, Grobbee DE, Pols HAP, Hofman A, Vermeer C. Nutritional Intake of vitamins K₁ (phylloquinone) and K₂ (menaquinone) in The Netherlands. J Nutr Environm Med 1999;9:115-122.
4. Conly JM, Stein K. Quantitative and qualitative measurements of K vitamins in human intestinal contents. Am J Gastroenterol 1992;87:311-316.
5. Shearer MJ. Vitamin K. Lancet. 1995;345:229-234.
6. Hauschka PV, Lian JB, Gallop PM. Direct identification of the calcium-binding amino acid, gamma-carboxyglutamate, in mineralized tissue. Proc Natl Acad Sci USA 1975;72:3925-3929.
7. Price PA, Baukol SA. 1,25-Dihydroxyvitamin D3 increases synthesis of the vitamin K-dependent bone protein by osteosarcoma cells. J Biol Chem 1980;255:11660-11663.

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8. Hauschka PV, Carr SA. Calcium-dependent alpha-helical structure in osteocalcin. *Biochemistry* 1982;21:2538-2547.
9. Holmes RP, Kummerow FA. The relationship of adequate and excessive intake of vitamin D to health and disease. *J Am Coll Nutr* 1983;2:173-199.
10. Dueland S, Helgerud P, Pedersen JI, Berg T, Drevon CA. Plasma clearance, transfer and distribution of vitamin D₃ from intestinal lymph. *Am J Physiol* 1983;245:E 326-E331.
11. Webb AR, Kline L, Holick MF. Influence of season and latitude on the cutaneous synthesis of vitamin D₃: exposure to winter sunlight in Boston and Edmonton will not promote vitamin D₃ synthesis in human skin. *J Clin Endocrinol Metab* 1988;67:373-378.
12. Kruse K. Pathophysiology of calcium metabolism in children with vitamin D-deficiency rickets. *J Pediatr* 1995;126:736-741.
13. Shearer MJ, Rahim S, Barkhan P, Stimmeler L. Plasma vitamin K1 in mothers and their newborn babies. *Lancet* 1982;ii:460-463.
14. Lane PA, Hathaway WE. Vitamin K in infancy. *J Pediatr* 1985;106:351-359.
15. Douglas AS. Seasonality of hip fracture and haemorrhagic disease of the newborn. *Scot Med* 1993;38:37-40.
16. Siest G, Pillot T, Régis-Bailly A, Leininger-Muller B, Steinmetz J, Galteau M, et. al. Apolipoprotein E: An important gene and protein to follow in laboratory medicine. *Clin Chem* 1995;41:1068-1086.
17. Davignon J. Apolipoprotein E polymorphism and atherosclerosis. Chap. 5 In: Born CVR, Schwartz C.J., eds. *New horizons in coronary heart disease*. Current Science 1993;5:1-21.
18. Shiraki M, Aikiguchi I, Itoh H, Kaneko T, Matuo M. Vitamin K in the aged, with special reference to the clinical significance of the urinary gamma-carboxy glutamic acid content as a new indicator for the vitamin K status. *Nippon Ronen Igakkai Zasshi* 1983;20:128-132.
19. Kuwada M, Katayama K. An important method for the determination of gamma-carboxyglutamic acid in proteins, bone, and urine. *Analyt Biochem* 1983;131:173-179.
20. Main BF, Jones PJH, MacGillivray RTA, Banfield DK. Apolipoprotein E genotyping using the polymerase chain reaction and allele-specific oligonucleotide primers. *J Lipid Res* 1991;32:183-186.
21. Saupe J, Shearer MJ, Kohlmeier M. Phylloquinone transport and its influence on γ -carboxyglutamate residues of osteocalcin in patients on maintenance hemodialysis. *Am J Clin Nutr* 1993;58:204-208.
22. Szulc P, Chapuy MC, Meunier PJ, Delmas P. Serum undercarboxylated osteocalcin is a marker of the risk of hip fracture in elderly women. *J Clin Invest* 1993;91:1769-1774.
23. Price, PA, Faus SA, Williamson MK. Warfarin causes rapid calcification of the elastic lamellae in rat arteries and heart valves. *Arterioscler Thromb Vasc Biol* 1998;18:1400-1407.

Improved bone metabolism in female elite athletes after vitamin K supplementation

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Summary

In female elite athletes strenuous exercise may result in hypoestrogenism and amenorrhoea. As a consequence a low peak bone mass and rapid bone loss are often seen in relatively young athletes. In postmenopausal women, increased intake of vitamin K may result in an increase of serum markers for bone formation, a decrease of urinary markers for bone resorption, and a decrease in urinary calcium loss. In the present paper we report an intervention study among eight female athletes, four of whom had been amenorrhoeic for more than one year, whereas the others had been using oral contraceptives. All participants received vitamin K supplementation (10 mg/day) during one month, and various bone markers were measured before and after treatment. At baseline the athletes not using oral contraceptives were biochemically vitamin K-deficient as deduced from the calcium binding capacity of the circulating bone protein osteocalcin. In all subjects increased vitamin K intake was associated with an increased calcium-binding capacity of osteocalcin. In the low-estrogen group vitamin K supplementation induced a 15-20% increase of bone formation markers and a parallel 20-25 % decrease of bone resorption markers. This results could suggest an improved balance between bone formation and resorption.

Introduction

Vitamin K functions as a cofactor in the synthesis of a group of calcium-binding proteins generally known as „Gla-proteins“¹, where Gla stands for the unusual amino acid γ -carboxyglutamic acid. The calcium-binding capacity of these proteins decreases dramatically during episodes of vitamin K-deficiency². Gla-proteins are involved in a number of physiological processes including blood coagulation³, vascular biology^{4,5} and bone metabolism⁶. The minimal daily vitamin K intake required to ensure normal production of blood clotting factors is 0.5-1 $\mu\text{g/kg}$ body weight, for newborns a 10-fold higher intake is recommended during the first three months of life. Recent evidence suggests that upon ingestion, vitamin K is not equally distributed, but selectively taken up by a number of tissues⁷. Notably at low vitamin K intake, the liver absorbs a high fraction of the total vitamin K available. Hence the nutritional vitamin K requirements to fulfill the needs of bone tissue are presumably higher than those of the liver, which may explain the observation that biochemical vitamin K-deficiency of bone tissue (as deduced from undercarboxylation of the bone

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Gla-protein osteocalcin) is common in large parts of the population^{8,9}. Hepatic vitamin K-deficiency affecting the synthesis of blood coagulation factors, on the other hand, is rarely seen in apparently healthy subjects³. We have developed a test to estimate the vitamin K status of bone. This test is based on the different affinities of normal and undercarboxylated osteocalcin for hydroxyapatite during extraction of serum. The ratio between the fraction bound to the hydroxyapatite and that left free in the serum (the B/F ratio) will be used in this paper as a marker for vitamin K status.

It is well known that the reduction of estrogen production which takes place after the onset of the menopause, induces rapid loss of bone mass and is a major cause for osteoporosis. Several other factors, like low calcium consumption and poor vitamin D status contribute to this problem. In this study we have placed the focus on poor vitamin K status as a potential factor contributing to a negative bone balance. Osteoporotic hip and vertebral fractures have been shown to be associated with depressed levels of circulating vitamin K¹⁰⁻¹², and several studies have confirmed that supplementation of postmenopausal women with 1-10 mg/day of vitamin K results in an increase of markers for bone formation (serum bone alkaline phosphatase, osteocalcin), and in a decrease of markers for bone resorption (urinary hydroxyproline, calcium)^{8,9,13,14}. Moreover, vitamin K supplementation has been shown to substantially reduce the loss of bone mass in a 2 year prospective trial among 650 osteoporotic Japanese women¹³.

In addition to the onset of the menopause several other causes for rapid loss of bone mass are known, including microgravity experienced by astronauts during space flights¹⁵ and hypoestrogenism induced by strenuous exercise^{16,17}. In this paper we will restrict ourselves to the bone loss seen in young female elite long distance runners. Regular exhausting endurance exercise may have a direct effect on the female hormonal system: the serum levels of the reproductive hormones are often low, whereas the cortisol level may be increased¹⁸⁻²⁰. As a consequence the menarche may be delayed, the menstrual cycle may come to a halt, the peak bone mass may be low, and substantial loss of bone mass may occur even at very young age²¹. These phenomena are most frequently observed in sports characterized by a high energy turnover and by the fact that a low lean body mass is advantageous, as in marathon running²². In these women lower limb stress fractures are one of the most common sport injuries, with an incidence of over 30% per year^{23,24}. In the study we investigated in a small group of female elite athletes a) whether the markers for bone metabolism would be affected favorably by vitamin K-supplementation and if

so, b) whether this effect would be more pronounced in low-estrogen subjects than in those receiving estrogen contraceptives.

Materials and Methods

Subjects

Eight female long distance runners, who were active on an international competition level, were included. Their mean age was 35 years (range: 20-44), and the selection was made by sports physicians of the Royal Dutch Athletics Union (K.N.A.U.). All participants completed a questionnaire to provide information on their training schedules, training background, nutritional habits and menstrual status. Since all women reported an ample intake of green vegetables and a relatively high consumption of dairy produce, their vitamin K intake was well above the RDA value (1 $\mu\text{g/day/kg}$ body weight). Four athletes had used ethinylestradiol-containing oral contraceptives (either bi- or triphasic) for the last 10 years or longer. In all four cases the dose of ethinylestradiol was 30 mg/day. The other four participants had never used oral contraception. Users and non-users of oral contraceptives are designated here as the OCC⁺ and the OCC⁻ group, respectively. All participants in the OCC⁻ group had been amenorrhoeic for at least one year preceding our study. Some characteristics of the athletes are summarized in table I. Reference groups were obtained from a cross-sectional study in which 230 Caucasian women between 20 and 90 years of age were randomly selected from the city population files of Maastricht. Inclusion criteria were: apparently healthy, no recent (< 1 year ago) fractures, and no use of oral anticoagulants or medication known to influence calcium metabolism.

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Table 1. Anthropometrical and physical characteristics of participants.

Variable	Range	Mean
Age (years)	20-44	35.6
Weight (kg)	45-56	50.4
Length (cm)	158-176	163
Body mass index (kg/m ²)	17.2-19.8	18.5
Age of menarche (years)	12-14	13.2
Training distance (km/day)	10-22	14.4
Best 10 km time (min)	32.1-37.4	35.1
Subjects on OCC (number)	4	
Duration of OCC use (years)	10-18	15

Abbreviation: OCC, oral contraceptive.

The subjects of the reference population were classified into age groups with 5-year intervals, each group consisting of at least 25 women. In our study we have characterized the athletes by comparing their baseline data with those of the reference population in their own age group, and we recorded the effects of vitamin K supplementation by comparing the pre- and post-treatment data in the athletes.

Study design

During the period selected for this study the athletes had a constant training schedule with no severe competition. Throughout it after the first blood and urine sampling they received vitamin K (Konakion[®], 10 mg/day) over 30 days, and blood and urine samples were collected once more on the last day of treatment. The study had been approved by the Medical Ethics Committee of the University Hospital Maastricht, and informed consent was signed by all participants.

Blood and urine sampling

Blood was taken by venipuncture after an overnight fast, and serum was prepared by incubating the samples for 2 h at room temperature, followed by centrifugation for 15 min at 2,000 g. Urine was collected as the 2-h morning void after a 14-h fasting period. All samples were immediately frozen and kept at -80 °C until use.

Biochemical analyses

Circulating osteocalcin was measured by radioimmuno assay (Incstar, Stillwater, MI). The total immunoreactive osteocalcin ($\text{irOC}_{\text{total}}$) was fractionated by selective adsorption onto hydroxyapatite as reported by Knapen et al. (8), resulting in a fraction with high ($\text{irOC}_{\text{bound}}$) and a fraction with low ($\text{irOC}_{\text{free}}$) affinity for hydroxyapatite. The ratio between $\text{irOC}_{\text{bound}}$ and $\text{irOC}_{\text{free}}$ is designated as the B/F ratio and will be used to estimate the vitamin K status of bone tissue. Total alkaline phosphatase (TAP), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were assessed using a Cobas EIA system (Roche, Basel, Switzerland) and commercial test kits (Roche). Bone alkaline phosphatase (BAP) was assessed by the wheat germ lectin precipitation technique²⁵. Intact parathyroid hormone (iPTH) and estradiol-2 were measured by radioimmuno assay (Incstar, Stillwater, MI). Urinary calcium concentrations were determined by atomic absorptiometry (Perkin Elmer, Norwalk, CN), and free γ -carboxyglutamate (Gla) by high performance liquid chromatography as described by Kuwada and Katayama²⁶. Hydroxyproline (OHPro, Organon Teknika, Oss, The Netherlands) and deoxypyridinoline (DPD, Metra Biosystems, Palo Alto, USA) were tested with commercial test kits.

Results

Hormonal status in female elite athletes.

The hormonal status of the athletes was evaluated by assessing their circulating estrogen, FSH, and LH levels. As is shown in fig. 1, the estradiol-2 levels in the long distance runners were comparable to those in postmenopausal women. FSH and LH were even well below the postmenopausal levels, and were found to be in the range of prepubertal girls or men. FSH in the OCC^+ group was substantially lower than in the OCC^- group, which is explained by the suppressive effect of progesterone (present in all oral contraceptives) on FSH-secretion. These data are consistent with the low estrogen production in female elite athletes, and with the amenorrhoeic state of the athletes in the OCC^- group.

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Figure 1. Comparison between circulating sex hormones in female athletes and postmenopausal women. The data from the postmenopausal women came from a reference population of 85 women between 60 and 80 years old. All data are given $\pm$ SEM. Open bars: follicle-stimulating hormone (FSH); hatched bars: luteinizing hormone (LH); closed bars: estradiol-2. Abbreviations: OCC, subjects not using oral contraceptives; OCC⁺, subjects using oral contraceptives; PMP, postmenopausal women.

Vitamin K status in female elite athletes.

Vitamin K status was assessed in the participants by measuring the ratio between iOC_{bound} and iOC_{free} (the B/F ratio, see table II, first line). The values obtained were compared with the means of the corresponding age groups in the reference population. From earlier studies it is known that the age groups included are generally vitamin K-sufficient, and that vitamin K-supplementation does not affect the B/F ratio⁹. It turned out that, based on the B/F ratio, five out of the eight athletes studied could be classified as vitamin K-deficient, three of whom belonged to the OCC⁻ group. In the OCC⁻ group the mean B/F ratio was 65%; in the OCC⁺ group the ratio was 105% to that of the age-matched controls. After vitamin K-supplementation (see also table III, first line) the B/F ratios in both groups were comparable (133 and 116% of the controls, respectively). From these data we conclude that the subjects in the OCC⁻ group were subclinically vitamin K-deficient.

Table II: Biochemical markers in female athletes compared with a reference population.

Marker tested	OCC ⁻ athletes		OCC ⁺ athletes	
	Reference value in control population	Relative value in athletes (% of reference $\pm$ SEM)	Reference value in control population	Relative value in athletes (% of reference $\pm$ SEM)
<i>In serum</i>				
B/F ratio	1.64	64.9 $\pm$ 17.0	1.59	105.6 $\pm$ 27.0
irOC _{total} (ng/ml)	2.49	142.2 $\pm$ 11.7	2.65	81.9 $\pm$ 18.3
irOC _{free} (ng/ml)	0.94	173.7 $\pm$ 16.7	1.03	76.8 $\pm$ 21.4
IrOC _{bound} (ng/ml)	1.55	112.0 $\pm$ 21.3	1.62	86.8 $\pm$ 24.6
BAP (U/L)	27.7	146.5 $\pm$ 18.0	32.9	80.8 $\pm$ 13.8
IPTH(pg/mL)	22.0	115.0 $\pm$ 8.8	18.5	73.1 $\pm$ 14.4
<i>In urine</i>				
Calcium (mmol/h)	128.5	32.0 $\pm$ 20.4	109.4	42.4 $\pm$ 37.8
OH-Pro (mg/h)	5.55	132.8 $\pm$ 17.0	5.55	60.3 $\pm$ 24.9
Gla (mmol/h)	1.45	101.3 $\pm$ 7.1	1.50	76.4 $\pm$ 20.3
DPD (nmol/h)	1.60	133.8 $\pm$ 13.8	1.55	68.4 $\pm$ 41.5

Abbreviations: irOC, immunoreactive osteocalcin; BAP, bone alkaline phosphatase; iPTH, intact parathyroid hormone; OHPro, hydroxyproline; Gla, γ -carboxyglutamate; DPD, deoxypyridinoline. The B/F ratio is the ratio between irOC with high (irOC_{bound}) and that with low (irOC_{free}) affinity for hydroxyapatite. For further details see text.

Table III: Vitamin K administration to female elite athletes: effect on bone markers.

Marker tested	OCC ⁻ athletes				OCC ⁺ athletes			
	before K	after K	$\Delta \pm \text{SEM}$	% change	before K	after K	$\Delta \pm \text{SEM}$	% change
<i>In serum:</i>								
B/F ratio	1.08	2.18	$+1.10 \pm 0.3$	+102	1.67	1.84	$+0.17 \pm 1.1$	+10
IrOC _{total} (ng/ml)	3.52	4.22	$+0.70 \pm 0.1$	+ 20	2.17	2.91	$+0.74 \pm 0.2$	+34
IrOC _{free} (ng/ml)	1.72	1.38	$- 0.34 \pm 0.2$	- 20	0.78	1.09	$+0.31 \pm 0.3$	+40
IrOC _{bound} (ng/ml)	1.80	2.83	$+1.03 \pm 0.08$	+ 57	1.40	1.82	$+0.42 \pm 0.5$	+30
BAP (U/L)	40.8	47.0	$+6.2 \pm 3.9$	+ 15	28.1	25.1	$- 3.0 \pm 0.2$	- 11
<i>In urine:</i>								
Calcium (mmol/h)	41.0	32.4	$- 8.6 \pm 11.8$	- 21	46.4	39.1	$- 7.3 \pm 6.3$	- 16
OH-Pro (mg/h)	7.5	5.9	$- 1.6 \pm 0.5$	- 21	4.2	2.9	$- 1.3 \pm 0.9$	- 31
Gla (mmol/h)	1.51	1.48	$- 0.03 \pm 0.2$	- 2	1.46	1.62	$+0.16 \pm 0.11$	+11
DPD (nmol/h)	2.04	1.51	$- 0.53 \pm 0.6$	- 26	2.72	1.79	$- 0.93 \pm 0.89$	- 34

Δ stands for the difference between the respective markers before and after vitamin K treatment. For further abbreviations see the legend to table II.

Baseline data on bone metabolism in female elite athletes.

We assessed two bone formation markers (BAP and $\text{irOC}_{\text{total}}$), two bone resorption markers (OHPro and DPD), serum iPTH, urinary calcium and Gla excretion in all participants. The data were compared with the reference population (see table II), and it is clear that the urinary calcium excretion in all athletes was extremely low (about one third of normal). A second observation was that bone turnover in the OCC^+ group was relatively low (all bone markers were approximately 25% below the reference group), whereas in the OCC^- group the bone turnover was high, with all bone markers about 30% higher than in the reference group.

Effects of vitamin K supplementation.

Immediately after the first blood and urine sampling the women received 10 mg/day of vitamin K during one month, on the last day of treatment a second set of samples was taken for assessment of the various markers. The data are summarized in table III and show a mild increase of the bone formation markers, combined with a more prominent decrease of the bone resorption markers. In an attempt to amplify the slight shifts in the various markers, we have expressed the vitamin K-induced differences as Z-scores for each marker separately. Subsequently we have calculated the sum of Z-scores as: $3Z = Z_{\text{OC}} + Z_{\text{BAP}} - Z_{\text{CTX}} - Z_{\text{DPD}}$; it can be seen that interventions resulting in a positive sum of Z-scores suggest a positive effect on the balance between bone formation and resorption, whereas negative sum of Z-scores indicate a negative effect. As can be seen in fig. 2, the effect of vitamin K-supplementation was much larger in the OCC^- group than in the OCC^+ group, with mean values of + 5.3 (± 1.4 SD) and +0.6 (± 1.9 SD), respectively. Urinary calcium loss - which was already low - had decreased further in both groups.

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Figure 2. Effect of vitamin K treatment on bone markers in female long distance runners. The accumulated changes are given as the sum of Z-scores for each subject individually.

Discussion

Progressive loss of bone tissue implies a long lasting imbalance between bone formation and resorption. The fact that increased vitamin K intake by postmenopausal women induces an increase of markers for bone formation, and a simultaneous decrease of markers for bone resorption suggests that vitamin K may contribute to the reduction of bone loss in this group^{8,9,13,14}. From a mechanistic point of view it is intriguing whether a similar effect can be observed in other forms of negative bone balance, such as glucocorticoid-induced and microgravity-induced bone loss, and in athletic osteoporosis. For reasons of health care and health costs, the most important question is obviously whether the positive effect of vitamin K on bone markers results in a beneficial effect on the rate of bone loss, on bone mass, and on fracture risk. However, the latter studies are expensive and require a follow-up of several years. Therefore, clinical trials on the effect of nutrients and food supplements on bone mass are generally preceded by one or more shorter studies in which only the effects on bone markers are recorded. Recently we completed an investigation in which we showed a substantial improvement of the bone balance after vitamin K-supplementation during a 6-month period of microgravity²⁷, while here we report the results of a small trial among female elite athletes.

The hydroxyapatite-binding capacity of circulating osteocalcin is the most sensitive marker for vitamin K status presently known. In this paper it is expressed as the B/F ratio. Whereas a biochemical vitamin K-deficiency as deduced from the B/F ratio is common among postmenopausal women^{8,9}, a more severe deficiency leading to reduced levels of the vitamin K-dependent coagulation factors is rarely found in healthy adults. One of the principal observations of this study was that the mean value of the marker for vitamin K status (the B/F ratio) in the estrogen supplemented athletes was within the normal range, but that the B/F ratio in athletes not using oral contraception was well below normal, which suggests a poor vitamin K status of their bone tissue. After vitamin K administration the B/F ratio increased in both groups to values slightly above the normal reference population. Consistent with the known effect of estrogen on bone metabolism^{28,29}, the bone turnover (as deduced from the bone formation and bone resorption markers) in the OCC⁺ group was low. In the OCC⁻ group, on the other hand, bone turnover was substantially higher. This is a well known risk factor for a high rate of bone loss.

A further difference between both study populations was that in the OCC⁻ group the increased vitamin K intake was paralleled by both an increase of bone formation markers and a decrease of bone resorption markers. In the OCC⁺ group the observed changes were mainly found in the markers for bone resorption. After vitamin K treatment the bone turnover remained high in the OCC⁻ group, but there was a clear shift showing improved bone formation and reduced bone resorption. In this respect vitamin K acts similarly in postmenopausal women and in amenorrhoeic athletes. Remarkably, urinary Gla excretion was hardly affected by vitamin K supplementation suggesting that osteocalcin degradation products only form a minor fraction of the total urinary Gla. The fact that the use of oral contraceptives influenced the impact of vitamin K treatment is consistent with the different effects of vitamin K in pre- and postmenopausal women, and should be taken into account when larger placebo-controlled trials are designed. For the present study it meant that the already small group of eight athletes had to be subdivided into two groups of four subjects each, which hampered further statistical analysis of the data.

Questions which remain to be answered are whether vitamin K supplementation will prove to be an alternative for established anti-osteoporotic therapies, and whether vitamin K will have an additional or synergistic effect if administered in combination with one of these therapies. In this respect it should be mentioned that all athletes had a very low urinary calcium excretion. This phenomenon may be explained by a combination of sweat calcium loss³⁰ and the usually low calcium intake by athletes³¹. Additionally, there might be a compensation mechanism for the high calcium loss immediately post exercise³², resulting in decreased calcium loss after an overnight

rest. Our data suggest that long distance runners are prone to develop a nutritional calcium deficiency, and we recommend that calcium supplementation (possibly in combination with vitamin D) is evaluated as one of the vitamin K-independent countermeasures to prevent further bone loss. It is clear that the size of the present study was too small to allow generalization of the data to the female athletic population as a whole, but the fact that the data are closely similar to those observed in postmenopausal women in earlier studies justifies further investigations among both groups.

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References

1. Vermeer C.: *Gamma-carboxyglutamate-containing proteins and the vitamin K-dependent carboxylase*. *Biochem J* 266: 625-636, 1990.
2. Vermeer C., Hamulyák, K.: *Pathophysiology of vitamin K deficiency and oral anticoagulants*. *Thromb Haemostas* 66: 153-159, 1991.
3. Furie B., Furie B.C.: *The molecular basis of blood coagulation*. *Cell* 53 :505-518, 1988.
4. Luo G., Ducy P., McKee M.D., Pinero G.J., Loyer E., Behringer R.R., Karsenty G.: *Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein*. *Nature* 386: 78-81, 1997.
5. Nakano T., Kawamoto K., Kishino J., Nomura K., Higashino K., Arita H. *Requirement of γ -carboxyglutamic acid residues for the biological activity of Gas6: contribution of endogenous Gas6 to the proliferation of vascular smooth muscle cells*. *Biochem J* 1997; 323: 387-392.
6. Hauschka P.V., Lian J.B., Cole D.E., Gundberg C.M.: *Osteocalcin and matrix Gla protein: vitamin K dependent proteins in bone*. *Phys Rev* 69: 990-1047, 1989.
7. Thijssen H.H., Drittij-Reijnders M.J., Fischer M.A.: *Phylloquinone and menaquinone-4 distribution in rats: synthesis rather than uptake determines menaquinone-4 organ concentrations*. *J Nutr* 126: 537-543, 1996.
8. Knapen M.H., Hamulyák K., Vermeer C.: *The effect of vitamin K supplementation on circulating osteocalcin (bone Gla-protein) and urinary calcium excretion*. *Ann Int Med* 111: 1001-1005, 1989.

9. Knapen M.H., Jie K.-S., Hamulyák K., Vermeer C.: Vitamin K-induced changes in markers for osteoblast activity and urinary calcium loss. *Calcif Tissue Int* 53: 81-85, 1993.
10. Hart J.P., Catterall A., Dodds R.A., Klenerman L., Shearer M.J., Bitensky L., Chayen J.: Circulating vitamin K₁ levels in fractured neck of femur. *Lancet* ii: 283, 1984.
11. Hart J.P., Shearer M.J., Klenerman L., Catterall A., Reeve J., Sambrook P.N., Dodds R.A., Bitensky L., Chayen J.: Electrochemical detection of depressed circulating levels of vitamin K₁ in osteoporosis. *J Clin Endocrinol Metab* 60: 268-269, 1985.
12. Hodges S.J., Akesson K., Vergnaud P., Obrant K., Delmas P.D.: Circulating levels of vitamins K₁ and K₂ decreased in elderly women with hip fracture. *J Bone Miner Res* 8: 1241-1245, 1993.
13. Orimo H., Fujita T., Onomura T., Inoue T., Kushida K., Shiraki M.: Clinical evaluation of Ea-0167 (Menatetrenone) in the treatment of osteoporosis. *Clin Eval (Japan)* 20: 45-100, 1992.
14. Plantalech L.C., Chapuy M.C., Guillaumont M., Chapuy P., Leclercq M., Delmas P.D.: Impaired carboxylation of serum osteocalcin in elderly women: Effect of vitamin K₁ treatment, in Christiansen C., Overgaard K. (eds.): *Osteoporosis 1990*. Copenhagen, Osteopress, 1990, pp 345-347.
15. Rambaut P.C., Goode A.W.: Skeletal changes during space flight. *Lancet* ii: 1050-1052, 1985.
16. Constantini N.W.: Clinical consequences of athletic amenorrhoea. *Sports Med* 17: 213-223, 1994.
17. Drinkwater B.L., Nilson K., Chesnut C.H., Bremner W.J., Shainholtz S., Southworth M.B.: Bone mineral content of amenorrheic and eumenorrheic athletes. *N Engl J Med* 311: 277-281, 1984.
18. Loucks A.B., Mortola J.F., Girton L., Yen S.S.: Alterations in the hypothalamic-pituitary-ovarian and the hypothalamic-pituitary-adrenal axes in the athletic women. *J Clin Endocrinol Metab* 68: 402-411, 1989.
19. Mansfield M.J., Emans S.J.: Anorexia nervosa, athletics, and amenorrhea. *Ped Clin N Am* 36: 533-549, 1989.
20. Prior J.C., Vigna Y.M., McKay D.W.: Reproduction for the athletic women. *New understanding of physiology and management*. *Sports Med* 14: 190-199, 1992.
21. Chilibeck P.D., Sale, D.G., Webber C.E.: Exercise and bone mineral density. *Sports Med* 19: 103-122, 1995.

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22. Sundgot-Borgen, J.: Eating disorders in female athletes. *Sports Med* 17: 176-188, 1994.
23. Highet R.: Athletic amenorrhoea: An update on aetiology, complications and management. *Sports Med* 7: 82-108, 1989.
24. Marti, B.: Health effects of recreational running in women: some epidemiological and preventive aspects. *Sports Med* 11: 20-51, 1991.
25. Brixen K., Nielsen H.K., Eriksen E.F., Charles P., Mosekilde L.: Efficacy of wheat germ lectin-precipitated alkaline phosphatase in serum as an estimator of bone mineralization rate: comparison to serum total alkaline phosphatase and serum bone Gla-protein. *Calcif Tissue Int* 44: 93-98, 1989.
26. Kuwada M., Katayama K.: An improved method for the determination of γ -carboxyglutamic acid in proteins, bone, and urine. *Analyt Biochem* 131: 173-179, 1983.
27. Vermeer, C., Wolf, J., Craciun, A.M., Knapen, M.H.J. Bone markers during 6-month space flight: effects of vitamin K supplementation. *Journal of Gravitational Physiology* 1998; 5(2): 65-69.
28. Gamero P., Sornay-Rendu E., Delmas P.D.: Decreased bone turnover in oral contraceptive users. *Bone* 16: 499-503, 1995.
29. Lindsay R.: Estrogens and osteoporosis: clinical, in Papapoulos S.E., Lips P., Pols H.A.P., Johnston C.C., Delmas P.D. (eds.): *Osteoporosis 1996*. Amsterdam, Elsevier Science, 1996, pp 251-255.
30. Brouns F., Saris W., Schneider H.: Rationale for upper limits of electrolyte replacement during exercise. *Int J Sport Nutr* 2: 229-238, 1992.
31. Van Erp-Baart A.M., Saris W.H., Binkhorst R.A., Vos J.A., Elvers J.W. Nationwide survey on nutritional habits in elite athletes Part II. Mineral and vitamin intake. *Int J Sports Med* 10: S11-16, 1989.
32. Dalsky G.P.: Effect of exercise on bone: permissive influence of estrogen and calcium. *Med Sci Sports Exerc* 22: 281-285, 1990.

Microgravity-induced changes in bone markers : effects of vitamin K supplementation

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Summary

Rapid bone loss is a serious health problem for astronauts during long lasting missions in space. We have recorded the changes of biochemical markers for bone metabolism in one of the astronauts during the 6-month space flight of the EUROMIR-95 mission. Immediately after launch both bone resorption markers and urinary calcium excretion increased about two fold, whereas bone formation markers remained unchanged. After 122 weeks the astronaut received vitamin K₁ (10 mg/day for 6 weeks). Vitamin K is known to be involved in the formation of γ -carboxyglutamate (Gla) in proteins, such as the calcium-binding bone Gla-proteins osteocalcin and matrix Gla-protein. Concomitant with the start of vitamin K treatment, the calcium-binding capacity of osteocalcin increased, and so did the urinary excretion of free Gla. This is suggestive for a subclinical vitamin K-deficiency in the astronaut before vitamin K-supplementation. During periods of high vitamin K status markers for bone formation (osteocalcin and bone alkaline phosphatase) had increased as compared to the first part of the flight. The mean increases were 14 and 23%, respectively. Our data suggest that increased intake of vitamin K may contribute to counteracting microgravity-induced loss of bone mass during long lasting space missions, but need confirmation in more astronauts.

Introduction

Microgravity-induced bone loss and muscle atrophy are well recognized complications of long-lasting space missions⁹⁻¹¹. The highest bone losses have been observed in the weight bearing bones, and although there are large inter-individual variations the reported loss seems to increase in rough proportion to mission length and may be as high as 19.8% during a 184-day space flight¹⁰. The total body calcium loss and the negative bone balance were reported to continue for at least one year¹⁴, but data from in-flight assessment of bone markers are rare. Progressive bone loss may be the most critical biomedical obstacle encountered by astronauts during still longer missions (e.g. to Mars). Countermeasures, such as in-flight physical training and increased vitamin D intake, may retard the rate of bone loss but thus far the process could not be stopped^{1,14}. The effects of increased intake of vitamin K have not yet been investigated. Vitamin K is involved in the posttranslational formation of γ -carboxyglutamate (Gla) residues in proteins. Gla-residues form strong calcium-binding groups in these proteins. Bone contains at least three Gla-proteins: osteocalcin, matrix Gla-protein, and protein S^{3,14}. Vitamin K may also have a second function, i.e. the inhibition of osteoclast activity and bone resorption^{2,4}. A number of

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clinical trials have been published showing that increased vitamin K intake by postmenopausal women leads to an increase in circulating bone formation markers, to a decrease in urinary bone resorption markers and calcium excretion^{5,6}, and to a substantial reduction of net bone loss⁸. In this paper we have recorded the effect of microgravity on bone formation and resorption markers via regular blood and urine sampling during a long-lasting space flight (180 days). Moreover, we have investigated whether vitamin K-supplementation may reduce the effects of microgravity on bone metabolism. Since the number of potential test subjects is limited, this study could only be performed in one astronaut thus far.

Methods

Experimental design.

Fasting blood and urine samples were collected before (n=3), during (n=13) and after (n=4) the 6-month Euromir-95 mission. Pre-flight baseline sample collection was accomplished at 180, 60, and 15 days before launch, in-flight sample collection was at days 10, 20, 31, 42, 55, 72, 86, 104, 125, 130, 146, 167, and 178. The return was at day 180. Post-flight samples were taken at 7, 14, 28 and 98 days after landing. During the first 12 weeks of microgravity the astronaut received no food supplements; at day 86 a 6-week period was started during which the astronaut received a pharmacological dose of vitamin K₁ (Konakion®, 10 mg/day); at day 130 the treatment was stopped and followed by another 7-week period of no treatment. The protocol for this study was approved by the University and Hospital Medical Ethics Committee, and by the ESA Medical Ethics Committee.

Sample handling.

Fasting blood (5 ml) was collected between 8 and 9 a.m. (board time) by venipuncture; after the blood clot had formed, serum was prepared by centrifugation (15 min, 2,000 x g) in the on-board centrifuge. Within 2 h after collection the samples were frozen at -40 °C. Urine was collected as the 2-h second void after an overnight fast, using a tight-fitting urine collection bag with bar-lines from which the volume could be read. Samples were collected with the aid of a syringe, and immediately frozen in the on-board freezer. Similar procedures were maintained for the pre- and post-flight baseline samples. The sample at day 178 was erroneously collected as the overnight void, and will not be taken into consideration. In-flight samples were brought back to earth with the STS-74 and -76 flights. They were transferred to our laboratory in a frozen state, and tested for all markers immediately after thawing.

Biochemical markers.

Osteocalcin and intact parathyroid hormone (Incstar), bone alkaline phosphatase (Tandem-R Ostase, Hybritech), deoxypyridinoline (Pyrilinks-D, Metra) and type I C-terminal collagen cross-linked peptides (CrossLaps, Osteometer) were purchased as commercial kits. Fractionation of osteocalcin was performed using hydroxyapatite extraction⁵. Creatinine concentrations were assessed with the Cobas centrifugal analyzer (Roche), calcium was determined by atomic absorption spectrophotometry (Perkin Elmer, Norwalk CN, USA). Gammacarboxyglutamate was measured in urine according to the method described by Kuwada and Katayama⁷. Urinary markers are expressed per mmol urinary creatinine throughout this paper. All data are presented as the means of duplicate measurements.

Results

Bone vitamin K status was recorded by measuring the affinity of circulating osteocalcin for hydroxyapatite⁵. The serum levels of normally carboxylated immunoreactive osteocalcin (irOC_{bound}) and undercarboxylated osteocalcin (irOC_{free}) were established for each sample, and the ratio between both values (Bound / Free ratio) is given in fig. 1 (top panel). The pre-flight baseline data show a B/F ratio of 1.8, which is in the normal range for healthy adults. Vitamin K status was found not to be affected by the change from normal gravity to microgravity, whereas a substantial increase of B/F ratio (showing the compliance of the astronaut) was observed at the start of oral vitamin K supplementation. The high B/F ratio persisted for at least 7 weeks after the intake of vitamin K was stopped. From these data it appears that during pre-flight baseline sampling as well as during the first part of the mission the astronaut's vitamin K status was in the normal range, and that the effect of vitamin K-supplementation was obvious from the increased affinity of circulating osteocalcin for hydroxyapatite.

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Figure 1. Changes of vitamin K status and urinary calcium excretion in time. Top panel: ratio between carboxylated ($i\text{OC}_{\text{bound}}$) and undercarboxylated immunoreactive osteocalcin ($i\text{OC}_{\text{free}}$) as a marker for bone vitamin K status. Each point represents the mean of duplicate measurements. Launch was at $t=0$, the start and the end of the vitamin K treatment are indicated with arrows. Bottom panel: calcium/creatinine concentration in 2-h fasting urine. Each point represents the mean of duplicate measurements. Inflight A and B represent the episodes of normal and high vitamin K status, respectively.

The period during which the effect of vitamin K remained detectable after discontinuation of the treatment, suggests a long tissue half-life time for vitamin K, which is in contrast with its short half-life time (about 2 h) in blood¹². This is in good agreement with studies in postmenopausal women in whom an increased vitamin K-status was found until 2-3 months after treatment with pharmacological doses of vitamin K⁶. Only after return to a 1 g environment did bone vitamin K status return to its pre-flight value. Based upon these data we have subdivided our experiment into four time periods: 1) the pre-flight baseline measurements; 2) the in-flight samplings during normal vitamin K-status (in-flight A, $n=7$); 3) the in-flight samplings in the period of high vitamin K-status (in-flight B, $n=6$); and 4) the post-flight baseline measurements. Figure 1 (bottom panel) shows the urinary calcium excretion at the various sampling points during the experiment. After entering microgravity the calcium excretion (expressed as the Ca / creatinine ratio) increased sharply, to become about twice the baseline level with a slow decline after the fifth week in

space. All urinary calcium values during the inflight period were above the pre-flight baseline mean, but the values were lower during the inflight-B period than during inflight-A. The mean calcium loss returned to normal after return to 1 g conditions.

To find the reason for the changes in calcium homeostasis, we measured a number of markers of bone metabolism. These include osteocalcin (OC) and bone alkaline phosphatase (BAP) (markers of bone formation) and Type I C-terminal collagen cross-linked peptides (CTX) and deoxypyridinoline (DPD) (markers of bone resorption). Time courses of these markers are given in fig. 2. Bone resorption markers (top panel) were about two-fold the pre-flight baseline mean during the entire in-flight period, and returned to pre-flight levels shortly after landing. Vitamin K administration had no effect. Bone formation markers (bottom panel) were slightly depressed during the in-flight A period (-7 and -9%, respectively), and were well above the pre-flight baseline mean during the in-flight B period (+7 and +13%, respectively).

Figure 2. Changes of markers for bone metabolism in time. Top panel: bone resorption markers deoxypyridinoline (•) and type I C-terminal collagen crosslinked peptides (○) both expressed per mol of creatinine. Bottom panel: bone formation markers bone alkaline phosphatase (•) and osteocalcin (○).

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Other markers tested were: urinary free γ -carboxyglutamate (Gla) and serum intact parathyroid hormone (iPTH). Urinary Gla-excretion remained normal during the in-flight A period, but markedly increased after the onset of vitamin K supplementation (see fig. 3, top panel). This suggests that during the earlier phase Gla-proteins had remained undercarboxylated, i.e. that the astronaut had been subclinically vitamin K-deficient. Microgravity had a large effect on serum iPTH, which remained depressed during the entire space flight and - except for the outlier immediately after landing - returned to the pre-flight baseline value after in the post-flight samples (fig. 3, bottom panel).

Figure 3. Changes of urinary Gla and serum iPTH in time. Top panel: γ -carboxyglutamate per mol of creatinine in 2-h fasting urine. Bottom panel: serum intact parathyroid hormone.

In most cases the in-flight changes of the various markers were relatively small. Their mean values ($\pm$ SD) during the four experimental periods are given in table I.

Table I: Effect of microgravity and vitamin K administration on markers for bone

Marker	Units	Pre-flight	In-flight A	In-flight B	Post-flight
BAP	$\mu\text{g/l}$	12.87 ± 1.10	11.53 ± 0.75	14.27 ± 1.51	15.00 ± 3.76
OC	$\mu\text{g/l}$	4.80 ± 0.03	4.49 ± 0.42	5.13 ± 0.39	5.01 ± 0.50
iPTH	ng/l	36.57 ± 5.08	22.94 ± 3.22	23.57 ± 2.50	37.22 ± 13.57
DPD/creat	$\mu\text{mol/mol}$	3.29 ± 0.36	5.76 ± 0.61	6.72 ± 0.72	4.40 ± 0.53
CTX/creat	g/mol	0.40 ± 0.10	0.73 ± 0.15	0.88 ± 0.16	0.43 ± 0.05
Ca/creat	mol/mol	0.14 ± 0.02	0.38 ± 0.05	0.29 ± 0.04	0.12 ± 0.02
Gla/creat	$\mu\text{mol/mol}$	1.75 ± 0.22	1.85 ± 0.29	3.39 ± 0.48	2.20 ± 0.20

Mean values $\pm$ standard deviation were calculated for the four episodes of the experiment. Abbreviations used: BAP, bone alkaline phosphatase; OC, osteocalcin (total antigen); iPTH, intact parathyroid hormone; DPD, deoxypyridinoline, CTX, type I C-terminal collagen crosslinked peptides; Gla, γ -carboxyglutamate.

Discussion

The most striking effect of living under microgravity conditions was the 2 fold increase of urinary calcium excretion, which continued at that level during the entire flight. The serum calcium was not measured in our experiment but, based on the studies performed in the Skylab series of experiments¹⁵, it was probably increased in our astronaut and likely accounts for the observed suppression of serum iPTH. The data in fig. 2 suggest that the increased calcium loss originated from a shift in the balance between bone formation and bone resorption in the favor of the latter. Like the Gla-content of serum osteocalcin, urinary Gla excretion remained unaffected by microgravity, but increased substantially after vitamin K-supplementation. These data are consistent with a subclinical vitamin K-deficiency in the astronaut during the pre-flight A period, and possibly also during the baseline data collection.

During the second part of the flight we induced an increased vitamin K status in the astronaut. It is clear that this had no effect on the circulating iPTH; although the urinary calcium excretion during the in-flight B period was lower than during in-flight A, a causal relation between the increased vitamin K status and decreased urinary

calcium is highly speculative at this time. In postmenopausal women an increased osteoclast activity due to the fall of estrogen levels is the main cause of accelerated bone loss. It has been shown that in this group vitamin K-supplementation may induce a decrease in markers for bone resorption^{5,6}. The fact that these markers remained unaffected during the in-flight B period suggests that the mechanism underlying microgravity-induced bone loss differs from that during the postmenopause. Like in postmenopausal women vitamin K administration was accompanied by an increase of markers for bone formation, but whether this effect is of physiological relevance remains to be established.

Another observation was that the vitamin K status remained high for about 2 months after the treatment was stopped. This provides the opportunity to substantially simplify the protocol for future experiments, with only pre-flight vitamin K treatment and in-flight sampling at 1 or 2 weeks after launch. Using this simplified protocol, a randomized placebo-controlled study could be performed among astronauts from a number of subsequent missions. In this way it will be possible to reach the group size required for statistical evaluation of a beneficial effect of vitamin K. At this time neither clinical nor experimental animal data are available demonstrating any risk of vitamin K supplementation in the dosages used. It is highly unlikely, therefore, that the proposed protocol will have undesired side-effects for the astronauts.

In summary we conclude that this experiment has shown that (i) microgravity induced substantial increases in markers for bone resorption and urinary calcium excretion, and a decrease in serum iPTH, (ii) vitamin K administration coincided with a change of the bone markers consistent with an improvement of bone balance, and with an increase in urinary Gla-excretion suggesting a subclinical vitamin K-deficiency in the astronaut before supplementation, and (iii) the experiment may be repeated among larger numbers of astronauts using a substantially simplified protocol.

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References

1. Convertino, V.A. *Exercise as a countermeasure for physiological adaptation to prolonged spaceflight. Med. Sci. Sports Exercise* 28: 999-1014, 1996.

2. Hara, K., Y. Akiyama, T. Nakamura, S. Murota, and I. Morita. The inhibitory effect of vitamin K₂ (menatetrenone) on bone resorption may be related to its side chain. *Bone* 16: 179-184, 1995.
3. Hauschka, P.V., J.B. Lian, D.E.C. Cole, and C.M. Gundberg. Osteocalcin and matrix Gla protein: vitamin K dependent proteins in bone. *Phys. Rev.* 69: 990-1047, 1989.
4. Kameda, T., K. Miyazawa, Y. Mori, T. Yuasa, M. Shiokawa, Y. Nakamura, H. Mano, Y. Hakeda, A. Kameda, and M. Kumegawa. Vitamin K₂ inhibits osteoclastic bone resorption by inducing osteoclast apoptosis. *Biochem. Biophys. Res. Commun.* 220: 515-519, 1996.
5. Knapen, M.H.J., K. Hamulyák, and C. Vermeer. The effect of vitamin K supplementation on circulating osteocalcin (bone Gla-protein) and urinary calcium excretion. *Ann. Int. Med.* 111: 1001-1005, 1989.
6. Knapen, M.H.J., K.-S.G. Jie, K. Hamulyák, and C. Vermeer. Vitamin K-induced changes in markers for osteoblast activity and urinary calcium loss. *Calcif. Tissue Int.* 53: 81-85, 1993.
7. Kuwada, M. and K. Katayama. An improved method for determination of gammacarboxy-glutamic acid in proteins, bone and urine. *Analyt. Biochem.* 131: 173-179, 1983.
8. Orimo, H., M. Shiraki, T. Fujita, T. Onomura, T. Inoue, and K. Kushida. Clinical evaluation of menatetrenone in the treatment of involutional osteoporosis - a double-blind multicenter comparative study with 1 α hydroxy vitamin D₃. *J. Bone Mineral Res.* 7: S122, 1992.
9. Rambaut, P.C. and A.W. Goode. Skeletal changes during space flight. *Lancet* ii: 1050-1052, 1985.
10. Schneider, V.S., A. Leblanc, and P.C. Rambaut. Bone and mineral metabolism. In: *Space physiology and medicine*, edited by E. Nicogossian, C. Leach Huntoon, and S.L. Pool. Philadelphia: Lea & Febiger, 1989, p. 214-221.
11. Seitzer, U., M. Bodo, P.K. Muller, Y. Acil, and B. Batgate. Microgravity and hypergravity effects on collagen biosynthesis of human dermal fibroblasts. *Cell Tissue Res.* 282: 513-517, 1995.
12. Shearer, M.J., Allan, V., Haroon, Y., and Barkhan, P. Nutritional aspects of vitamin K in the human. In: *Vitamin K metabolism and vitamin K-dependent proteins*, edited by J.W. Suttie. Baltimore: University Park Press, p. 317-327.
13. Tipton, C.M., J.E. Greenleaf, and C.G.R. Jackson. Neuroendocrine and immune system responses with spaceflights. *Med. Sci. Sports Exerc.* 28: 988-998, 1996.

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14. Vermeer, C., K.-S.G. Jie, and M.H.J. Knapen. Role of vitamin K in bone metabolism. *Annu. Rev. Nutr.* 15: 1-22, 1995.
15. Whedon, G.D., Lutwak, L., Reid, J., Rambaut, P., Whittle, M., Smith, M., Leach, C. Mineral and nitrogen metabolic studies on Skylab orbital space flights. *Trans. Assoc. Amer. Phys.* 87: 95-110, 1974.

CHAPTER 10

SUMMARY AND DISCUSSION

The group of compounds with vitamin K activity comprises a major plant form (phyloquinone, K₁) and several members of a series synthesized by bacteria (menaquinones, K₂). All are involved in protein synthesis at the stage of posttranslational modification, in which they participate as a coenzyme in the gammacarboxylation of peptide-bound glutamate residues into gammacarboxy glutamate (Gla). Within the Gla-containing proteins the Gla-residues form Ca-binding groups, and where their function is known, Gla is essential to their biological activity (for instance in blood coagulation factors), whereas it is assumed to be essential in Gla-proteins of unknown function. The importance of Gla-proteins for bone development was demonstrated in animal studies using knock-out mice for osteocalcin¹ and for matrix Gla-protein², as well in rats³ and lambs⁴, in which extrahepatic gammaglutamate carboxylation was blocked by treatment with high doses of warfarin combined with low doses of vitamin K₁.

Chapter 1 reviews our current knowledge on vitamin K. The classical definition of vitamin K-deficiency is a decreased intake or impaired uptake of the vitamin leading to an increased hemorrhagic tendency and spontaneous bleeding. In healthy adults this severe form of deficiency is rarely seen, but occasionally it is found in non-supplemented children during the first five days of life. In approximately 1:5,000 cases this may lead to gastrointestinal and intracranial bleeding with high morbidity. Subclinical vitamin K-deficiency has been defined as a state in which undercarboxylated prothrombin (PIVKA II) is detectable in plasma. The rationale behind this definition is that a reduced vitamin K supply will result in the production of undercarboxylated species of Gla-proteins, which - if released into the blood stream - can be assayed. Vitamin K-restricted diets were shown to induce PIVKA II in healthy volunteers after 2 weeks⁵. Also during the hemorrhagic states associated with acute hepatitis, liver cirrhosis and hepatocellular carcinoma, PIVKA II may be found in plasma; in these cases its presence is due to impaired liver function rather than to low vitamin K intake^{6,7}. After the discovery of extrahepatic Gla-proteins, accumulating evidence suggests that full carboxylation of these proteins requires higher vitamin K intake than needed for clotting factor synthesis. A significant fraction (about 20%) of circulating osteocalcin (bone Gla-protein) appeared to be undercarboxylated in the majority of healthy adults, and an even higher fraction of undercarboxylated osteocalcin (ucOC) was found in newborns⁸ (prone to develop vitamin K-deficiency) and in elderly subjects. Moreover, ucOC seem to be inversely correlated with bone mineral density, and elevated levels of ucOC appeared to be a strong risk factor for hip fracture⁹. Since osteocalcin is the Gla-protein most sensitive to impaired vitamin K-status, a modern definition for vitamin K-deficiency is a state in which incompletely carboxylated osteocalcin is found in the circulation.

In chapters 2 and 3 we describe experiments in the two existing animal models for vitamin K-deficiency: rats fed a vitamin K-deficient diet, and rats treated with vitamin K-antagonists (coumarins). In chapter 2 we demonstrate that both models are different. In the nutritionally deficient animals the vitamin K epoxide (the metabolite formed during one cycle of glutamate carboxylation) generated is efficiently re-used 500-1,000 fold by the action of the enzyme KO-reductase, whereas in the coumarin-treated model KO-reductase is blocked. This explains why in the latter model at least 500 times more vitamin K is required to maintain normal hemostasis. A second difference was that in the deficient model MK-9 had substantially higher bioactivity than K₁ and MK-4, whereas in the coumarin-treated model MK-9 had no measurable effect. One explanation for this phenomenon is that the intestinal absorption or hepatic uptake of the highly lipophilic MK-9 is poor and does not reach the high levels required to compensate for the blockade of KO-reductase. A second possibility is related to the fact that in the nutritionally deficient animals the reduction of vitamin K quinone into the active cofactor (K-hydroquinone) is accomplished by the membrane-bound KO-reductase, whereas in the coumarin-treated model this function is taken over by the cytosolic DT-diaphorase. In this model the bioactivity of the three K-vitamins tested is strictly correlated to their water solubility: MK-4>K₁>MK-9, which may just reflect their possibility to reach the reducing enzyme system.

In chapter 3 we have defined the minimal daily requirement of vitamin K (MDR) in rats by measuring plasma prothrombin procoagulant activity at increasing intake of vitamin K. Plateau levels for prothrombin activity were obtained at 0.3 mmol MK-4 per day. Control rats received 1 mmol/day, whereas other groups received 20 mmol of either MK-4 or K₁. Whereas prothrombin levels were similar in all groups, the urinary Gla-excretion had increased significantly at high vitamin K intakes. This suggests a higher vitamin K requirement for extrahepatic carboxylases. When comparing Gla-excretion in vitamin K-deficient and in coumarin-treated animals we found that at 10% remaining prothrombin procoagulant activity, urinary Gla-excretion in the deficient animals had only slightly changed from its pre-treatment value, whereas in the coumarin-treated model Gla-excretion had decreased to 17% of the starting values. An explanation for this apparent discrepancy is that prothrombin procoagulant activity does not decrease proportional to the prothrombin Gla-content. During vitamin K-deficiency a wide range of undercarboxylated coagulation factors (e.g. prothrombin containing 0-9 Gla residues/molecule) are formed which have very low procoagulant activity, but will produce free Gla upon degradation. During coumarin treatment Gla-formation was essentially blocked, so that the anticoagulated state was reached much faster, and virtually all vitamin K-dependent coagulation factors were synthesized in their non-carboxylated form. In the latter case Gla-

proteins with long half-life times (e.g. those captured in bone tissue) will probably form a major part of the urinary Gla-content.

Chapters 4 and 5 must be regarded as the development of tools for human studies. In chapter 4 we compare a number of markers for vitamin K-status in humans: plasma descarboxyprothrombin (PIVKA II), serum undercarboxylated osteocalcin (ucOC), and urinary Gla. Pregnant women and their newborn babies form risk groups for developing vitamin K-deficiency. Part of the women was treated during the last 4 weeks of pregnancy with vitamin K (1 mg/day). Nevertheless, PIVKA II could not be detected in any of the participating 44 women shortly before delivery nor in their newborn babies (cord blood). On the other hand, ucOC was unusually high during pregnancy, and in cord blood the level was even comparable to that in anticoagulated subjects. Vitamin K treatment completely corrected the levels in the mothers, and partly in the cord serum (due to the poor placental passage of vitamin K). Also in a second group at risk of developing subclinical vitamin K-deficiency (postmenopausal women) we found that a 4-week treatment with vitamin K significantly decreased the circulating ucOC. In this group also urinary Gla-excretion was measured, but this marker remained unchanged by vitamin K treatment. It is concluded, therefore that among the used markers, the ratio between ucOC and total osteocalcin is the most sensitive one for vitamin K-status. This conclusion is shared by other researches in the field. A possible explanation for the quick response of osteocalcin to low vitamin K status was given by Chu et al., who demonstrated that the Ala¹⁰ in the propeptide of Gla-protein precursors is of major importance for their affinity towards carboxylase. Osteocalcin is the only known Gla-protein lacking an alanine at this position, and based on a comparable mutation in the propeptide of factor IX the authors conclude that the resulting low affinity between osteocalcin propeptide and carboxylase explains why osteocalcin is the first protein to experience poor vitamin K status.

In chapter 5 we describe the development of the first automated test for serum osteocalcin. This so-called coated beads assay may be implemented on fully automated analyzers, which may reduce imprecision and increase the applicability of osteocalcin as a marker for bone metabolism in the routine clinical setting. The test performance was good as compared to a number of commercially available ELISA-based biomarkers for bone metabolism. Unfortunately, this coated beads assay was not taken in production by our industrial partner (Roche Diagnostics), because after we completed our studies a merger between Roche and Boehringer Mannheim changed their interest and strategy. Therefore, most of our clinical studies had to be performed with standard ELISA-based techniques.

Chapter 6 provides the problem statement underlying the human volunteer studies in this thesis. Vitamin K is a cofactor in the synthesis of proteins regulating blood coagulation and bone proteins. The bioavailability of vitamin K depends on a number of factors including the type of food and food matrix, the dietary fat content, the type of vitamin K, and the apoE genotype. Undercarboxylation of blood coagulation factors is extremely rare in healthy adults, demonstrating that the hepatic vitamin K supply is adequate. The undercarboxylated bone Gla protein osteocalcin is frequently found in healthy subjects, with high levels in postmenopausal women. This strongly suggests that bone (and possibly other extrahepatic tissues as well) receives insufficient vitamin K to cover the amount required for full carboxylation of all Gla-sites. Supplementation of elderly women with pharmacological doses of vitamin K has shown markers for bone formation to increase and, occasionally, markers for bone resorption to decrease¹¹. We postulate the concept of tissue-defined vitamin K-deficiency: subjects who have adequate levels of vitamin K intake to cover their hepatic requirements, may be (subclinically) vitamin K-deficient with respect to bone or other tissue. One group who may particularly be in need of vitamin K supplementation are postmenopausal women. In this thesis we have tried to find other groups at risk for developing poor vitamin K status: children in Romanian children's homes, young female elite athletes, and astronauts during their stay in space.

In **chapter 7** we describe investigations among 127 institutionalized Romanian children who are known to be prone of developing vitamin malnutrition. A first screening for rickets was performed in January 1998 among children between 4 - 48 months of age. At that time 46 cases of rickets were diagnosed. Using urinary Gla as marker for vitamin K status, a subclinical vitamin K deficiency was assessed in this group as well. Since both vitamin K and vitamin D deficiencies have comparable seasonal fluctuations with highest incidence in the colder season, the remaining 81 children were followed during the rest of the winter. It turned out that during the subsequent three months 13 subjects developed rickets, whereas in those without biochemical signs of rickets in the control group, both serum vitamin D concentrations and urinary Gla excretion decreased and reached the levels of rickets children by the month of April. The vitamin deficiencies were independant of the anthropometric indices at birth or at present, and neither on dietary protein or calory intake. These data demonstrates the necessity of daily vitamin supplementation of the children living in Romania's childrens homes. We also determined the apolipoprotein E genotype in a sample of the Romanian population and in a number of former vitamin K deficiency (VKDBN) and rickets patients. Apo E is known to be involved in the plasma transport of vitamins K and D. No clear correlation with any

apo E genotype was found, suggesting that the observed deficiencies were primarily caused by low intake and not by a plasma transport-related factor.

In chapter 8 we have focussed on female elite athletes the strenuous exercise of whom may result in hypoestrogenism and amenorrhoea. As a consequence low peak bone mass and rapid bone loss are often seen in relatively young athletes. In this relatively small intervention study we describe the effects of 1 month of vitamin K supplementation in eight female athletes, four of whom had been amenorrhoeic for more than one year, whereas the others had been using oral contraceptives. All participants received vitamin K supplementation (10 mg/day), and various bone markers were measured before and after treatment. At baseline the athletes not using oral contraceptives were biochemically vitamin K-deficient as deduced from the calcium binding capacity (i.e. Gla-content) of circulating osteocalcin. In all subjects the use of vitamin K supplements was associated with an increased calcium-binding capacity of osteocalcin. In the low-estrogen group vitamin K supplementation induced a 15-20% increase of bone formation markers and a parallel 20-25 % decrease of bone resorption markers. This shift is suggestive for an improved balance between bone formation and resorption. The preliminary study reported here has formed the basis for a ZON research grant, which has facilitated a 3-year clinical trial among 120 elite female athletes from Belgium, Germany and The Netherlands. The latter study, in which also the rate of bone loss (DEXA) is recorded, is currently in progress and will be completed in December 2001.

In chapter 9 we report about a unique experiment in the Russian space station MIR. Rapid bone loss is a serious health problem for astronauts during long lasting missions in space. We had the possibility to record the changes of biochemical markers for bone metabolism in one of the astronauts during the 6-month space flight of the EUROMIR-95 mission. Immediately after launch bone formation markers decreased, and bone resorption markers increased. As a result, the excretion of urinary calcium increased about two fold. After 12½ weeks the astronaut received vitamin K₁ (10 mg/day for 6 weeks). Concomitant with the start of vitamin K treatment, the Gla-content of the circulating osteocalcin increased, and so did the urinary excretion of free Gla. This strongly suggests a subclinical vitamin K-deficiency of the astronaut before vitamin K supplementation. During periods of high vitamin K status, markers for bone formation were significantly higher than during the first part of the flight. Parathyroid hormone (PTH, which was low during microgravity) and markers for bone resorption (high during microgravity) remained unaffected by vitamin K supplementation. The data suggest that increased vitamin K intake may contribute in counteracting microgravity-induced bone loss, and we provide a greatly

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simplified design to prove this hypothesis in a larger number of astronauts using a minimal amount of flight time.

In conclusion we have demonstrated in this thesis that subclinical vitamin K-deficiency of other tissues than liver is common among the healthy population. This may result in undercarboxylation of extrahepatic Gla-proteins such as osteocalcin and matrix Gla-protein. Although their mechanism of action is not yet known precisely, several of these proteins are involved in the regulation of calcium deposition. Knock-out mice have demonstrated that extrahepatic proteins are essential for normal bone formation (osteocalcin) and for preventing arteries and other soft tissues from rapid calcification (MGP). Since both proteins mentioned have been reported to occur in an undercarboxylated form in the majority of the population, the nutritional vitamin K intake is too low to ensure proper carboxylation (and thus: function) of these proteins. Since the mean vitamin K intake in the adult population is well above the present RDA value (1 µg/day/kg body weight), we recommend that these RDA-values should be re-evaluated.

References:

1. Dacy, P., Desbois, C., Boyce, B., Pinero, G., Story, B., Dunstan, C., Smith, E., Bonadio, J., Goldstein, S., Gundberg, C., Bradley, A., Karsenty, G. Increased bone formation in osteocalcin-deficient mice. *Nature*, 1996; 382: 448-452.
2. Luo, G., Dacy, P., McKee, M.D., Pinero, G.I., Loyer, E., Behringer, R.R. Spontaneous calcification of arteries and cartilage in mice lacking matrix Gla protein. *Nature*, 1997; 386: 78-81.
3. Price, P.A., Williamson, M.K., Haba, T., Dell, R.B., Jee, W.S.S. Excessive mineralization with growth plate closure in rats on chronic warfarin treatment. *Proc.Natl.Acad.Sci.* 1982; 79: 7734-7738.
4. Pastoureau, P., Vergnaud, P., Meunier, P.J., Delmas, P.D. Osteopenia and bone-remodeling abnormalities in warfarin-treated lambs. *J. Bone Miner Res.* 1993; 8(12): 1417-1426.
5. Ferland, G., Sadowski, J.A., O'Brien, M.E. Dietary induced subclinical vitamin K deficiency in normal human subjects. *J. Clin. Invest.* 1993; 91: 1761-8.
6. Blanchard, R.A., Furie, B.C., Jorgensen, M., Kruger, S.F., Furie, B. Acquired vitamin K-dependent carboxylation deficiency in liver disease. *New Engl. J. Med.* 1981; 305: 242-8.
7. Okuda, H., Nakanishi, T., Furukawa, M., Obata, H. Vitamin K-dependent proteins and hepatocellular carcinoma. *J. Gastroenterol. Hepatol.* 1991; 6: 392-9.

8. Jie, K.-S.G., Hamulyák, K., Gijsbers, B.L., Roumen, F.J., Vermeer, C. Serum osteocalcin as a marker for vitamin K-status in pregnant women and their newborn babies., *Thromb. Haemost.* 1992; 68: 388-91.
9. Szulc, P., Chapuy, M.-C., Meunier, P.J., Delmas, P.D. Serum undercarboxylated osteocalcin is a marker of the risk of hip fracture in elderly women. *J. Clin. Invest.* 1993; 91: 1769-1774.
10. Chu, K., Wu, S.M., Stanley, T., Stafford, D.W., High, K.A. A mutation in the propeptide of Factor IX leads to warfarin sensitivity by a novel mechanism. *J. Clin. Invest.* 1996; 98(7): 1619-1925.
11. Knapen, M.H.J., Jie, K.-S.G., Hamulyák, K. & Vermeer C. Vitamin K-induced changes in markers for osteoblast activity and urinary calcium loss. *Calcif. Tissue Int.* 1993; 53: 81-85.

CHAPTER 11**SAMENVATTING EN CONCLUSIES**

De groep van verbindingen met "vitamine K-activiteit" bestaat vooral uit fyloquinon (vitamine K₁), dat voorkomt in groene planten (bladgroenten), en daarnaast uit een groep verwante verbindingen die gesynthetiseerd worden door bacteriën (menaquinonen, vitamine K₂). Alle K-vitamines zijn betrokken bij de synthese van eiwitten op het niveau van de posttranslationale modificatie. De rol van vitamine K daarbij is dat het fungeert als co-enzym in de gammacarboxylering van glutaminezuur (Glu) residuen welke onderdeel zijn van de peptide-keten. Deze Glu-residuen worden daarbij omgezet in gammacarboxyglutaminezuur (Gla). Gla-residuen vormen calcium-bindende groepen in de Gla-bevattende eiwitten (kortweg Gla-eiwitten genaamd). In alle gevallen waarin de functie van deze Gla-eiwitten bekend is, is de aanwezigheid van de Gla-residuen essentieel voor hun biologische activiteit (bijvoorbeeld de procoagulante activiteit van een aantal stollingsfactoren). Van sommige Gla-eiwitten is de precieze functie nog niet bekend, maar algemeen wordt verondersteld dat ook in die gevallen Gla belangrijk is voor een correcte functie. Het belang van Gla-eiwitten voor de ontwikkeling van bot werd aangetoond in transgene ("knock-out") muizen welke deficiënt gemaakt waren voor ofwel osteocalcine (bone Gla-protein) dan wel matrix Gla-proteïne (MGP). Ook in ratten en lammeren waarin alleen de Gla-vorming in de bot-eiwitten was geblokkeerd met behulp van de vitamine K-antagonist warfarine werd een pathologische botvorming vastgesteld.

Hoofdstuk 1 is een literatuuroverzicht waarin de huidige kennis van vitamine K wordt samengevat. De klassieke definitie van vitamine K-deficiëntie is een verminderde inname of een verstoorde opname van het vitamine leidend tot een verhoogde bloedingsneiging, en spontane bloedingen. In gezonde volwassenen komt deze ernstige deficiëntie niet of uiterst zelden voor, maar een enkele keer wordt dit wel gevonden bij pasgeborenen. Indien geen vitamine K gesupplementeerd wordt kan dat in ongeveer 1 op de 5000 gevallen leiden tot gastrointestinale en intracraniale bloedingen met een hoge morbiditeit. Subklinische vitamine K-deficiëntie wordt gedefinieerd als een staat waarin ondergecarboxyleerd protrombine (PIVKA II) detecteerbaar is in plasma. Deze definitie is gebaseerd op de aanname dat een te geringe toevoer van vitamine K naar de diverse weefsels zal leiden tot onvolledig gecarboxyleerde vormen van de in die weefsels geproduceerde Gla-eiwitten, welke - indien uitgescheiden in de bloedstroom - kunnen worden bepaald. In proefpersonen is aangetoond dat een vitamine K-beperkt dieet binnen 2 weken leidt tot aantoonbare concentraties PIVKA II in het bloed. Ook gedurende de verhoogde bloedingsneiging welke geassocieerd is met acute hepatitis, lever cirrhose, en hepatocellulair carcinoom, wordt vaak PIVKA II aangetoond in plasma; in deze gevallen wordt dat veroorzaakt door een verstoorde leverfunctie, en niet door een te lage inname van

vitamine K. De eerste Gla-eiwitten die ontdekt werden behoorden allemaal tot de in de lever gemaakte stollingsfactoren (hepatische Gla-eiwitten). Na de ontdekking dat er ook extra-hepatische Gla-eiwitten bestaan, is in toenemende mate duidelijk geworden dat voor volledige carboxylering van deze eiwitten een hogere vitamine K-inname vereist dan voor de stollingsfactoren. Een aanzienlijke fractie (ongeveer 20%) van het circulerend osteocalcine, bijvoorbeeld, is ondergecarboxyleerd in de meeste gezonde volwassenen, terwijl in pasgeborenen en ouderen deze fractie zelfs nog aanzienlijk hoger was. De concentratie ondergecarboxyleerd osteocalcine (ucOC) in serum was ook op inverse wijze gecorreleerd met botdichtheid, terwijl een verhoogde serum spiegel ucOC een sterke risicofactor is voor heupfracturen. Aangezien osteocalcine het Gla-eiwit is dat het meest gevoelig is voor een lage vitamine K-status, stellen wij voor om als nieuwe definitie voor vitamine K-deficiëntie te hanteren een staat waarin onvolledig gecarboxyleerd osteocalcine gevonden wordt in de circulatie.

In de hoofdstukken 2 en 3 beschrijven we experimenten in twee bestaande proefdiermodellen voor vitamine K-deficiëntie: ratten gevoed met een vitamine K-deficiënt dieet en ratten behandeld met vitamine K-antagonisten (coumarines). In hoofdstuk 2 tonen we aan dat beide modellen van elkaar verschillen. In de dieren op vitamine K-deficiënt voedsel wordt het vitamine K epoxide dat ontstaat tijdens de Glu-carboxylering, gemiddeld 500 tot 1000 maal hergebruikt door de activiteit van het enzym KO-reductase, terwijl in coumarine-behandelde dieren KO-reductase geblokkeerd is. Dit verklaart waarom in het laatste model ten minste 500 maal meer vitamine K nodig is om een normale hemostase te garanderen. Een tweede verschil was dat in het deficiënt-dieet model menaquinon-9 (MK-9) een aanzienlijk hogere biologische activiteit had dan vitamine K₁ of MK-4, terwijl in coumarine-behandelde dieren MK-9 geen meetbaar effect vertoonde. Een verklaring voor dat fenomeen is dat de intestinale absorptie of de opname door de lever van het zeer lipofiele MK-9 beperkt is, en niet de hoge waarden bereikt die nodig zijn om de blokkade van KO-reductase te compenseren. Een tweede factor die een rol kan spelen is dat in het deficiënt-dieet model de reductie van vitamine K quinon tot het hydroquinon (de actieve co-factor) verloopt via het membraan-gebonden KO-reductase, terwijl in het coumarine-behandelde model deze omzetting wordt overgenomen door het cytologische enzym D-diaforese. In het laatste geval moet verwacht worden dat de biologische activiteit van de drie geteste K-vitamines recht evenredig zijn met hun oplosbaarheid in water (MK-4 > K₁ > MK-9), welke een afspiegeling is van de mate waarin zij het reducerende enzymstelsel kunnen bereiken. Dit komt overeen met onze bevindingen.

In hoofdstuk 3 hebben we de minimale dagelijkse dosis (MDR) vitamine K gedefinieerd die nodig is voor een optimale protrombine synthese in ratten. Plateauwaarden voor plasma protrombine werden verkregen boven 0.3 mmol vitamine K per dag. Deze waarde werd gehanteerd als de MDR. In een volgend experiment ontvingen ratten gedurende 3 weken een betrekkelijk lage dosis (1 mmol/dag = 3 x MDR) of een hoge (20 mmol/dag = 60 x MDR) dosis van ofwel vitamine K₁ dan wel MK-4. Terwijl de plasma-protrombine niveaus vergelijkbaar waren in alle groepen, was de Gla-excretie via de urine significant verhoogd in de groepen met hoge vitamine K-inname. Dit is een sterke aanwijzing voor een verhoogde vitamine K-behoefte van extra-hepatische carboxylases. Wanneer we de Gla-excretie van deficiënt-dieet ratten vergelijken met die van coumarine-behandelde dieren zien we dat in de eerste groep de urinaire Gla-excretie slechts weinig verschilt van de waarde vóór aanvang van de proef, terwijl in de coumarine-behandelde groep de Gla-excretie gedaald was tot ongeveer 17% van de startwaarde. Een verklaring voor dit verschil is dat de procoagulante activiteit van protrombine (normaal 10 Gla-residuen per molecule) veel sterker dan evenredig daalt wanneer het aantal Gla-residuen per molecule afneemt. Tijdens vitamine K-deficiëntie wordt een groot aantal soorten protrombine (en andere stollingsfactoren) met 9, 8, 7, 6, tot 0 Gla-residuen per molecule gevormd, die allen een zeer lage procoagulante activiteit hebben, maar die wel een aanzienlijke bijdrage leveren aan de vrije Gla productie tijdens hun degradatie. Tijdens coumarine behandeling is de vorming van Gla totaal geblokkeerd, zodat de geantistolde staat veel eerder bereikt wordt, en alle vitamine K-afhankelijke stollingsfactoren in hun ongecarboxyleerde staat (0 Gla-residuen per molecule) gevormd worden. In het laatste geval zal degradatie van Gla-eiwitten met lange biologische halfwaarde-tijden (bijvoorbeeld eiwitten die gevangen zitten in de botmatrix) waarschijnlijk de belangrijkste bijdrage leveren aan de urinaire Gla-excretie.

De hoofdstukken 4 en 5 moeten beschouwd worden als de ontwikkeling van technieken voor studies in mensen. In hoofdstuk 4 vergelijken we een aantal merkstoffen voor de vitamine K-status in mensen: plasma descaboxypotrombine (PIVKA II), serum ondergecarboxyleerd osteocalcine (ucOC) en het Gla-gehalte van urine. Zwangere vrouwen en pasgeborenen vormen een risico-groep voor het ontstaan van vitamine K-deficiëntie. Van een eerder beschreven groep 44 zwangere vrouwen was een deel behandeld met extra vitamine K (10 mg/dag) gedurende de laatste 4 weken van de zwangerschap. Kort voor de bevalling kon in geen van de deelnemsters PIVKA II aangetoond worden. Ook bij de baby's bleef PIVKA II in het navelstrengbloed in alle gevallen beneden de detectiegrens. Wel werd gevonden dat in de onbehandelde zwangeren vlak vóór de bevalling het ucOC onverwacht hoog

was. In het navelstreng bloed van hun baby's was het zelfs nog hoger: het was vergelijkbaar met het niveau in personen die langdurig worden geantistold. De behandeling met vitamine K had het circulerend ucOC bij de moeders volledig genormaliseerd, terwijl in hun baby's het ucOC sterk verlaagd was ten opzichte van de onbehandelde groep. Het feit dat toch nog ucOC aantoonbaar was komt waarschijnlijk door de slechte passage van vitamine K door de placenta. In een tweede groep met verhoogd risico op een subklinische vitamine K-deficiëntie (postmenopausale vrouwen) vonden we dat een 4-weekse behandeling met vitamine K het circulerend ucOC significant verlaagde. In deze groep werd tevens de urinaire Gla-excretie gemeten, maar deze merkstof was niet significant veranderd door de behandeling met vitamine K. Op basis van deze gegevens hebben we daarom geconcludeerd dat van de door ons geteste parameters, de verhouding tussen ucOC en totaal osteocalcine de meest gevoelige maat is voor vitamine K-status. Deze conclusie wordt op dit moment gedeeld door de meeste andere onderzoekers in het vakgebied. Een mogelijke verklaring voor de gevoeligheid van osteocalcine voor een lage vitamine K-status wordt gegeven door Chu en medewerkers, die aantoonen dat het Ala⁻¹⁰ in het propeptide van precursors van Gla-eiwitten van belang is voor hun affiniteit voor carboxylase. Osteocalcine is het enig bekende Gla-eiwit dat geen alanine op deze positie heeft, en gebaseerd op een vergelijkbare natuurlijke mutatie in het propeptide van stollingsfactor IX concluderen de auteurs dat de te verwachten lage affiniteit tussen het osteocalcine propeptide en carboxylase geheel verklaart waarom osteocalcine het eerste eiwit is dat het effect ondervindt van een teruglopende vitamine K-status.

In hoofdstuk 5 beschrijven we de ontwikkeling van de eerste geautomatiseerde test voor serum osteocalcine. Deze zogenaamde "coated beads" test kan toegepast worden op volledig geautomatiseerde pipetteerstations, hetgeen de nauwkeurigheid verbetert, en de toepasbaarheid van osteocalcine als merkstof voor botmetabolisme in de routine sterk vergroot. Vergeleken met een aantal commerciële ELISA tests waren de resultaten met onze nieuw ontwikkelde procedure zeer goed, maar helaas werd de test niet in productie genomen door onze industriële sponsor omdat na een fusie (Hoffmann-La Roche en Boehringer Mannheim) deze lijn niet meer tot het interessegebied behoorde. Daarom zijn de meeste van de hieronder beschreven klinische studies uitgevoerd met de commerciële standaard ELISA-techniek.

Hoofdstuk 6 beschrijft de probleemstelling voor de humane studies in dit proefschrift. Vitamine K is een co-factor bij de synthese van eiwitten die een belangrijke rol spelen bij de regulatie van de bloedstolling en van de mineralisatie van bot. De biologische beschikbaarheid van vitamine K hangt af van een aantal factoren zoals het soort voedsel dat gegeten wordt, de voedsel matrix, de

hoeveelheid vet van de maaltijd, het soort vitamine K (fylloquinon, menaquinon) dat gegeten wordt, en het apoE genotype. Aangezien ondercarboxylering van stollingsfactoren buitengewoon zeldzaam is in gezonde volwassenen, nemen we aan dat de vitamine K voorziening van de lever adequaat is. Ondergecarboxyleerd osteocalcine (bot Gla-proteïne) wordt echter waargenomen bij het overgrote deel van alle volwassenen, met zeer hoge waarden in postmenopausale vrouwen. Dit suggereert dat botten (en mogelijk ook andere extrahepatische weefsels) onvoldoende vitamine K ontvangen om alle in dat weefsel geproduceerde Gla-eiwitten volledig te carboxyleren. Supplementatie van oudere vrouwen met farmacologische doses vitamine K heeft laten zien dat de carboxyleringsgraad van osteocalcine toeneemt, en dat andere merkstoffen voor botvorming in gunstige zin beïnvloed worden. Wij postuleren hier het concept van weefsel-gedefinieerde vitamine K-deficiëntie: personen die voldoende vitamine K innemen om de behoefte van de lever te dekken, kunnen (subklinisch) vitamine K-deficiënt zijn wat betreft bot of ander weefsel. Een groep die met name extra vitamine K nodig zou kunnen hebben wordt gevormd door postmenopausale vrouwen. Hieraan is en wordt reeds door anderen uit onze groep en elders aandacht besteed. In dit proefschrift hebben we getracht andere risico-groepen te vinden voor een slechte vitamine K-status: kinderen in Roemeense kindertehuizen, jonge vrouwelijke atleten, en astronauten tijdens hun verblijf in de ruimte.

In hoofdstuk 7 beschrijven we een onderzoek onder 127 geïnstitutionaliseerde Roemeense kinderen van 4 tot 48 maanden oud; hiervan is bekend dat ze vaak een vitamine tekort ontwikkelen. Bij een eerste screening op rachitis (t.g.v. vitamine D-tekort) in januari 1998 werden 46 gevallen van rachitis gediagnosticeerd. Op basis van urinair Gla als merkstof werd in deze groep tevens een vitamine K-deficiëntie geconstateerd. Vitamine K- en vitamine D-deficiëntie vertonen een vergelijkbaar circadiaan patroon, met een hoogste incidentie in het koude seizoen. Daarom werden de resterende 81 kinderen vervolgd gedurende de rest van de winter. Het bleek dat in april nog eens 13 kinderen rachitis hadden ontwikkeld, terwijl in de kinderen zonder aantoonbaar rachitis zowel de serum vitamine D concentratie als de urinaire Gla-excretie gedaald waren tot een niveau vergelijkbaar met de rachitis groep. De vitamine deficiënties waren onafhankelijk van de antropometrische gegevens bij geboorte of ten tijde van het onderzoek, en van de dagelijkse eiwit en calorie inname. Deze gegevens tonen de noodzaak aan van vitamine suppletie bij kinderen die leven in Roemenië's kindertehuizen. We hebben ook het apolipoproteïne E genotype bepaald van een representatieve groep uit de Roemeense bevolking zowel als van een groep voormalig vitamine K-deficiënte kinderen en van de rachitis patiënten. ApoE is betrokken bij het transport van de

vitamines D en K in de bloedstroom. Er werd echter geen duidelijke correlatie gevonden tussen het voorkomen van genoemde ziekten en het apoE genotype, hetgeen suggereert dat de gevonden deficiënties voornamelijk veroorzaakt werden door een voedingstekort.

In hoofdstuk 8 hebben we ons gericht op vrouwelijke top-atleten, bij wie de zeer zware trainingsinspanning kan resulteren in verminderde oestrogeenproductie en menstruatiestoornissen. Als gevolg daarvan worden een lage piek-botmassa en snel botverlies vaak gezien bij jonge atleten. In deze betrekkelijk kleine interventiestudie onderzochten wij het effect van vitamine K suppletie (10 mg/dag) gedurende 1 maand bij acht vrouwelijke atleten waarvan er vier al langer dan 12 maanden niet meer hadden gemenstrueerd, terwijl de andere vier orale voorbehoedmiddelen (met als resultaat onttrekkingsbloedingen) gebruikten. Alle deelnemsters ontvingen vitamine K, en diverse merkstoffen voor botstofwisseling werden gemeten voor en aan het einde van de behandeling. Vóór de behandeling waren de atletes die geen orale voorbehoedmiddelen gebruikten subklinisch vitamine K-deficiënt (verhoogd serum ucOC). In alle deelnemsters was behandeling met vitamine K geassocieerd met een verlaging van het serum ucOC. In de groep niet-pilgebruiksters resulteerde de behandeling met vitamine K in een 15-20% verhoging van de merkstoffen voor botvorming, en in een 20-25% verlaging van de merkstoffen voor botresorptie. Deze verschuiving suggereert een verbeterde balans tussen botvorming en botafbraak, maar is op zichzelf te klein om harde conclusies aan te verbinden. De studie diende echter als pilot-onderzoek voor een 3-jarige door het Praeventiefonds / ZON gesubsidieerde trial onder 120 vrouwelijke sporters uit Nederland, België en Duitsland. Deze studie, waarin ook de snelheid van botverlies (DEXA) wordt gemeten, zal niet voor december 2001 voltooid zijn.

In hoofdstuk 9 beschrijven we een uniek experiment in het Russische ruimtestation MIR. Snel botverlies is een groot gezondheidsprobleem voor ruimtevaarders tijdens langdurige ruimtevluchten. Wij hadden de mogelijkheid om de veranderingen te meten die optreden in de biochemische merkstoffen voor botstofwisseling in één van de ruimtevaarders tijdens de 6 maanden durende vlucht van de EUROMIR-95 missie. Onmiddellijk na de lancering daalden de merkstoffen voor botvorming, terwijl die voor botresorptie sterk toenamen. Dit is indicatief voor een veranderde botbalans, met een netto verlies aan botmassa, en verklaart ook de waargenomen verdubbeling van de calcium uitscheiding in de urine. Na 122 weken kreeg de astronaut een vitamine K₁ supplement (10 mg/dag gedurende 6 weken). Direct na de start van de vitamine K toediening zagen we een sterke verhoging van de carboxyleringsgraad van het serum osteocalcine, en ook van de uitscheiding van vrij Gla in de urine. Beide waarnemingen zijn indicatief voor een subklinische vitamine K-deficiëntie

tijdens het eerste deel van de vlucht, en een verbetering van de vitamine K-status tijdens de suppletie-periode. Tijdens en na de verhoogde inname van vitamine K waren de merkstoffen voor botvorming duidelijk hoger dan tijdens het eerste deel van de vlucht. Parathormoon (PTH) was laag tijdens de gehele periode van gewichtloosheid, terwijl ook de merkstoffen voor botresorptie onveranderd bleven door toediening van vitamine K. Hoewel dit experiment slechts werd uitgevoerd bij één astronaut, ondersteunen de verkregen gegevens de hypothese dat een verhoogde vitamine K inname door astronauten kan bijdragen aan het probleem van botverlies tijdens gewichtloosheid. Natuurlijk is het niet mogelijk om dit experiment te herhalen als een placebo-gecontroleerde studie bij een grote groep ruimtevaarders tijdens een serie ruimtevluchten. Daarom stellen we een gewijzigd, sterk vereenvoudigd protocol voor waarmee deze hypothese getoetst kan worden onder ruimtevaarders m.b.v. metingen voor en na de vlucht, zodat de tijdsbelasting tijdens de vlucht minimaal zal zijn. Op die wijze zou zonder noemenswaardige belasting een prospectieve klinische trial kunnen worden opgezet onder bijvoorbeeld alle bewoners van het International Space Station gedurende de komende 2-3 jaar.

De conclusie van dit proefschrift is dat een subklinische vitamine K-deficiëntie van andere weefsels dan de lever vóórkomt in grote delen van de gezonde, volwassen bevolking. Dit kan resulteren in ondercarboxylering van extrahepatische Gla-eiwitten zoals osteocalcine en matrix Gla-proteïne. Ofschoon hun werkingsmechanisme nog niet in alle gevallen op moleculair niveau bekend is, weten we dat verschillende van deze eiwitten betrokken zijn bij de regulatie van calcium depositie. Knock-out muizen hebben laten zien dat extrahepatische Gla-eiwitten van essentieel belang zijn voor normale botvorming (osteocalcine, MGP), en ter voorkoming van snelle verkalking van diverse zachte weefsels, zoals bloedvaten en kraakbeen (MGP). Aangezien van beide genoemde eiwitten bekend is dat ze vóórkomen in een ondergecarboxyleerde vorm bij het grootste deel van de bevolking, moeten we vaststellen dat de inname van vitamine K via de voeding onvoldoende is om de adequate carboxylering (en dus: de correcte functie) van deze eiwitten te verzekeren. De huidige aanbevolen inname (RDA-waarde) voor vitamine K is $1 \mu\text{g}/\text{dag}/\text{kg}$ lichaamsgewicht, en deze waarde wordt over het algemeen ruimschoots bereikt via de normale voeding. Deze waarde is echter vastgesteld aan de hand van de behoefte van de lever voor de aanmaak van bloedstollingsfactoren. Kennelijk is de RDA-waarde onvoldoende voor ten minste een aantal andere organen, en wij bevelen daarom met klem aan dat die waarde binnen korte tijd herzien wordt.

References:

1. *Ducy, P., Desbois, C., Boyce, B., Pinero, G., Story, B., Dunstan, C., Smith, E., Bonadio, J., Goldstein, S., Gundberg, C., Bradley, A., Karsenty, G. Increased bone formation in osteocalcin-deficient mice. Nature, 1996; 382: 448-452.*
2. *Luo, G., Ducy, P., McKee, M.D., Pinero, G.I., Loyer, E., Behringer, R.R. Spontaneous calcification of arteries and cartilage in mice lacking matrix Gla protein. Nature, 1997; 386: 78-81.*
3. *Price, P.A., Williamson, M.K., Haba, T., Dell, R.B., Jee, W.S.S. Excessive mineralization with growth plate closure in rats on chronic warfarin treatment. Proc.Natl.Acad.Sci. 1982; 79: 7734-7738.*
4. *Pastoureau, P., Vergnaud, P., Meunier, P.J., Delmas, P.D. Osteopenia and bone-remodeling abnormalities in warfarin-treated lambs. J. Bone Miner Res. 1993; 8(12): 1417-1426.*
5. *Ferland, G., Sadowski, J.A., O'Brien, M.E. Dietary induced subclinical vitamin K deficiency in normal human subjects. J. Clin. Invest. 1993; 91: 1761-8.*
6. *Blanchard, R.A., Furie, B.C., Jorgensen, M., Kruger, S.F., Furie, B. Acquired vitamin K-dependent carboxylation deficiency in liver disease. New Engl. J. Med. 1981; 305: 242-8.*
7. *Okuda, H., Nakanishi, T., Furukawa, M., Obata, H. Vitamin K-dependent proteins and hepatocellular carcinoma. J. Gastroenterol. Hepatol. 1991; 6: 392-9.*
8. *Jie, K.-S.G., Hamulyák, K., Gijsbers, B.L., Roumen, F.J., Vermeer, C. Serum osteocalcin as a marker for vitamin K-status in pregnant women and their newborn babies., Thromb. Haemost. 1992; 68: 388-91.*
9. *Szulc, P., Chapuy, M.-C., Meunier, P.J., Delmas, P.D. Serum undercarboxylated osteocalcin is a marker of the risk of hip fracture in elderly women. J. Clin. Invest. 1993; 91: 1769-1774.*
10. *Chu, K., Wu, S.M., Stanley, T., Stafford, D.W., High, K.A. A mutation in the propeptide of Factor IX leads to warfarin sensitivity by a novel mechanism. J. Clin. Invest. 1996; 98(7): 1619-1925.*
11. *Knapen, M.H.J., Jie, K.-S.G., Hamulyák, K. & Vermeer C. Vitamin K-induced changes in markers for osteoblast activity and urinary calcium loss. Calcif. Tissue Int. 1993; 53: 81-85.*

CURRICULUM VITAE

Alexandra Crăciun was born on 4 May 1965 in Cluj, Romania. In 1984 she attended the Faculty of Medicine in Cluj, six years later obtaining her medical doctor degree, in 1990. Till 1993 she worked as physician in Cluj-Napoca at Medical Clinic III. During 1994-1996 she received from the Nuffic organization two scholarships in order to carry out research in the Department of Biochemistry at Maastricht University, under the supervision of Dr. Cees Vermeer.

In 1996 she was appointed as assistant at the Department of Biochemistry at the University of Medicine and Pharmacy Cluj-Napoca, Romania. In 1998 she finalized her residency in clinical laboratory, becoming a clinical chemist. In 1999 she was appointed part-time at the laboratory of Medical Clinic II in Cluj as well, while working at the university. During the last two years she continued her research in Cluj, receiving a grant from the Ministry of Education in Romania, allowing her to conduct several trials, the methodology of which she learned in Maastricht. From the collaboration with Dr. Vermeer several articles resulted, the abstract of one receiving in 1999 – "Reach The World Travel Award", at the XVIIth International Congress on Thrombosis and Haemostasis"- in Washington D.C. In order to continue the research of vitamin K in Romania she founded in 1996 a non-governmental organization, Scientific Society Provitamine, which aims to support the research of liposoluble vitamins.

LIST OF PUBLICATIONS

1. Vermeer, C., Knapen, M.H.J., Jie, K.-S.G., Crăciun, A.M. (1995) Circulating osteocalcin and urinary Gla as markers for vitamin K status. In „Vitamin K in infancy” (Ed. A.H. Sutor). Schattauer Verlag, Stuttgart, 111-122.
2. Vermeer, C., Gijbers, B.L.M.G., Crăciun, A.M., Groenen-van Dooren, M.M.C.L., Knapen, M.H.J. (1996) Effects of vitamin K on bone mass and bone metabolism. J. Nutr. 126, 1187S-1191S.
3. Crăciun, A.M., Groenen-van Dooren, M.M.C.L., Vermeer, C. (1997) Nutritional vitamin K intake and urinary γ -carboxyglutamate excretion in the rat. Biochim. Biophys. Acta 1334, 44-50.
4. Crăciun, A.M., Groenen-van Dooren, M.M.C.L., Thijsen, H.H.W., Vermeer, C. (1998) Induction of prothrombin synthesis by K-vitamins compared in vitamin K-deficient and in brodifacoum-treated rats. Biochim. Biophys. Acta 1380, 75-81.
5. Crăciun, A.M., Wolf, J., Knapen, M.H.J., Brouns, F., Vermeer, C. (1998) Improved bone metabolism in female elite athletes after vitamin K supplementation. Int. Sports Med. 19; 479-484.
6. Vermeer, C., Wolf, J., Crăciun, A.M., Knapen, M.H.J. (1998) Bone markers during 6 –month space flight: effects of vitamin K supplementation. Journal of Gravitational Physiology. 5(2): 65-69.
7. Crăciun, A.M., Vermeer, C., Eisenwiener, H.G., Drees, N., Knapen, M.H.J. (2000) Evaluation of a bead-based enzyme immunoassay for the rapid detection of osteocalcin in human serum. Clin. Chem. 46:2, 252-257.
8. Crăciun, A.M., Dican, L., Diaconescu, L., Jebeleanu, G., Miu, N. (2000) Biochemical bone markers variation after vitamin D3 treatment in rickets children. BMJ: submitted for publication.
9. Crăciun, A.M., Miu, N., Vermeer, C. (2000) Association of vitamin D and vitamin K deficiency in nonsupplemented children. J of Pediatrics: submitted for publication.

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