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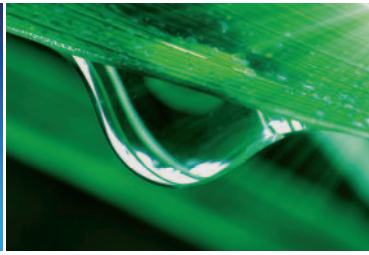
GIAN PAOLO LITTARRU

VITAMIN K2





FACTS AND PERSPECTIVES IN BIOLOGY AND MEDICINE

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Vitamin K2:
Facts and Perspectives in Biology and Medicine

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Introduction

Vitamin K is a fat soluble vitamin whose discovery, about 80 years ago, was related to a blood clotting disorder produced in the chicken while testing the effect of a diet impoverished in cholesterol.
The bleeding phenomenon was not due to lack of cholesterol, but to the deprivation of an antihemorrhagic factor contained in the lipid fraction of the diet, a “Koagulation Vitamin”, thereafter named vitamin K.
This factor was essential for the activity of prothrombin, a plasma protein necessary for blood clotting.
The mechanism by which vitamin K allows prothrombin to be functional was elucidated several decades later, and the fascinating story of this discovery is summarized in the first part of this book.
The role of vitamin K in coagulation is firmly established, and its biological function as a cofactor in the reaction of gamma-glutamyl carboxylation was found to extend to other proteins deeply involved in bone and vascular physiology.
The common denominator of these proteins acting in different fields is the capability of “properly” handling calcium in the phenomenon of bone calcification, which is essential for the skeleton health, and for arterial calcification, which is detrimental for vascular health.
Recent research focused on vitamin K leads us to hypothesize that the vitamin requirement sufficient for proper blood clotting may not be the optimal one for bone and vascular integrity; this is clearly indicated by epidemiological studies disclosing a significant correlation between vitamin K intake and bone density, and between vitamin K intake and decreased cardiovascular risk.
These facts might be of great concern for public health, as vitamin K intake could have a preventive role on diseases of huge social impact.
Meanwhile pharmacokinetics studies have shown that menaquinones (vitamin K2), a group of chemical subtypes of vitamin K which are found in fermented foods and are also synthesized by intestinal bacteria, are endowed

with superior bioavailability in comparison to phylloquinone (vitamin K1), which is mainly present in vegetables.
MK7 is the most studied among menaquinones and there are clear experimental data indicating that it is also more effective in the carboxylation of vitamin K-dependent proteins.
On the basis of most recent finding we can foresee an important contribution of MK7 to human health.

1. Vitamin K development and milestones

1.1 Vitamins and metabolism

Metabolism (from the Greek word *metabolè*, change) is the set of life-sustaining chemical transformations within a living organism. If we consider the human body, various organic substances must be supplied, through our food, in order to provide energy or to serve as building units. In the field of Nutrition dietary components such as sugars, lipids and proteins are called macronutrients, as they must be introduced in our bodies in considerable amounts.

For a proper functioning of our body we must also have a sufficient daily intake of water, minerals and vitamins. The term "Vitamins" includes a group of organic substances that are essential in minute quantities to the nutrition of most animals and some plants. Vitamins cannot, generally, be synthesized in amounts sufficient to meet metabolic needs and therefore must be obtained from the diet or from some synthetic source. For this reason, vitamins are called essential nutrients. If a vitamin is not present in an adequate amount in our diet, or is not properly absorbed by the body, a specific deficiency disease may develop.

Certain vitamins (e.g., B vitamins, vitamin K) can be synthesized by microorganisms normally present in the intestines of some animals. Both plants and animals are important natural vitamin sources for human beings. Vitamins are not distributed equally in foods, so that a diversified diet is needed in order to provide adequate amounts of different vitamins. Some vitamins can be assembled by our body, starting from vitamin precursors that must be introduced through our diet. For instance vitamin A, called also retinol, is synthetized in our gut and live cells starting from beta-carotene, the vitamin A precursor which is present in different foods.

Vitamins usually perform their essential activities once proper chemical reactions transform them into "coenzymes", the active forms of vitamins. Coenzymes are non-proteinaceous organic molecules which allow apoenzymes (made of proteins) to really act as enzymes (Fig. 1).

Deficiencies of vitamins can be either primary or secondary. A primary deficiency

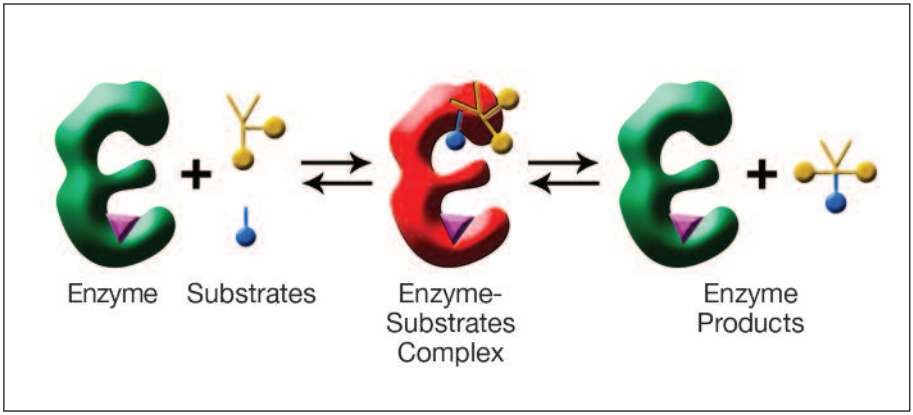


Figure 1. The apoenzyme, combined with the coenzyme, constitutes the holoenzyme, which catalyzes the enzymatic reaction transforming the substrates into products.

occurs when an organism does not get enough of the vitamin in its food. Secondary deficiencies are those due to a derangement that interferes with the absorption of the vitamin or determines greater requirement of a certain vitamin due to a "lifestyle factor", such as smoking, excessive alcohol consumption, or the use of medications that hamper the absorption of the vitamin.

In developing countries vitamin deficiencies are relatively common, because of the limited access to nutrients. In developed populations vitamin deficiencies can be the result of wrong, unbalanced eating behaviors, often due to poverty or food faddism. Elderly people, especially if institutionalized, are exposed to vitamin deficiency; alcoholism is another cause of deficiency. Patients undergoing parenteral nutrition can develop vitamin deficiencies if their supply is not adequate.

It is necessary to mention that vitamins are usually classified as either water soluble (like vitamins B and C) or fat soluble (vitamins A, D, E and K). Fat soluble vitamins are absorbed in the small intestine together with fat, therefore their absorption can be hampered if fat intake is too low, or in the presence of some malabsorption syndrome.

1.1.1 Vitamins: some history notes

Long before the identification of vitamins the essential role of certain foods in maintaining health was known. The ancient Egyptians knew the value of feeding

liver to cure a person affected by night blindness, a disease now recognized to be caused by vitamin A deficiency. The progress of ocean trips after the discovery of America implied long months with a diet devoid of fresh fruit and vegetables, making illnesses from vitamin deficiency common among ships' crews. Almost 300 years ago, after the intuition of the Scottish Surgeon Lind, the British Royal Navy adopted the practice of using lemons and limes to prevent scurvy, a deadly disease now recognized to be the effect of vitamin C deficiency.
During the last two centuries research focused on experimental deprivation models allowed scientists to isolate and identify a number of vitamins. These studies can be considered among the milestones of modern medicine and a number of high ranking researchers in the field were awarded the Nobel Prize.

1.2 The discovery of vitamin K and K2

The discovery of vitamins has proceeded through experiments focused on the "essentiality" of certain nutrients. In the case of vitamin K the Danish biochemist Henrik Dam, in the early 1930s, was exploring the effect of diets which had been extracted with non polar solvents, in order to remove cholesterol. Chicks fed with these diets underwent brain and muscular hemorrhages and their blood was shown to clot slowly (1). Similar observations were made by different American researchers and the initial interpretation of the phenomenon was that those diets were somehow deficient in vitamin C, or some toxic factors present in the protein component of the diet was causing the bleeding (2).
It was also discovered that, when fish meal was used as the protein source in the diet, the hemorrhagic disease did not develop if the fish meal had been dried slowly: this observation pointed out that a possible bacterial growth in the moist diet had allowed bacteria to synthetize a protective factor. At the same time it became clear that also some alfalfa extracts were capable of counteracting the bleeding effects of the deficient diets. In fact by 1939 Dam and coll. isolated the vitamin as a yellow oil from alfalfa and it was soon discovered that the active compound was a quinone. In the same years Edward Adelbert Doisy succeeded in synthetizing vitamin K1. In 1943 Dam and Doisy were awarded the Nobel Prize for the "discovery of vitamin K" and "discovery of the chemical nature of vitamin K" respectively. Dam was also the one who suggested the term vitamin K for the antihemorragic factor, mainly referring to the word "koag-

ulation" according to the Scandinavian and German spellings.
Discovery and characterization of the chemical structure of vitamin K proceeded together with the elucidation of the biochemical steps of blood clotting. In addition to prothrombin (factor II) three additional procoagulants were identified in plasma; all of them are known as "vitamin K-dependent clotting factors". Thereafter the essentiality of vitamin K for the synthesis of these proteins became clear. At one moment it was hypothesized that vitamin K was a component of the mitochondrial respiratory chain, and its functionality was indispensable in order to generate enough energy to sustain the synthesis of these proteins, but it was not a bioenergetic role. It still took a few decades to generate the data that established the precise role of vitamin K in the process of gamma-glutamyl carboxylation, the biochemical step necessary to activate prothrombin, to make this protein capable of starting the clotting process.
This is just the beginning: during these last years, research has discovered more about K vitamins and especially about the difference between vitamin K1 and vitamin K2 (Fig. 2).

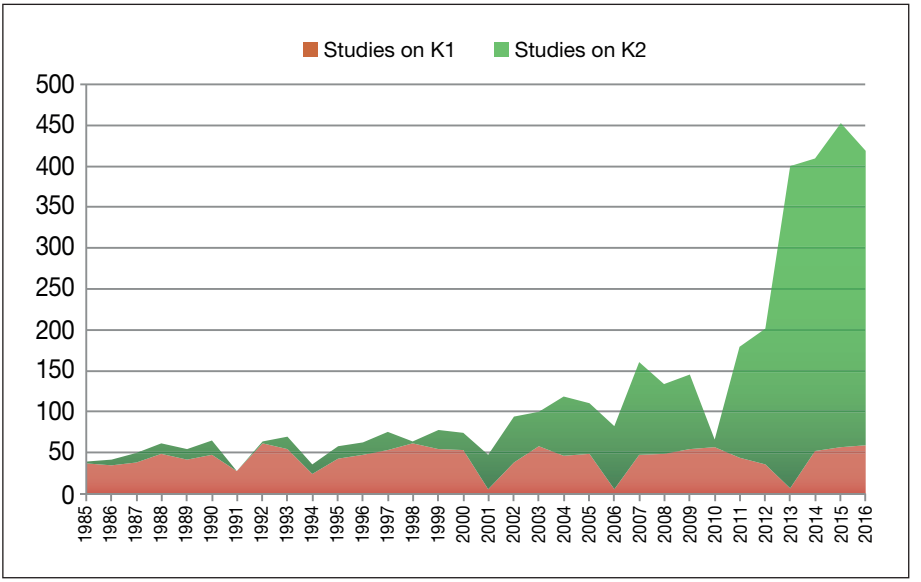


Figure 2. Annual trend of studies mentioning "vitamin K1" or "vitamin K2" (source: PubMed 21/12/2016 query with "vitamin K1" and "vitamin K2").

1.3 The chemical transformation that makes prothrombin work

At the time of vitamin K discovery the different steps of blood coagulation had not yet been fully elucidated, but it was well known that the presence, in sufficient amounts, and the integrity of prothrombin was essential. On the basis of the data gathered in the animal models of vitamin K deficiency it was postulated that the vitamin was necessary to make prothrombin, as a component of the mitochondrial respiratory chain that yielded the energy sustaining the biosynthetic process, or had some role in the regulation of the rate of synthesis. Therefore research efforts were also directed towards exploring the possibility that vitamin K works in a process that converts the inactive form of prothrombin to the biologically active protein. In particular, the abnormal protein was somewhat unable to bind calcium ions, a step necessary for the activation of prothrombin by factor X. Different research groups eventually succeeded in isolating and characterizing several modified glutamic acid (Glu) residues in active prothrombin: specifically, the 10 Glu residues in the first 33 residues of prothrombin are transformed, by the action of vitamin K, into gamma-carboxyglutamic acid (Gla) residues (Fig. 3).

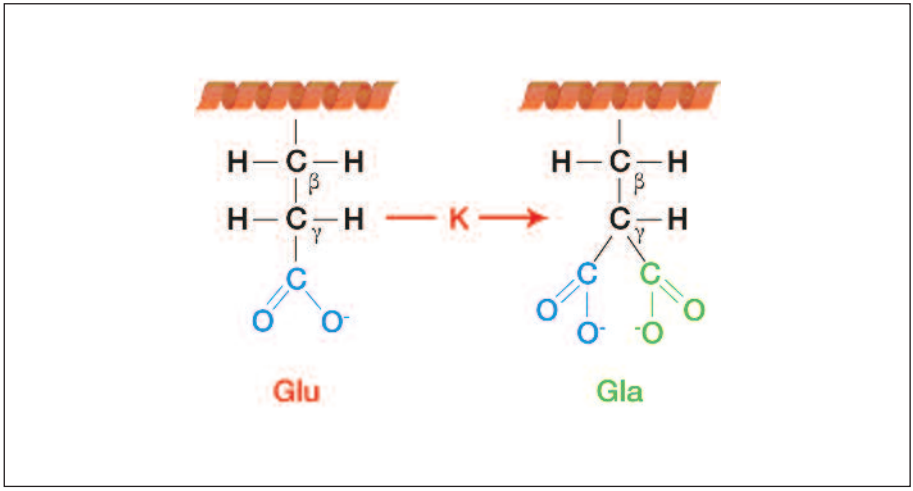


Figure 3. Vitamin K-dependent gamma-glutamyl carboxylase.

Gla residues can bind calcium ions, and Ca^{++} binding by the protein is essential in order to produce conformational changes that allow the interaction with phospholipids necessary to produce biological activity, in this case the transformation of prothrombin into thrombin.

Therefore vitamin K was recognized to have an essential role in a previously unknown carboxylase reaction: the existence of a vitamin K-dependent gamma-glutamyl carboxylase was postulated. The enzyme was eventually purified, in 1991, many years after the discovery of vitamin K, and the human carboxylase was cloned and expressed by Stafford and his group (3,4).The

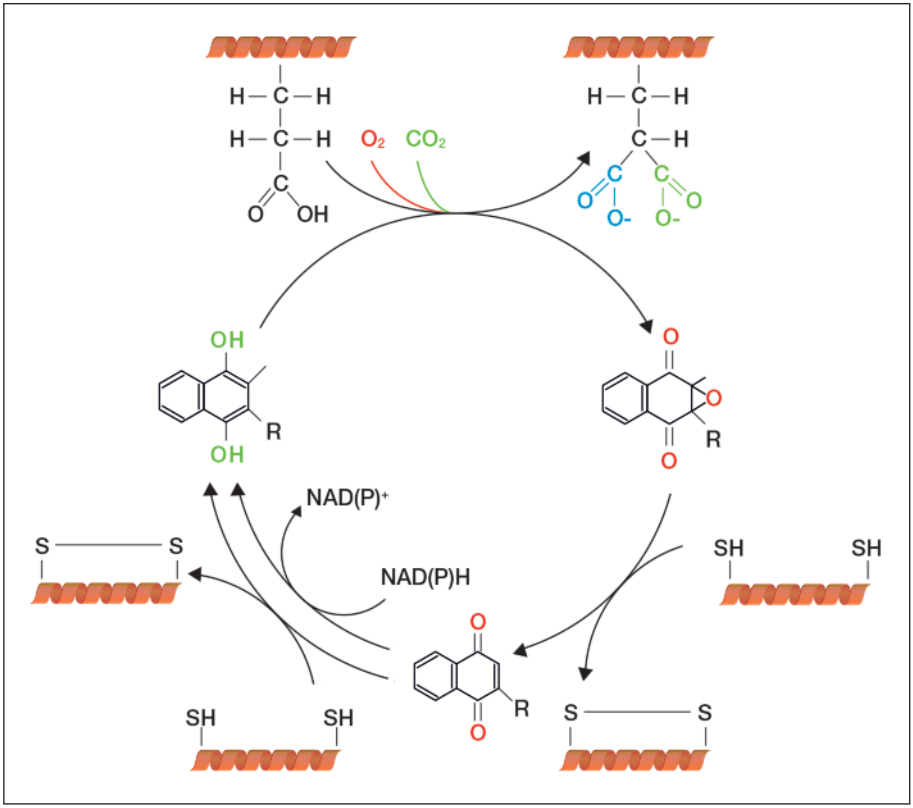


Figure 4. In the process of γ-glutamyl carboxylation vitamin K is oxidized to vitamin K 2,3-epoxide (KO), which is reduced back to active vitamin K by vitamin K-epoxide reductase (VKOR), through a two step reaction (see text).

carboxylation reaction does not require ATP: the energy that drives the reaction derives from an oxidation, namely the oxidation of reduced vitamin K (KH_2) to K-2,3 epoxide.

Vitamin K, according to the general mechanism of vitamin action, acts as a cofactor in the reaction, but it is truly a substrate of the enzymatic reaction. The products of the reaction are GLA and vitamin K 2,3-epoxide (KO). The reason why the vitamin is necessary in tiny amounts is that the product of the reaction, vitamin K epoxide, is regenerated to active vitamin K by another enzyme, vitamin K epoxide reductase (VKOR). As shown in figure 4 this is a two-step reaction, where the epoxide is first converted to naphtoquinone, which is further reduced to hydronaphtoquinone, the fully regenerated, active form of vitamin K. The reducing equivalents of the first step are donated by a reduced dithiol; the second reduction, to hydronaphtoquinone, can also be driven by the same dithiol dependent reductase, or by one of several NADH or NADPH-linked quinone reductases (Fig. 4).

2. Vitamin K: structural aspects, diffusion and physiology

2.1 Structural aspects

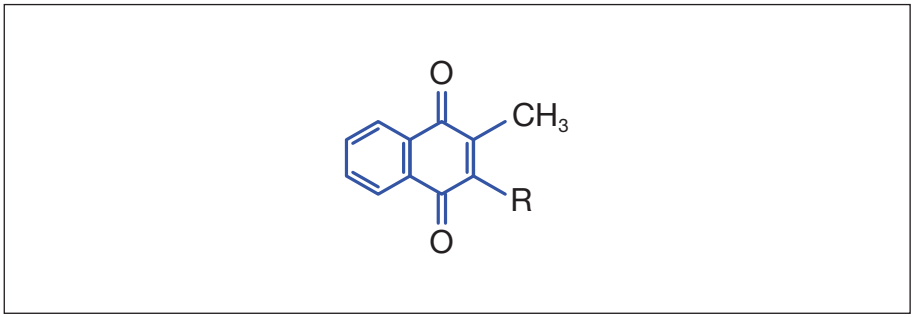


Figure 5. A generic form of vitamin K. Different side chains characterize the different vitamers.

The term vitamin K refers to a series of structurally related compounds having in common the methylated naphtoquinone ring system and an aliphatic side chain characterized by a varying number of isoprenoid units (Fig. 5). This side chain is also responsible for the lypophilic nature of these vitamins, which is what makes them fat soluble. Vitamin K1, also called Phylloquinone, has a side chain of 4 isoprenoid residues, only one of which is unsaturated. **Vitamin K2 is constituted by a group of compounds with a side chain of 4 to 13 isoprenoid units, most of them unsaturated** (Fig. 6). **They are also called menaquinones, and generally denoted as MKn, where n stands for the number of the isoprenoid in the side chain.** Vitamin K1 is abundant in green vegetables, especially spinach and broccoli. The role of vitamin K1 in plants is to act as an essential electron carrier in their photosynthetic systems, a function exerted by its naphtoquinone ring. Most bacteria are able to synthetize menaquinones, which share with vitamin K1 the 2-methyl-1,4 naphtoquinone ring: menaquinones (MK) fulfill a key role in microbial respiratory electron transport chain. Phylloquinone in plants and MK in bacteria are therefore essential for their bioenergetic role. Moreover, MK in their

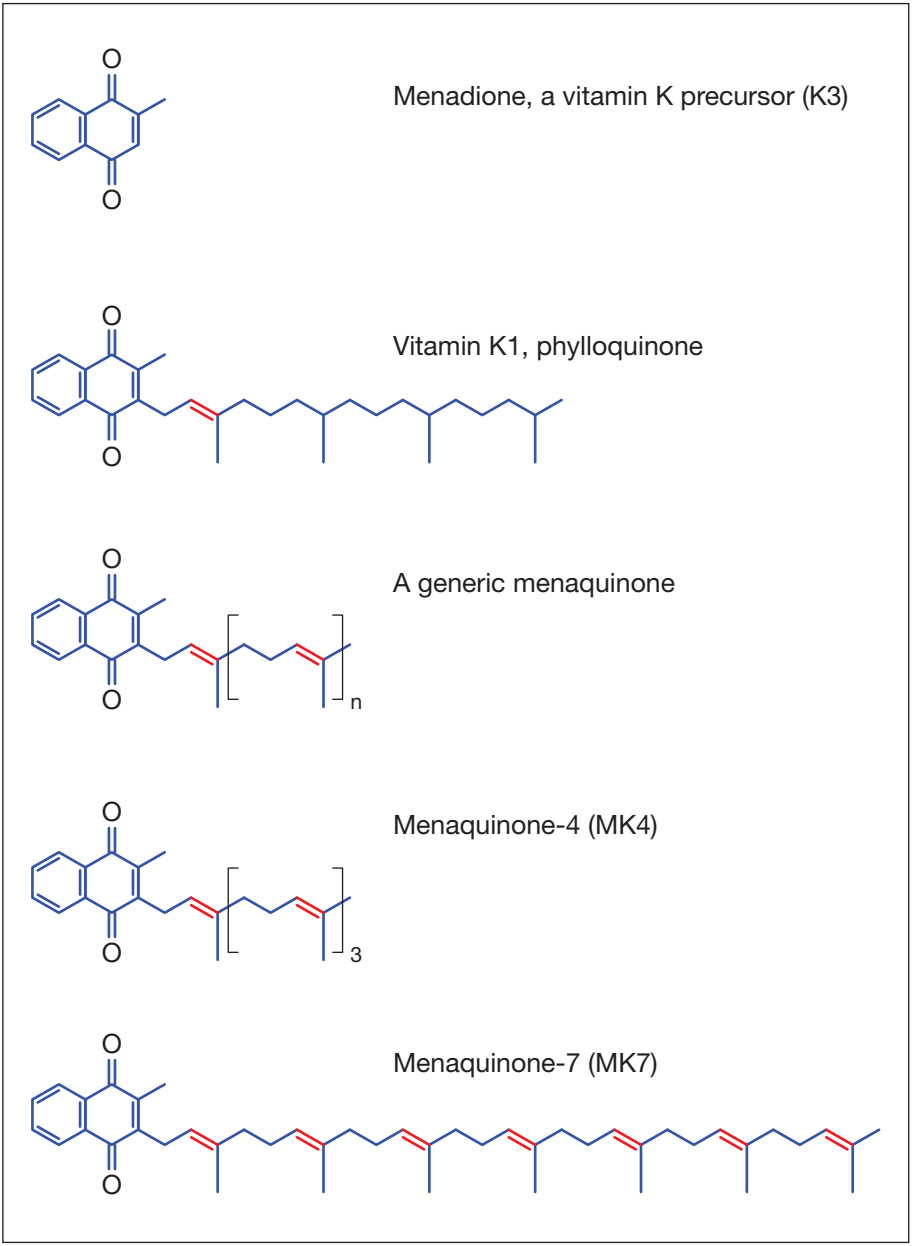


Figure 6. Menadione, a vitamin K precursor and different forms of vitamin K.

reduced form are also endowed with antioxidant properties, like many other quinol structures. In mammals and other vertebrates these compounds act as a vitamin involved in blood coagulation (the first discovered role) but also in the gamma carboxylation of proteins essential for bone and vascular health.
Menaquinones or K2 are of microbial origin; they are present in fermented cheese, curd and, in particularly high concentrations, in natto (a traditional Japanese breakfast food made of fermented soy beans). Therefore, vitamin K1 and all menaquinones share a common function: they act as a cofactor for the enzyme gamma-glutamate carboxylase, which transforms certain glutamate residues of a group of proteins into gamma-carboxy glutamate. These are generally known as Gla proteins and include seven proteins involved in the process of blood coagulation, plus other proteins not related to the clotting factors, the most studied of which are osteocalcin, MGP and Gla-rich protein. As we will explore in the following chapters they play a role in the field of bone formation and regulation of vascular calcification.

2.2 Content in foods and dietary intake

Vitamin K1 is particularly abundant in green vegetables, as shown in Table 1 (5). The authors point out that there are substantial differences in the reported Phylloquinone content of some foods; climate, soil or growing conditions might be at the basis of these differences. The first four items appearing in the table are the main contributors to vitamin K intake in the U.S.

Food item	µg/100 g
Collards	440
Spinach	380
Salad greens	315
Broccoli	180
Cabbage	145
Soybean oil	193
Olive oil	55

Table 1. Vitamin K1 content of some foods (Suttie, 2009; p. 41).

According to the "Framingham Offspring" study, the mean daily intake of vitamin K1 in 2900 U.S. adult subjects was 162±114 µg, data referring to European populations being not much different (6).

Plasma fasting levels of Phylloquinone are around 0.5 ng/ml (1.1 nmol/l).

Menaquinones are generally of microbial origin and they are present in substantial amounts in fermented cheese, yogurt, curd; remarkable concentrations are found in natto, a traditional Japanese breakfast meal derived from fermented soy beans, where it is produced by *Bacillus* natto. Natto contains about 1000 µg/100 g of MK7. According to a study by Kamao et al. (7) the dietary intake of menaquinones in a group of young Japanese women was 57.4 (S.D.83.7) µg/day, almost 100% constituted by MK7, a data heavily influenced by the widespread consumption of natto: in fact almost 50% of the studied population regularly consumed natto. Data appearing in some Dutch studies indicate a daily intake of menaquinones between 20 and 30 µg/day, while the estimated intake in the UK was between 36 (female adults) and 54 (male teenagers) µg/day. Menaquinones present in the diet are mainly constituted by MK4 (animal products) and by bacterially produced long-chain menaquinones such as MK7, MK8 and MK9. As menadione, vitamin K3, can be prenilated to become MK4, this is present in remarkable amounts in chicken meat (30-60 µg/100 g) and in egg yolk (about 40 µg/100 g); in fact menadione is often used as a source of vitamin K in poultry feed but is banned in supplements because of its potential toxicity in human use. Regarding long chain

Food item	µg/100 g
Natto	998
Hard cheese	1.3
Soft cheese	1.0
Pork steak	0.5
Eel fish	0.4
Curd cheese	0.3
Sauerkraut	0.2
Buttermilk	0.1

Table 2. Vitamin K2 content of some foods (Kamao 2007, Schurgers 2000, Manoury 2013).

menaquinones they are present in considerable amounts in soft and in hard cheese (50-70 µg/100 g when considering the sum of MK5, MK6, MK8, MK9 and MK10). MK7 content of cheese is about 1 µg/100 g (Tab. 2) (7-9).
Plasma levels of MK4 and MK7 have been investigated in several studies. For MK4 levels ranging between 0.1 and 0.3 ng/ml have been reported. MK7 plasma concentrations are highly variable between individuals, being mainly dependent on the consumption of fermented cheese, and on natto in Japan. In some studies these levels ranged between 0.3 and 0.5 ng/ml (10).

2.3 Absorption and bioavailability

Vitamin K is a lipophilic compound, its intestinal absorption via the lymphatic system implies its incorporation into lipid micelles, which are formed through the intervention of the pancreatic lipolytic enzymes and of bile salts. Therefore, the absorption of vitamin K requires the presence of a certain amount of fat in the diet (or in nutraceutical formulations of vitamin K given as a food supplement), and it is hampered in case of biliary insufficiency or intestinal malabsorption. Normal human subjects, upon ingestion of 1 mg of phylloquinone, excrete less than 20% of the dose in the feces, but this portion increases to nearly 80% in the case of impaired fat absorption due to obstructive jaundice, pancreatic insufficiency or celiac disease (11).
It had previously been assumed that fat-soluble vitamins, such as vitamin K, as well as dietary cholesterol, were absorbed by a passive diffusion process through plasma membrane of enterocytes. More recent studies demonstrated the relevance of several membrane transporters in the absorption of cholesterol, vitamin E and vitamin K (12). A remarkable contribution to the knowledge of these mechanisms has been generated by the development of ezetimibe, an inhibitor of cholesterol absorption clinically used in hypercholesterolemia.
Niemann-Pick C1 Like 1 (NPC1L1) is a transporter playing a key role in intestinal cholesterol absorption. This protein is highly expressed in the brush border membrane of enterocytes in the proximal intestine and is recognized to be a molecular target of ezetimibe. NPC1L1 has also the ability to transport, and therefore to absorb, vitamin K. Several studies have shown that the uptake of vitamin K1 is inhibited by ezetimibe in a concentration-dependent manner, and administration of ezetimibe significantly potentiates the anticoagulant effect of warfarin. Therefore it was ascertained that ezetimibe determines a vitamin K1 malabsorption, which

leads to a depletion of hepatic vitamin K levels: in these condition the effect of warfarin is more pronounced, as resulting from a longer prothrombin time. The conclusions suggested by these animal experiments have been strengthened by a retrospective survey conducted at the University of Tokyo Hospital (13): in more than 85% of warfarin-treated patients the Prothrombin time expressed as International Normalized Ratio (INR) increased after starting the co-treatment with ezetimibe. Moreover, this effect was more frequent in patients who also took statins (14). In fact, inhibition of cholesterol synthesis by statins determines a compensation mechanism which leads to an increase of NPC1L1 transporters. In this conditions NPC1L1-dependent vitamin K1 absorption, and the inhibitory effect of ezetimibe might become more relevant. The same review by Yamanashi et al. (12) points out that other molecular transporters involved in cholesterol absorption are, at least in part, involved in the intestinal uptake of vitamins E and K. Regarding the subject of this book we one should reflect on the fact that molecules such Ezetimibe, aimed at curbing cholesterol absorption, could as well determine a subclinical vitamin K deficiency. This “not optimal” vitamin K status could be not sufficient to determine a coagulation defect, but potentially dangerous as capable of undermining the functions of osteocalcin and of MGP, with serious long term consequences on bone and vascular health.

Bioavailability of vitamin K, as for other lipophilic compounds, is rather limited. Speaking about absolute bioavailability, less than one fifth of a certain dose of vitamin K is absorbed. According to a study by Jones et al. (15) absorption of oral, deuterated vitamin K1 is 13 (±9)%.

As pointed out in different studies, for the absorption of all vitamin K forms, including menaquinones, a certain amount of fatty matrix is necessary. As I will discuss in the next pages all the bioavailability trials with the highly absorbable K2 were conducted using appropriate lipid carriers.

Following intestinal absorption vitamin K is transported in plasma predominantly by chylomicrons and very low density lipoproteins, which deliver it to various tissues. MK7 oral supplementation leads to plasma concentrations which are higher and longer lasting than the corresponding peaks obtained by administering phylloquinone.

The total human body pool of phylloquinone is very small and studies conducted by the infusion of a very small amount of tritiated vitamin K1 indicate that the

turnover time for phylloquinone in human subjects is about 1.5 days (16). Urinary excretion of tritiated metabolites was about 35% of the administered dose. The faecal excretion of phylloquinone and its metabolites was 32% of the administered dose when the volunteers were kept on a control diet and 13% when they had been treated with a low vitamin K diet.
Even though the above mentioned data suggest a small-sized pool of phylloquinone, it has been hypothesized that there may be pools of vitamin K in fat deposits that are subjected to a slower turnover (17).

2.4 Menaquinones in the human gut

Another possible source of vitamin K for our body is long-chain menaquinones produced by different species of bacteria present in the human gut. Data from different studies indicate that the total amount of menaquinones present in the intestinal tract is equivalent to several mg and provide direct evidence for the absorption of vitamin K2 from the distal small bowel, supporting a definite role for bacterially synthesized vitamin K2 in contributing to the human nutritional requirements of this vitamin (18). This view is supported by the numerous reports of antibiotic-induced hypoprothrombinemia, which could be corrected by vitamin K (19). In that review it was suggested that the potential of antiobiotics to kill intestinal bacteria, which are a source of vitamin K, may not be the only cause of hypoprothrombinaemia. Certain antibiotics, which contain thiol-leaving groups, may produce hypoprothrombinaemia because the thiol group inhibits the vitamin K-dependent step in clotting factor synthesis. The difficulty in producing a clinically significant hypoprothrominemia, in adults, by limiting the dietary intake of vitamin K, is also regarded as an indication that the contribution of intestinal menaquinones to the vitamin K status may be relevant.

3. Elucidating vitamin K role in metabolism

3.1 Vitamin K and coagulation

Blood coagulation is the protective mechanism by which bleeding is prevented, following vascular injury, through the formation of a blood clot. A balance exists between the pathways leading to clot formation and the mechanisms which make coagulation possible only at the injury site. The formation of a blood clot (thrombus) that sticks to the wall of the arteries or of the veins is called thrombosis. Arterial thrombosis can be the cause of myocardial infarction and stroke, and in clinical situations with increased proneness to thrombosis the prevention of blood coagulation is necessary.

Molecules endowed with anticoagulant capacity are widely used in clinical practice, both for long term prevention of thrombotic events and for the acute treatment of arterial and venous thrombosis. On the basis of their inhibitory action on the vitamin K epoxide reductase warfarin and other hydroxycoumarin derivatives are the most commonly used oral anticoagulants.

Warfarin also has an inhibitory, negative effect on other vitamin K-dependent proteins, such as osteocalcin and MGP. This fact has greatly stimulated the study of Direct Oral Anticoagulants (DOACs), which determine anticoagulation by other mechanisms, without perturbing vitamin K status.

3.2 Vitamin K – bone and vascular health

3.2.1 Vitamin K-dependent proteins not involved in coagulation

Gla residues are present also in proteins other than in the vitamin K-dependent clotting factors. In fact, besides seven proteins involved in blood coagulation ten more members of this Gla protein family are currently known. Osteocalcin and MGP (Matrix Gla Protein) are two of these proteins for which a clear physiological role has been elucidated. Both of them are involved in the process of calcification, osteocalcin for bone formation and MGP mainly in the cartilage and the vessel wall, where it acts as an inhibitor of soft tissue calcification.

GRP (Gla Rich Protein) is another vitamin K-dependent protein also involved in regulation of osteogenesis.
In the process of bone formation a cartilage template is replaced by mineralized bone. Osteoblasts, osteoclasts and osteocytes are the bone cells involved in the active process of bone formation and remodeling.
Type 1 collagen is the main component of the organic bone matrix, whereas hydroxyapatite, composed of calcium and phosphate, constitutes the main mineral component.

3.2.2 Osteocalcin

Osteocalcin is the most abundant noncollagenous protein in bone. Its concentration in serum reflects some aspects of bone metabolism and serves as a biological marker for the clinical assessment of bone disease.
Osteocalcin, when carboxylated, has a high affinity for calcium, a feature which is probably related to its capability of modulating hydroxyapatite morphology and growth.

In addition to binding to hydroxyapatite, osteocalcin functions in cell signalling and in the recruitment of osteoclasts and osteoblasts, which have active roles in bone resorption and deposition, respectively (20).
Today there are methods to quantify serum osteocalcin levels, which are believed to be a sensitive marker of osteoblast activity. Moreover, it is possible to evaluate the concentration of undercaboxylated osteocalcin (ucOC), which are commonly regarded as a measure of vitamin K status in bone.

3.2.3 Matrix GLA Protein (MGP)

MGP is abundant in cartilage and in calcified tissues. Physiological calcification occurs in bone and allows the conversion of the extracellular matrix into a rigid material which is essential to exert mechanical force. This is a dynamic process, tightly controlled by mechanisms which make it happen to the right extent for that anatomical district and MGP is a molecule necessary to avoid an excess of mineralization. In 1982 Price et al. (21) observed that rats, maintained for 8 months on a level of warfarin sufficient to decrease the vitamin K-dependent protein of bone to 2% of normal, have an excessive mineralization disorder characterized by complete fusion of the proximal tibial

growth plate and cessation of longitudinal growth. A similar abnormality was also seen in humans. In fact newborns from mothers treated with warfarin show excessive mineralization of the tibial growth plate. These excessive mineralization disorders were recognized to be caused by the inactivation of Matrix GLA Protein (MGP), a vitamin K-dependent inhibitor of mineralization whose presence in cartilage was demonstrated by Hale et al. in 1988 (22). Calcification of the extracellular matrix can be physiological or pathological. A brilliant demonstration of the essentiality of MGP for a proper calcification was given by Luo et al. in 1997 (23). The authors demonstrated that in a genetic strain of mice lacking MGP inappropriate calcification of various cartilages was present, leading to short stature. More relevant, mice lacking MGP died within two months as they underwent massive arterial calcification resulting in blood vessel rupture. Besides controlling calcification at bone level MGP is necessary to prevent calcification in soft tissues. In order to exert its inhibitory effect on calcification MGP must have its Gla residues. In fact these are necessary to directly bind hydroxyapatite and to allow interaction of MGP with bone morphogenetic protein-2 (BMP-2). BMP-2 is a potent inducer of bone formation and MGP modulates its activity.

3.2.4 Pathological calcification

Calcification in anatomical districts, except for bone and teeth, can lead to devastating clinical consequences. In particular, calcification of blood vessels, mainly present in the arterial walls, determines impaired arterial elasticity which interferes with circulation and blood supply. Heart attack and stroke are the main consequences of this altered hemodynamics, along with vascular disease of the aorta and peripheral vessels. Among the key aspects of this critical issue, formation of the atherosclerotic plaque and its calcification constitute two fundamental steps.

3.2.5 The atherosclerotic plaque

Atherosclerosis is a pathological modification of the arterial walls characterized by plaques which progressively narrow the lumen of the arteries. The intrusion of the plaque into the lumen leads to eventual alterations in blood flow; moreover several facts can trigger plaque rupture and thrombosis, with acute frightening consequences when this happens at the level of coronary arteries (myocardial infarction) or of brain arteries (stroke). Development of atheroscle-

rosis starts early in childhood, even though most individuals become symptomatic many decades later. The rate of this progression depends on many biochemical mechanisms which can accelerate or slow down the formation of the plaque, its calcification and its propensity to rupture (vulnerability). Following rupture of a vulnerable plaque blood clotting occurs on top of the site, resulting in arrest of blood flow.

The atherosclerosis issue is of huge importance for cardiovascular disease and has tremendous impact on public health. Many factors are responsible for development of the atherosclerotic plaques and myriads of research groups are involved in elucidating the related biochemical and clinical aspects. Great emphasis is put on dyslipidemia, oxidative stress and inflammation. It is firmly established that elevated blood levels of Low Density Lipoproteins (LDL), a class of lipoproteins which

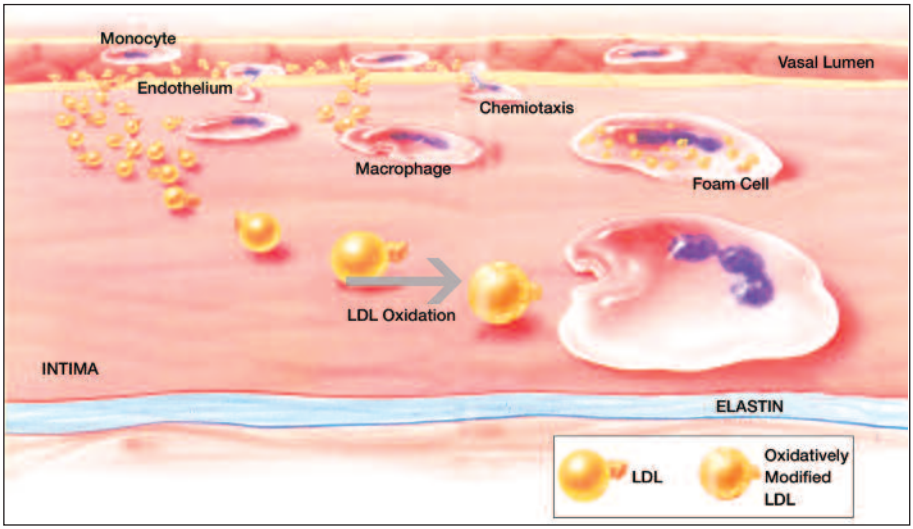


Figure 7. Schematic representation of the vascular scenario in which foam cells and the atheromatous plaque are formed. LDL migrate from blood to the subendothelial space, where they are probably oxidized by endothelial cells and other unknown factors. Oxidatively modified LDL are capable of activating platelets and chemotactically attract circulating monocytes, which also migrate to the subendothelial space, where they become macrophages. Oxidation of LDL involves modifications of the lipidic as well of the proteic moiety of lipoproteins: the modified apoprotein becomes recognizable by a special receptor of the macrophages, the "scavenger receptor". By this mechanism macrophages assume large quantities of oxidatively modified LDL, to eventually become foam cells, responsible for the formation of the atherosclerotic plaque.

are the main transporters of plasma cholesterol, play a key role in the formation of foam cells, which constitute the basis of the atheroma itself. As illustrated in figure 7 oxidized LDL are the ones which are recognized by the scavenger receptors of the macrophages; following phagocytosis of oxidized LDL these macrophages become lipid laden foam cells. This process depends on a subtle balance between prooxidant stimuli, oxidizability of the LDL particles and antioxidants present in plasma and within LDL. From a pathomorphological point of view these lesions appear as intimal or subintimal lipid deposits forming fatty streaks; these plaques determine a progressive lumen obliteration and are more or less vulnerable. In fact thrombosis can occur over plaques, because of erosion of the endothelial surface or a true disruption or tear in the cap of the plaque: in this case blood from the lumen enters the lipid core of the plaque, where thrombus is formed.

3.2.6 Vascular calcifications

Another factor is represented by arterial calcification and this is the aspect which we will explore a little more deeply, as some vitamin K-dependent proteins are involved in calcification, also in calcification outside the bone district, the so called pathological or ectopic calcification.

In particular, vascular calcification is highly prevalent, as shown by the Multi-Ethnic Study of Atherosclerosis (MESA study), reported and discussed in ref 24. In that study, conducted by computed tomography (CT) scanning on almost 7000 participants, prevalence of coronary calcification was near to 70% in Caucasian men and to 45% in women, with lower prevalence in people of different ethnicity. The risk for a major coronary event (myocardial infarction or death from coronary disease) was 6.84 for individuals with a calcium score >300, significantly higher than that found in people with a calcium score up to 100. Coronary Artery Calcium score (CAC score) is an established method for quantifying the extent of coronary artery calcification.

Vascular calcification can be present as intimal calcification, in the inner lining of the artery, or medial calcification, at the level of the tunica media (Fig. 8).

At intimal level calcification can have the aspect of spotty microcalcification or more diffused calcium deposit (macrocalcification). Intimal calcification is at level of the atherosclerotic plaque. Microcalcification are thought to originate from apoptotic smooth muscle cells of the nearby medial layer of the artery or from

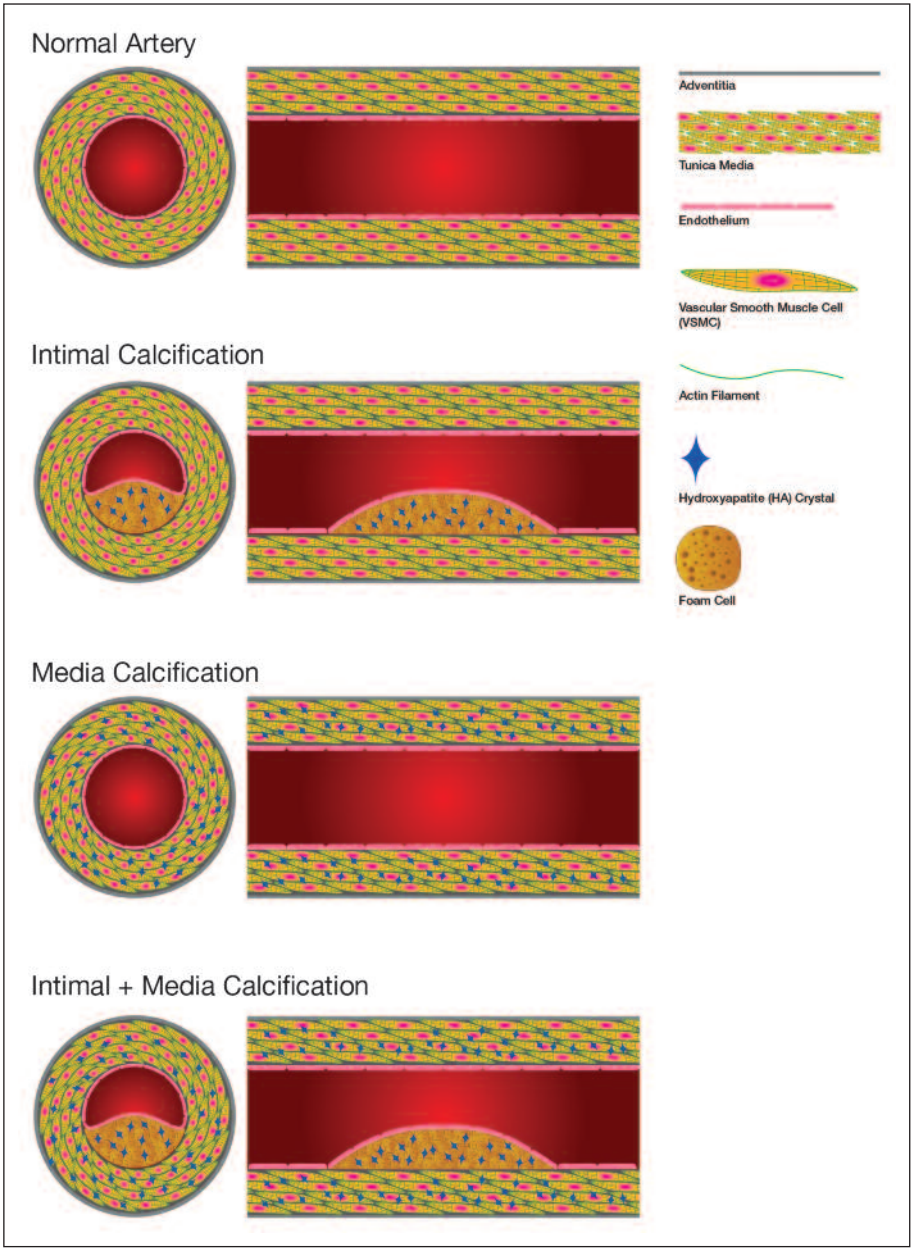


Figure 8. Schematic features of arterial calcification.

matrix vesicles released by these cells. Microcalcifications in the fibrous cap of coronary arteries contribute to plaque instability.

Medial calcification is characterized by amorphous calcium deposits along the elastin fibers of the tunica media. Medial calcification can be widely diffused in all arterial vessels and becomes more common with aging and the presence of diabetes or chronic kidney disease. Mineral deposits become evident along the elastin fibers of the tunica media, the medial layer of blood vessels.

Calcification along the elastic elements of the medial layer leads to loss of elasticity and consequent arterial stiffness; this results in impaired hemodynamic regulation and increased cardiac post load, the pressure against which the heart must work to eject blood.

Vascular Smooth Muscle Cells (VSMC) are highly specialized cells which constitute a good part of this medial layer in the arterial wall. Being able to contract they regulate the tone and the caliber of the arteries, and thus blood pressure and blood flow distribution.

VSMC can undergo osteoblastic differentiation becoming osteoblast-like cells (Fig. 9). Influx of VSMC would therefore be a requirement also for intimal calcification.

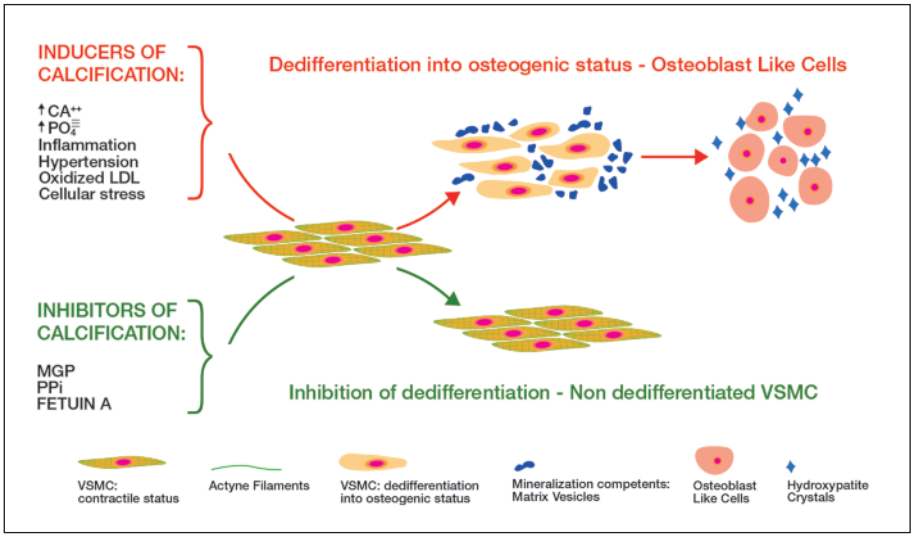


Figure 9. Dedifferentiation of Vascular Smooth Muscle Cells (VSMC) towards osteoblasts. In this process VSMC become mineralization-competent.

VSMC release Matrix Vesicles (MV), extracellular vesicles which are mineralization-competent but are normally protected from mineralization by the presence of calcification inhibitors. A series of biochemical mechanisms can induce differentiation of VSMC towards osteoblast-like cells and create an environment permissive for the nucleation of basic Ca and P minerals. Some of the major risk factors for cardiovascular disease also heavily influence osteogenic differentiation of VSMC and arterial calcification (25). Inflammation, for instance, is a potent inducer of calcification, and dyslipidemia also influences osteogenic differentiation of VSMC. In this respect High Density Lipoproteins (HDL) were shown to inhibit differentiation.

At molecular level some biochemical events are a prerequisite for arterial calcification. In VSMC some smooth muscle-specific genes are down regulated, whereas genes associated with osteogenesis are upregulated. An increased calcium phosphorus product is an inducer of mineralization of the arterial wall.

MGP plays a fundamental role in preventing or mitigating differentiation and subsequent vascular calcification. As mentioned above, MGP deficient mice and warfarin treated rats undergo massive vascular calcification. Carboxylation is necessary for proper MGP functioning, therefore a good vitamin K status is also responsible for optimal MGP control of vascular calcification, mainly through inhibition of BMP-2 activity; as mentioned above BMP-2 promotes osteoblast differentiation and mineralization.

3.2.7 GLA-Rich Protein (GRP)

GLA-Rich Protein (GRP) is another vitamin K-dependent protein which has been suggested to act as a negative regulator of osteogenic differentiation (26). A clear relationship, at gene and protein level, was established between GRP and vascular calcification. A relevant GRP accumulation was found in the ateromatous regions of calcified aortic valve samples. Notably, undercarboxylated GRP was the predominant protein form in foam cells. GRP was clearly upregulated, in the calcified aortic samples, concomitantly with MGP and osteocalcin.

4. Vitamin K2 clinical aspects

4.1 Possible causes of vitamin K deficiency

The most well known vitamin K deficiency status, resulting in serious bleeding problems, is the Hemorrhagic Disease of the Newborn, whose causes and clinical aspects are discussed in the next chapter. Vitamin K consumption of adult human population is generally sufficient to maintain normal hemostasis, so that vitamin K deficiency in the adult population is considered as rare (27). Impairment of vitamin-dependent coagulation, revealed by hypoprothrombinemia, can be found in malabsorption syndromes, also depending from liver disease. As pointed out earlier in this book, liver and biliary tract function are essential for vitamin K absorption from the gut. Moreover, a high percentage of people with advanced cancer showed some degree of vitamin K deficiency. Vitamin K-responsive hemorrhagic events have also been reported in patients receiving antibiotics, and this is generally ascribed to decreased menaquinone availability from the gut (49). Particular attention is needed with patients under VKA (Vitamin K Antagonists) therapy who are treated with antibiotics. In fact an excessive anticoagulant effect can be seen in these conditions.

It is generally recognized that very small intakes of vitamin K are necessary in order to avoid bleeding, but the impact of suboptimal vitamin K intake on bone and vascular health has not yet been quantified.

4.2 Hemorrhagic disease of the newborn

Newborn infants are prone to develop vitamin K deficiency, with consequent coagulation defect and serious bleeding. During the uterine life transplacental transfer of vitamin K from maternal circulation to fetus is very limited, and the fetal liver is unable to build up appropriated stores. Moreover, breast milk has a low content of vitamin K and a sterile gut is also among the causes of the disease, as the production of menaquinones by the intestinal microbial flora also contributes to the vitamin K status, although at a limited extent.

Classic vitamin K deficiency bleeding will develop in up to one out of 59 newborns

if the vitamin is not immediately administered at birth. When intramuscular vitamin K1 is administered, the risk of vitamin K deficiency bleeding is reduced to 1/100000.

Since the time of its discovery vitamin K was used to prevent the hemorrhagic disease of the newborn. At the beginning 1 mg of vitamin K3 (menadione) was given at delivery. Natural, fat soluble vitamin K1 (phylloquinone) was soon adopted, and the intra muscular (IM) administration of 1 mg of K1 at birth became an established routine prophylaxis. Vitamin K oral formulations constitute an onus for the parents as they imply the responsibility of having to administer the medication to the infant for 3 months daily or weekly, so the IM way of administration is preferred (28).

Even though neonatal vitamin K prophylaxis is an effective well-established intervention, there is still a certain, albeit low percentage of parental refusal. A recent, population-based study highlighted that vitamin K refusal was associated with a 14.6 higher relative risk of having no recommended childhood vaccines at 15 months.

Poor diffusion of the prophylaxis is still a problem in developing countries. The possibility of analyzing the blood content of undercarboxylated prothrombin (PIVKA-II) a sensitive marker of vitamin K insufficiency, allowed to explore and quantify the risk of vitamin K deficiency bleeding in these countries (29). Results strongly support the need for universal vitamin K prophylaxis strategies, capable of counteracting this serious but preventable cause of mortality or permanent disability.

4.3 Vitamin K2 and bone health

4.3.1 Vitamin K status and bone mineral density

Like many other tissues, bone is subjected to rather fast remodeling, i.e., it is continuously destroyed and rebuilt. Osteoporosis occurs when the bone construction is not fast enough to compensate for the loss of old bone. In this condition bones become weak and brittle and the risk of breaking a bone from a simple fall is higher. In serious cases fractures can happen with minor stresses or even spontaneously. Bone mineral density (BMD) is the amount of mineral mass per volume of bone, and is usually evaluated by a radiology procedure called densitometry. Therefore densitometry is an indirect indicator of bone den-

sity and of fracture risk; measurements are usually performed over the wrist, the lumbar spine or the upper hip. Osteoporosis becomes more common with increasing age, its prevalence is higher in women, especially in postmenopause. There are many diseases that increase the probability of osteoporosis and also numerous medicines may cause bone loss. Diet and physical exercise play a fundamental role in osteoporosis prevention. Calcium and vitamin D are two dietary constituents heavily involved in bone health, and so is vitamin K.
As pointed out in the former pages, several vitamin K-dependent proteins, such as osteocalcin and MGP, are fundamental in bone metabolism.
Osteocalcin is produced by mature osteoblasts and the small amounts of the protein circulating in plasma are considered to reflect bone turnover; in fact its concentration is almost five times higher in children compared to adults. Also circulating osteocalcin is not fully carboxylated and the percentage of undercarboxylated osteocalcin has been linked to decreased mineralization.
It has been more than 20 years since carboxylation of osteocalcin is related to bone mineral density and to bone quality. A series of papers reports the association between a deteriorated vitamin K status, represented by higher ucOC (under carboxylated osteocalcin) concentrations, and the presence of lower bone mineral density and higher hip fracture risk (30).

4.3.2 Vitamin K2 and bone fractures

In a paper by Kaneki et al. published in 2001 (31) serum concentrations of vitamin K2 (MK7) were analyzed in Japanese postmenopausal women from different geographical areas. MK7 levels were remarkably higher in Tokyo women compared to Hiroshima women. Natto contains a large amount of MK7 and is eaten frequently in eastern Japan (Tokyo) but seldom in the western part (Hiroshima). Moreover, a statistically significant inverse correlation was found between incidence of hip fractures in women and natto consumption in each prefecture throughout Japan. Although other nutritional regional differences may have contributed to this finding, high natto consumption is likely responsible for the improvement in bone health. Of course, other molecules present in natto might also contribute to this finding, but its high content in MK7 is probably one of the causes of the observed effect.
Therefore in that study a clear correlation was found between natto consumption

and lower incidence of hip fractures. On the basis of the known effects of vitamin K on bone metabolism one might reasonably suppose that this natto effect is linked to an improvement in bone mineral density (BMD). This issue was addressed in the Japanese Population-based Osteoporosis Study, JPOS study (32). The BMD was measured at the spine, hip, and forearm in 944 women (20-79 y old) at baseline and at a follow-up conducted 3 y later. In postmenopausal women there were highly significant positive associations between natto intake and the rates of changes in BMD at the femoral neck and at the distal third of the radius.

4.3.3 Menaquinones and osteoporosis

MK7 is the most active form of vitamin K2 due to the prolonged lateral chain that enhances its lipophilicity (fat solubility). Therefore, not surprisingly, not only natto but also supplementation with MK7 (90-360 µg) has been demonstrated to increase plasma MK7 concentrations and to dose-dependently reduce (from 31% to 74%) the levels of uncarboxylated proteins (ucOC and dephoshorylated ucMGP) (33,34).

A meta-analysis was recently conducted to verify the hypothesis that vitamin K2 plays a role in the prevention and treatment of osteoporosis in postmenopausal women (35). Nineteen randomized controlled trials were included in the analysis. Subgroup analysis of postmenopausal women with osteoporosis revealed a significant improvement of vertebral BMD for both medium-term and long-term results favoring vitamin K2 group. Undercarboxylated osteocalcin was significantly reduced and there was an osteocalcin increment.

Another study showed that vitamin K2 supplementation at high dose (15 mg MK4) is able to counteract the negative effects of glucocorticoids on bone density, mainly by increasing a cytokine, osteoprotegerin, an inhibitor of osteoclastogenesis which is down-regulated by steroids (36).

Recently, Italian researchers demonstrated in ex *in vivo* human osteoblasts that the osteopenic effects of Advanced Glycated Endproduct (AGE) accumulation in bone might be attenuated and/or reversed by the presence or supplementation of vitamins D3 and K2 (37). Therefore, these vitamins could be very useful tools in diabetic subjects in the prevention of bone loss.

4.4 Vitamin K2 and vascular protection

4.4.1 Menaquinones and vascular calcification

As pointed out in the chapter dealing with Matrix GLA Protein (MGP) vitamin K fulfills an essential function in carboxylating MGP. We also mentioned that in a genetic strain of mice lacking MGP death occurred just two months after birth because of massive arterial calcification resulting in blood vessel rupture. It was hypothesized that undercarboxylation of MGP, resulting from inadequate intake of the vitamin, could result in enhanced calcification of atherosclerotic lesions, leading to increased risk of coronary heart disease (CHD). This hypothesis was verified in the Rotterdam study, a prospective, population based study which was conducted, in the years between 1990 and 2000, on a population of about 8,000 people aged 55 y and over, living in a defined district of Rotterdam (38). Accurate dietary data were obtained in almost 5,000 people, for whom a baseline X-ray for assessing calcification of the abdominal aorta was also available. The mean time of the follow up was 7.2 y. Menaquinone intake was lower in subjects with severe aortic calcification than in subjects with moderate or mild calcification. Risk of CHD incident (fatal and non fatal events combined) was strongly and significantly reduced in the upper tertile of menaquinone intake, as were also risk of CHD mortality (RR=0.43) and all cause mortality (RR=0.74). Intake of vitamin K1 was not significantly associated with risk of CHD incident, CHD mortality and all cause mortality. The authors of the Rotterdam study highlight the fact that the intake of menaquinone, in contrast to phylloquinone, is not associated to a particularly healthy diet, making it unlikely that the observed results are related to a confounding effect of a favorable lifestyle.

Gast et al. reported similar results, obtained from data collected in the Prospect-EPIC cohort consisting of 16,057 women, enrolled between 1993 and 1997 and aged 49-70 years, who were free of cardiovascular diseases at baseline (39). After a mean follow up of 8 years an inverse association between vitamin K2 and risk of CHD with a Hazard Ratio (HR) of 0.91 [95% CI 0.85-1.00] per 10 µg/d vitamin K2 intake was observed. Practically vitamin K2 was found to decrease the risk of CHD by 9% for every 10 µg of its daily consumption. This association was mainly due to vitamin K2 subtypes MK7, MK8 and MK9. Similarly to the Rotterdam study vitamin K1 intake was not significantly related to CHD.

Also in a cross-sectional study among 564 post-menopausal women conducted

by Beulens et al. (40), menaquinone intake was associated with decreased coronary calcification with an RR of 0.80 (95%-CI: 0.65-0.98; p (trend)=0.03). Phylloquinone intake was not associated with coronary calcification.

Calcifications in the arteries of the breast have also been associated with cardiovascular risk, and a cross-sectional study among 1689 women aged 49-70 years was conducted (41) to assess a possible relationship between vitamin K status and breast arterial calcification (BAC). BAC was assessed on standard screening mammograms and dietary vitamins K1 and K2 intake was calculated from a validated food frequency questionnaire. Although BAC was less common in the highest (9%) quartile of vitamin K2 intake, compared to the lowest (13%) after adjustment for aging, smoking, diabetes and dietary factors the association of mean vitamin K2 intake with BAC was no longer significant.

A recent paper by Knapen et al. (42) reports the results of long term treatment with menaquinone-7 (180 µg/day for three years) on arterial stiffness in a group of healthy postmenopausal women. Arterial stiffness significantly decreased in the whole group of the MK7 treated women, compared to the placebo controls, an effect which was particularly evident in subjects with elevated arterial stiffness at baseline. Furthermore, MK7 decreased dp-ucMGP by 50% compared to placebo. The vascular progress was likely due to an improvement of the carboxylation and phosphorylation status of MGP, a typical biochemical effect of vitamin K.

Similarly, 180-360 µg MK7 reduced dephosphorylated ucMGP (dp-ucMGP) with no effect on phosphorylated (43).

The supplementation of MK7 (180 µg) was also able to attenuate the loss in lumbar bone mineral density in people undergoing cardiac surgery (which is associated with increased risk of osteoporosis (44).

The association of dp-ucMGP with aortic stiffness in a large group of a general population was also recently reported by Mayer et al. (43).

A recent paper published by Vissers et al. in Atherosclerosis (45) reports the results of a study which investigated the association between the intake of vitamin K1, as well as menaquinones, and the occurrence of peripheral arterial disease (PAD). In a cohort of almost 37,000 participants baseline intake of phylloquinone and menaquinones was estimated using a validated food-frequency questionnaire. During 12 years of follow up results showed a reduced risk of PAD associated with high menaquinone intake. The association was particularly strong in

participants with hypertension or diabetes. High intake of phylloquinone was not associated with a reduced risk of PAD.

4.4.2 Vitamin K2 and Chronic Kidney Disease (CKD)

Patients affected by Chronic Kidney Disease (CKD) very commonly undergo mineral and bone disorders. In these patients the risk of hip fracture is four times higher compared to the general population. Moreover, vascular calcification is also more common, and is associated with a higher prevalence of vertebral fractures. CKD patients have two to five times more coronary artery calcification than healthy age-matched subjects. High concentrations of inorganic phosphate are one of the most relevant inducing factors in the pathogenesis of vascular calcification in CKD patients. In fact, human smooth muscle cell cultures treated with elevated inorganic phosphate concentrations lose the smooth muscle lineage markers with simultaneous gain of osteogenic markers, such as osteocalcin (46). The extent of calcification is the result of a subtle balance of inducers, such as phosphate, and calcification inhibitors, among them MGP, one of the vitamin K-dependent GLA proteins.

The vitamin K Italian (VIKI) dialysis study (47) explored the prevalence of vitamin K deficiency and the relationship between vitamin K status, vertebral fractures, vascular calcification, and survival in 387 patients in hemodialysis for at least one year. A relevant percentage of patients showed deficiency of MK7 (35,4%), vitamin K1 (23,5%) and MK4 (14,5%). More than 50% of the patients had vertebral fractures, nearly 80% had abdominal aorta calcification and 56% iliac calcification. Vitamin K1 deficiency was particularly associated with vertebral fractures, MK4 deficiency was a predictor of aortic calcification, whereas MK7 deficiency was a predictor of iliac calcification. Dyalisis patients showed higher levels of total MGP, however total MGP was lower in the subgroup of nonsurvivors. This finding might be interpreted as an overexpression of MGP as a protective measure, which is clearly not sufficient in non survivors. As pointed out by the authors, this is the first study relating K1 and K2 deficiencies directly to vertebral fractures and to vascular calcification. On the basis of these data they also believe that “adequate intake of vitamin K together with calcium and vitamin D should be recommended”.

These results were also confirmed by other studies conducted in non dialyzed renal patients where supplementation with 90 µg MK7 improved the carboxylation status of MGP in subjects with grade 3-5 kidney failure (48).

4.4.3 Ongoing clinical studies

The above mentioned studies point out that patients on chronic hemodialysis are particularly prone to vascular calcification. Within this category, treatment with vitamin K antagonists, because of atrial fibrillation, may further worsen this vulnerable condition. A recent paper by Caluwé et al. (49) describes the protocol of a clinical study designed to evaluate the effects of high doses of MK7 supplementation on progression of vascular calcification, in patients on chronic hemodialysis treated with vitamin K antagonists or with rivaroxaban, an anticoagulant belonging to the DOACs category. The paper also summarizes series of ongoing randomized clinical trials, conducted in different nations, aimed at verifying the effect of vitamin K supplementation on vascular calcification. The primary endpoints of these studies are generally related to changes in coronary artery calcification, thoracic aorta calcification, arterial stiffness and to cardiovascular events.

The same author reported that pharmacological doses of MK7 (360-1080 µg/daily) dose-dependently reduced dp-uc-MGP in dialyzed subjects (50).

4.5 Future perspectives for non classical vitamin K2 indications

4.5.1 Neuroprotection

A number of experimental studies support the hypothesis that different vitamin K vitamers may exert potential neuroprotective role in the CNS. Menaquinone-4 (MK4) accumulates in human brain; different K vitamers facilitate the survival of CNS neurons during the later stages of embryogenesis *in vivo* (51) and potently inhibit glutathione depletion-mediated oxidative cell death in neuronal cultures independently of their carboxylase activity. At this regard, the side chain of vitamin K appears to be of relevance despite the reaction being independent of the vitamin K cycle. Lifelong low phylloquinone intake is associated with cognitive impairment in elderly age both in rats and in humans. Gas6, a vitamin K-dependent protein, is decreased in aged rats and significant positive correlations between

vitamin K status and cognitive function have also been found in older persons with and without Alzheimer's disease (52).

Thus, vitamin K may be an important nutritional factor contributing to cognitive health during aging in humans (53,54). Further:

1. vitamin K may exert protective effects on **multiple sclerosis**. It appears to interact with myelin, supporting the production of one of its components (sulfatide), while the vitamin K-dependent protein Gas6 is involved in myelination and in amyloid generation in different experimental models. Noteworthy, CSF concentration of Gas6 is inversely correlated with the severity of relapse in relapsing/remitting multiple sclerosis (55);
2. vitamin K participates in the nervous system through its involvement in **sphingolipid metabolism**, a class of lipids widely present in brain cell membranes. Vitamin K preferentially accumulates in brain regions rich in white matter and positively correlates with certain sphingolipids. In the last two decades, studies have linked alterations in sphingolipid metabolism to age-related cognitive decline and neurodegenerative diseases such as Alzheimer's disease. The strong associations reported between brain MK4 and sphingomyelin, sulfatides and gangliosides suggest that this vitamer may play an important role in the brain (56).

4.5.2 Inflammation

While MK4 has been reported to decrease LPS-induced IL-6 secretion in experimental models (57,58), MK7 has been demonstrated to modulate immune and inflammatory reactions in the dose-response inhibition of TNF-α, IL-1α, and IL-1β gene expression (59).

This antinflammatory activity has also been studied in rheumatoid arthritis (RA), a common autoimmune disease leading to chronic joint inflammation, severe destruction of cartilage and disability. Menaquinones induce apoptosis in RA synovial cells and inhibit the proliferation of fibroblast-like synoviocytes and the development of collagen-induced arthritis in a dose-dependent manner in rats (60). MK4 (45 mg/day), administered in 70 patients with a serum undercarboxylated osteocalcin level of >4.5 ng/ml or with decreased bone mineral density in spite of the treatment with other anti-osteoporosis medications, regardless of RA disease activity, significantly decreased serum CRP and other

inflammatory markers (61). Similarly, supplementation with 100 µg/day of MK7 for three months improved disease activity in RA patients (62).
All these findings suggest that vitamin K2, especially MK7 vitamer even at low dosage, could be considered a potential anti-inflammatory therapeutic agent.

4.5.3 Joint health

Preliminary lines of evidence indicate that vitamin K may affect joint health. Subjects with low vitamin K status or low phylloquinone dietary intake display a greater risk for incident knee osteoarthritis (63); chondrocytes isolated from persons with osteoarthritis secrete less carboxylated MGP (64) which is involved in the regulation of mineralization; MGP accumulates not only in bone and dentin but also in cartilage where calcium depots parallel with arterial calcification in mice that are deficient in MGP (23); lastly, genetic defects in MGP in humans are also associated with cartilage calcification (65) and polymorphisms associated with osteoarthritis. Future studies will clarify if vitamin K2 supplementation could be an useful tool in preventing osteoarthritis in humans.

5. MK7: the innovation in vitamin K

5.1 Vitamin K2 MK7

5.1.1 Bioavailability

Whereas food supplements containing vitamin K1 have been commercially available since many years, MK7 diffusion is more recent, and it was necessary to obtain data on the pharmacokinetics, the absorption and the biochemical efficacy of MK7 compared to K1, not only for general purposes, but also to provide hematologists with the proper information regarding possible interference of MK7 with the management of patients treatment. The first important study was conducted in 2007 by Schurgers et al. (66). Maximal serum concentrations of both K1 and MK7 were seen at approximately 4 hours after intake but, whereas K1 declined to its baseline values within a few hours, MK7 persisted well above its basal level for at least 4 days. Practically, K1 declined with a half life of about two hours, while MK7 showed a half life near to 3 days. When the volunteers were administered with a daily dose of 143 µg/day, MK7 showed an accumulation pattern, and its serum level reached a plateau of

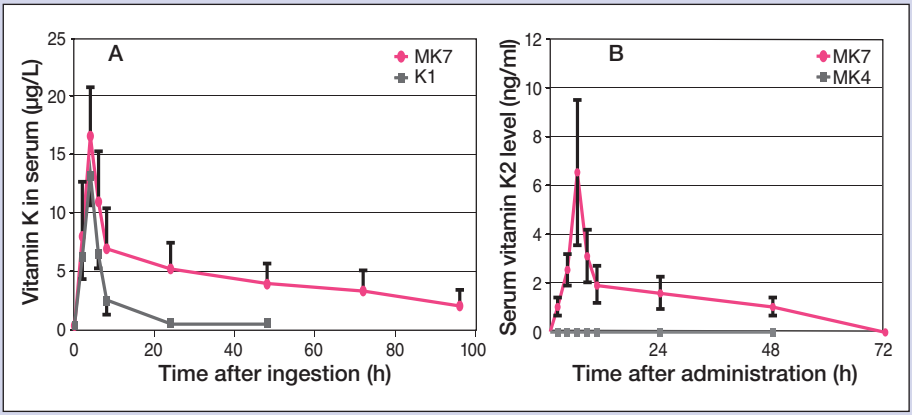


Figure 10. Comparison of bioavailability of vitamin K2 as MK7 versus vitamin K1(A) and MK4 (B) [(A) Schurgers, 2007; (B) Sato, 2012].

about 6 µg/l in 2 weeks. When K1 was administered at the same dose its serum level went up to 1 µg/l. The extent of osteocalcin (OC) carboxylation, a well-known marker of vitamin K status, is often expresses as the carboxylated OC/undercarboxylated OC ratio (cOC/ucOC ratio). At the end of six weeks of treatment the change in the cOC/ucOC ratio was 3 times higher for MK7 than for K1.

It was confirmed that the amount of MK7 in the blood increased when 420 µg of vitamin K2 was taken at once however MK4 was not detected in the blood. MK4 can get possibly broken before it gets absorbed by intestinal canal and reaches liver either because MK4 absorption is low or because it is very frail (Fig 10) (66,67).

MK7 is definitely more lipid soluble than vitamin K, and is more readily transported by plasma lipoproteins to other body districts, where it exerts its peculiar function in the carboxylation of osteocalcin and MGP (67). The bioavailability features of MK7, its capability of distributing to extrahepatic tissues, together with the epidemiological and clinical data, make this K vitamer a natural indication for prevention of bone vulnerability and of vascular calcification.

5.1.2 Natural-derived MK7

MK7 was isolated for the first time from natto, a traditional fermented soybean food in Japan, that has been found to have a stimulatory effect on calcification in the femoral tissues obtained from normal young rats *in vitro* (68). Currently used by Japanese people natto has a history of dietary consumption in Japan dating back at least 1,000 years (69) to the days of ancient samurais, whose daily diet included natto. They believed natto increased their strength and reaction time in battle. Ancient Japanese records point out that the leading medical professionals of the day insisted that pregnant women take a daily allowance of natto to ensure healthy offspring.

Natto is produced via the natural fermentation of boiled soybeans through the action of a specific bacteria, the *Bacillus subtilis* spp. natto (70,71).

Today in Japan *Bacillus subtilis* spp. natto is considered as the sole bacterium needed to produce good natto (72). One hundred grams of natto contains approximately 1,000 µg of MK7 (69).

Over the years, several epidemiological studies have reported evidences of the significant improvement in K2 vitamin status associated with the dietary intake of natto and have shed light on the safety of natto and MK7.
In 2000, changes in serum MK7 level with the frequency of dietary natto intake were examined in 134 healthy adults without and with occasional, and frequent dietary intake of regular natto including MK7 (73). In 2001, Kaneki et al. (29) evaluated natto consumption in postmenopausal women. The study analyzed the relation between the regional difference in natto intake and fracture incidence. A statistically significant inverse correlation was found between incidence of hip fractures in women and natto consumption in each prefecture throughout Japan. Then in 2004, Katsuyama et al. observed 73 healthy pre-menopausal women randomly allotted to four different fermented soybean (natto) consumption regimes finding that an increase in natto intake may have contributed to the promotion of bone formation in pre-menopausal women (74).
Although historical and clinical evidence reports major health benefits associated with a daily consumption of natto, western people find it very unappetizing, due to its strong texture, paving the way to nutritional use of natural-derived MK7.
In the last decades different industrialization methods have been developed. Since the strains of *Bacillus subtilis* natto used for manufacturing natto food is edible, the fermentation-derived production method has been recognized as the most advantageous. It provides a reproducible source of MK7, following the laws of nature, resembling the characteristics and chemical profile of MK7 as in the natural-enriched K2 foods.
Nowadays, the fermentation-derived method coexists with the chemical productions but - excluding guesswork and marketing speculation – the fermentative process from *Bacillus subtilis* natto remains the first choice and the golden standard.
Furthermore, all the clinical trial data and outcomes were performed with fermented vitamin K2 as MK7.

5.1.3 Fermentation-derived quality

The biological activity of K2 vitamins is strictly linked to their chemical structure, made up of naphthoquinone, the recognized functional group, and the different

aliphatic side chains, that influences absorption, transport, tissue distribution and bioavailability of menaquinones.

MK7 is the primary constituent of *Bacillus subtilis* natto, with Menaquinone-6 (MK6) present to a smaller but defined extent. The typical value 0.3 - 3.0% for MK6 is found in natto as described above and reported in literature. All the clinical trials performed with vitamin K2 did involve MK7 with a minor extent of MK6 as present in the natto food. The observation of the benefits of this relatively new vitamin came in fact from Japan where natto has been historically used together with other fermented foods.

Allowing higher values of MK6 can potentially lead to less purified products, other menaquinones or *cis* forms that are not preferred.

In natural environments, bacteria produce MK7 only in *trans* forms, where "*trans*" identifies the spatial configuration of the isoprenoid side chain double bonds. *All-trans* structure is peculiar to all menaquinones and it allows them to play a key role in prokaryotic respiratory electron transport chains by functioning as electron carriers in the cytoplasmic membrane (75,76). In addition to a role in microbial respiration, MK forms exhibit antioxidant properties and can play a role in protecting cellular membranes from lipid oxidation (77).

In humans as well, the *trans* isomer of MK7 is essentially responsible for the vitamin's biological activity and plays a crucial role in bone and cardiovascular health. *Cis*-analogs are considered biological inactive (78).

The industrial manufacturing of MK7 has historically been centered on the fermentation-derived process because it guarantees a final product as nature intended, with high reproducibility and good scalability. Very recently, synthetic methods have been proposed for a small scale production.

Nevertheless, the fermentation-derived process remains superior in terms of assay of final product, *all-trans* assay and purity profile. Bioprocesses take advantages from the same enzymatic pathways of bacteria to execute MK7 production. The process requires the optimization of production medium to maximize the metabolite yield by using a wide range of techniques respecting the natural biosynthetic route of *Bacillus subtilis* natto. Other key elements of fermentation-derived processes are the optimization of downstream processing for extracting, concentrating and purifying the product from a diluted fermentation broth, with gentle technologies. Finally, fermentation-derived process is

based on renewable resources and offers green, clean and sustainable products. It is considered as a reuse technology because compounds are easily degradable, the process requires less energy and creates less waste during production. Bioproduction processes respect the stereochemical structure of MK7, guaranteeing the highest *all-trans* isomeric purity, highest assay and lowest content of *cis*-isomers and natural impurities. Moreover, all the clinical trials available have been performed with fermentation-derived MK7 from *Bacillus subtilis* natto.
On the contrary, synthetic routes produce a mixture of both *trans* and *cis* isomers by default, with potential presence of different impurities.
In fact, the process counts the use of complex chemistry reactions and involves the use of organic solvents both in the synthetic and purification steps. The synthetic method generates a certain percentage of byproducts, in part constituted by the *cis* form, in part constituted of other regioisomers, whose activity and safety has not been proved. Moreover, high levels of side products (impurities), often not present in nature environment, are produced by synthetic procedures and require aggressive purification processes to drive away. The final synthetic-derived vitamin K2 as MK7 shows a complex not defined profile with mixed and not fully characterized impurities, often of unknown origin.

5.1.4 *All-trans* isomerism

The biological activity of MK7 is strictly linked to its natural, structural *all-trans* configuration. *Cis*-analogs of vitamin K2 are biological inactive. In natural environments, bacteria produce MK7 only in the *all-trans* form whereas the synthetic methods produce a mixture of both *trans* and *cis* isomers by default, with potential presence of different impurities. Only trans isomer of MK7 is responsible for its biological function. MK7 in fact is the primary constituent of *Bacillus subtilis* natto, with MK6 present in a smaller but defined extent. For this reason approximatively 1% of MK6 is the typical value found in natto menaquinones, as reported in literature. The historical use of the Japanese fermented food, natto, and the observation of the benefits of vitamin K2 as MK7 have driven the scientific world to clinically evaluate the fermentation-derived MK7 obtained from *Bacillus subtilis* natto, the only clinically tested, where MK6 is always present in minor extent (Fig. 11).
Analysis of MK7 finished products present in the market reveals that their isomeric

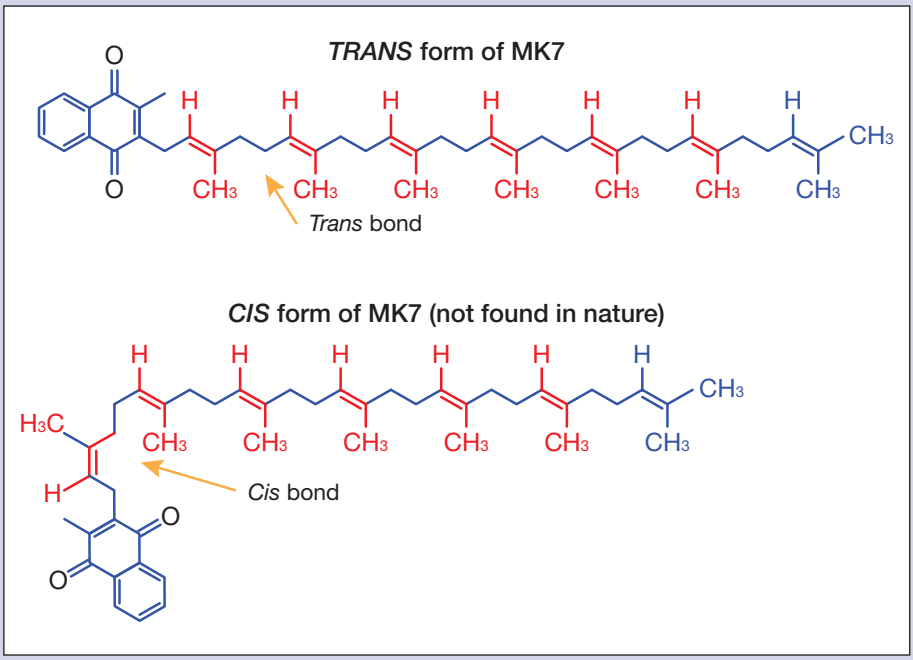


Figure 11. The biological activity of MK7 is strictly linked to its natural, structural all-trans configuration. Cis-analogs of vitamin K2 are biologically inactive.

purity is variable and can influences biological responses.

The analysis of isomeric purity of a marketed MK7 can tell us a lot about the origin of the products and can establish if they have been produced by natural (fermentative) or synthetic process. In fact, it is the same type of production process to leave an indelible mark on the final product.

The combination of different advanced chemical analyses can highlight the isomeric purity and the impurities profile of MK7: the presence of relevant percentage of *cis* analogs and unnatural impurities is characteristic of the synthetic ones while the fermentation-derived form generally has a cleaner profile with traces of MK6, with noticeable advantages. In fermentation-derived processes the side chain isomerization of *all-trans* structure may happen for poor and inefficient downstream process, where UV light has a role (Fig. 12).

The natural method based on *Bacillus subtilis* natto harvests only MK7 in the *all-trans* form.

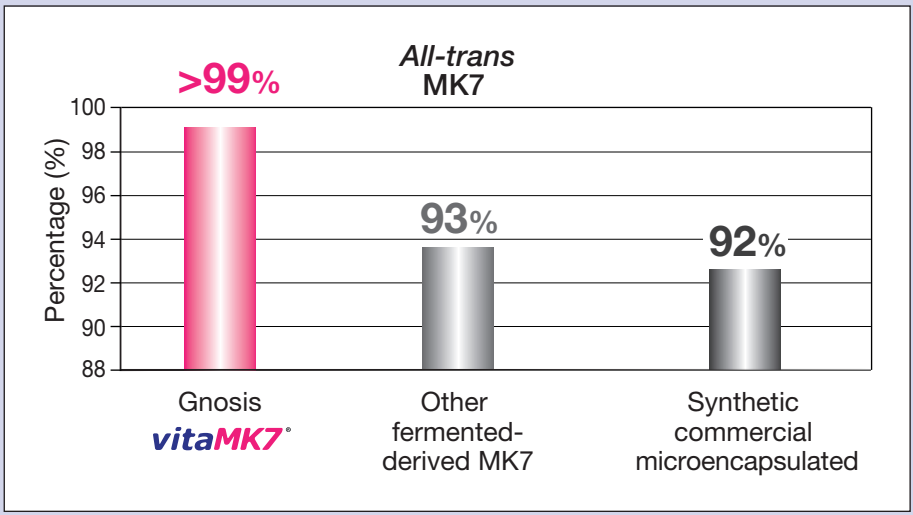


Figure 12. Declared all-trans MK7 contents in synthetic or fermented MK7.

Gentle, controlled and state-of-the-art purification processes can preserve *all-trans* isomer avoiding the possible (photo)-oxidative modification of the side chain of MK7. MK6 is a natural byproduct of the biofermentative process found in foods, also produced by intestinal bacteria and recovered in human tissues. MK6 is recognized as a marker of natural production and is regarded by chemical analysts as a tangible proof of fermentation-derived origin (Fig. 13).

However, the synthetic methods produce a mixture of both *trans* and *cis*-isomers by default and chemical solvents are used to extract the *trans* isomer and drive away the *cis*-isomers and the other impurities.

The analysis of isomeric purity evaluates the identity and the real content of the menaquinone-7 isomer discriminating and quantifying the other *cis*-isomers and regioisomers. It is commonly determined by High Performance Liquid Chromatography (HPLC), combining this technique with Mass Spectroscopy (MS) and Nuclear Magnetic Spectroscopy (NMR) methods, which can reveal not only the presence of *cis* analogs but also natural/unnatural impurities. HPLC is carried out using a chiral separation; however, chiral separations can be time consuming and typically involve the use of expensive chiral columns.

These chemical analyses are valuable tools for natural product identification, as

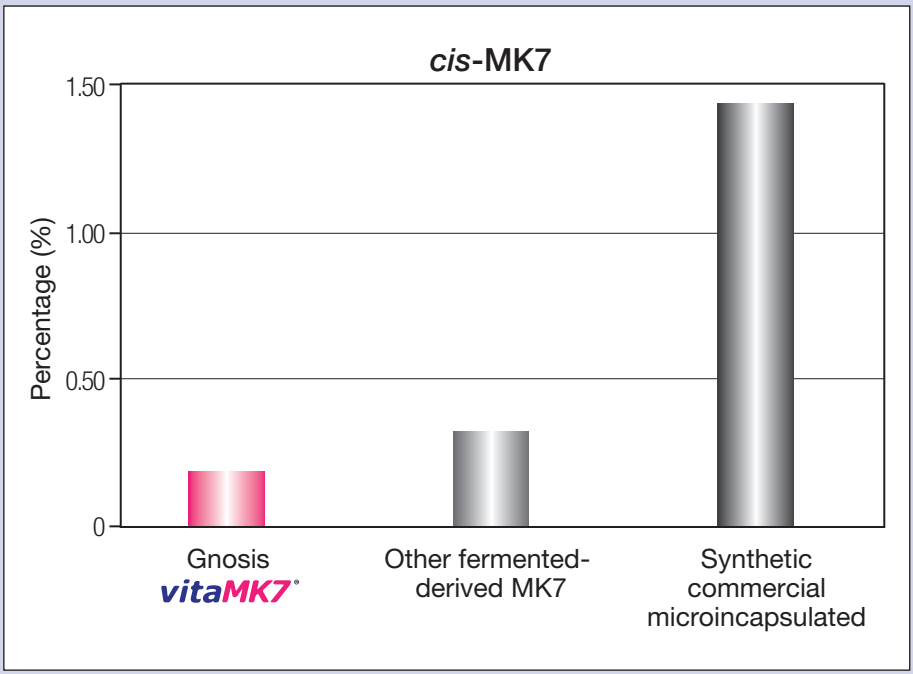


Figure 13. Fermented, high quality MK7 is characterized by negligible quantities of cis isomers and only MK6 marker of production.

well as the authentication, distribution, and quantification of constituents in biogenic raw materials, and are currently used to test natural medicines and biological materials obtained from model organisms, animals and humans.

5.1.5 Quality and impurity overview

The structural elucidation of known and unknown impurities plays an important role in the evaluation of an active ingredient, both in terms of quality of substances and safety of administration. MK7 is not an exception.

Generally, high levels of side products are characteristics of synthetic-derived MK7 and poor production method of fermentation-derived MK7. The chemical structure, function and biological activity are generally unknown as well as their potential side effects. The use of solvents can degrade the product and produce other impurities (Fig. 14, Tab. 3).

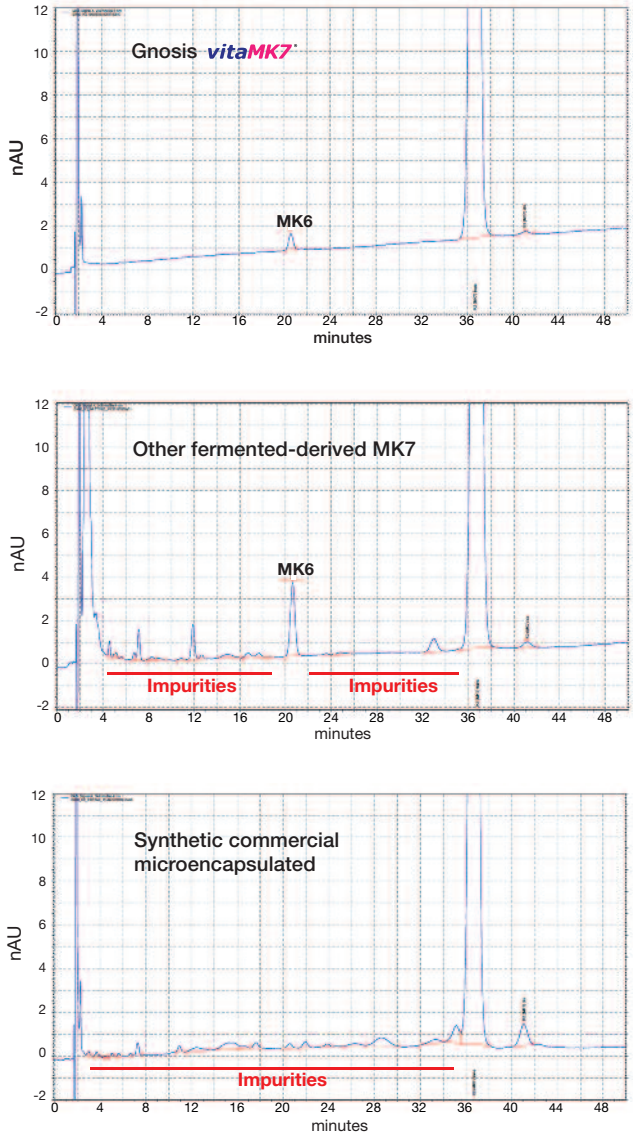


Figure 14. High quality fermented MK7 is only in the trans form whereas the synthetic methods produce a mixture of both trans and cis isomers by default, with the presence of several different impurities.

Manufacturer	Sample	Results			
	All-trans MK7 (area)	Known other compounds		Unknown impurities	
		Cis-MK7 (area %)	MK6 (area %)	Total unknown (area %)	n° of unknown impurities
Gnosis	99.27%	0.18%	0.56%	0.0%	0
Other fermented-derived MK7	93.67%	0.31%	2.50%	3.52%	19
Synthetic commercial microincapsulated	92.73%	1.40%	0.00%	5.67%	23

Table 3. Isomeric purity and impurities profile of different fermentation- and synthetic-derived MK7.

5.1.6 USP (United States Pharmacopeia) monograph

The recently market booming of MK7 in western countries and the increasing number of manufacturing players have highlighted the need to characterize and define its quality standards, in a global market where different sources and quality of vitamin K2 coexist.

In 2015, a first Official Compendia Monograph of Dietary Supplement for vitamin K2 as menaquinone-7 has been published in USP 38-NF33 that defines high quality standards for an optimal vitamin K2 ingredient. The Australian Health Authority TGA (Therapeutic Goods Administration) has also recognized the USP MK7 monograph for the Australian market shortly after.

The US Pharmacopeial Convention (USP) was established in 1820 for the purpose of setting standards for food and drug ingredients as part of its mission to improve food and drug quality and safety in international trading and provides specific standards for botanical and other dietary ingredients (including: tests, assays, and other specifications) for raw ingredient quality control in the manufacturing process of dietary supplements. USP monograph describes a MK7 of fermentative origin, derived from its *all-trans* form *Bacillus subtilis* natto, without the use of organic solvents.

The commercialization of synthetic-derived raw materials with the presence of mixtures of different compounds (byproducts) and high levels of side products (impurities), often not present in nature environment, has recently driven to the development of new methods of analysis to discriminate the impurity profile of MK7, also when it is obtained from synthetic process with high selectivity towards

such similar compounds. Up to 2015, the majority of scientific papers dealing with the analytics of menaquinones was concerned with their determination in biological fluids, food or plant material (79).
Determination of chemical purity and related substances in the MK7 active substances represents a closer pharmaceutical approach that can be applied both for the MK7 samples of high quality and the samples of lower quality, including crude samples before the purification step in raw materials, and can avoid adulteration and the related potential risks for health.

5.1.7 Fermented- and synthetic-derived MK7 (Tab. 4)

Fermentation key benefits	Synthesis considerations
• It delivers the biological MK7 through the natural *Bacillus subtilis* natto enzymatic production following the laws of nature.	• The production of structural mixtures of several isomers, with both trans and *cis* structure. The cis-form of MK7 is deemed to be biologically inactive.
• It releases pure vitamin K2 as MK7, with the desired *all-trans* stereochemical structure, the only active form of the vitamin.	• The use of chemical solvents - needed to purify the menaquinone-7 from *cis* analogs, byproducts and impurities - can partially remain in final products. Synthetic derived MK7 cannot reach *all-trans* purity and it stands around 90%.
• It does not produce *CIS* forms in natural environments.	• The production/presence of mixtures of different compounds (byproducts) and high levels of side products (impurities), which have different nature and structure, often not present in nature environment.
• The very low impurities are made up of the known MK6 (a marker of fermentation process) which is an homologous of MK7, also produced by intestinal bacteria and found in human tissues.	• The purification process cannot efficiently eliminate these impurities. They are often unknown and uncharacterized compounds, with unidentified biological effects;
• The more the product is pure, the more it is stable.	• Due to the quantity of *cis* isomer, the synthetic MK7 is NOT *ALL-TRANS* (only 92% of trans MK7).
• It is based on renewable resources and offers green, clean, and sustainable product. The process is considered as a reuse technology: compounds are easily degradable, the process requires less energy and creates less waste during production.	

Table 4. Main characteristics and differences of fermentation- and synthesis-derived MK7.

5.2 Gnosis vitaMK7®

A vitamin K2 as MK7 that stands out in the global market for its quality features and controlled origin is the branded vitaMK7®, obtained through a patent granted biofermentation process of *Bacillus subtilis* natto cultures by Gnosis Spa, an Italian-based biotechnology company which is leading in the manufacturing and sales

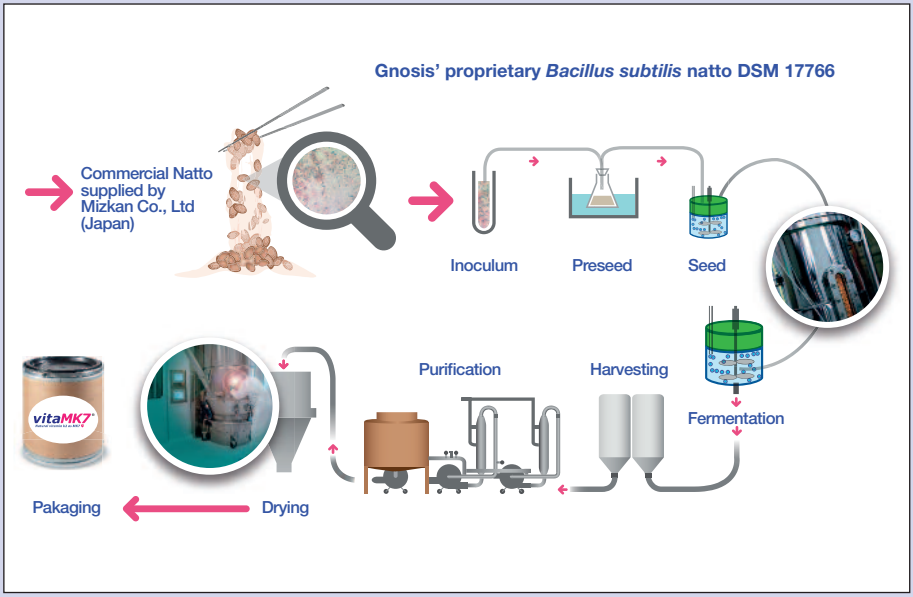


Figure 16. Production and purification of natural MK7 (vitaMK7®).

of fermentation-derived ingredients. *Bacillus subtilis* natto is the only bacterium used to produce good natto, Gnosis process does nor use chemicals or solvents. It is carried out in its own cGMP manufacturing site that works in compliance with cGMP (current Good Manufacturing Practise), ISO and HACCP rules and inspected by FDA, a warranty of origin, quality and safety of all vitaMK7® products. Gnosis was also able to patent a *Bacillus subtilis* proprietary strain (GN 13/72-DSM 17766) with high productivity, ranking from 1000 to 25000 ppm of dry matter. Extraction from the matrix is accomplished by Supercritical Carbon Dioxide (sCO_2) (Fig. 16).

This fluid state of CO_2, which is obtained at appropriate temperature and pressure, constitutes an extraction solvent with unique lack of toxicity and low environmental impact features. Separation from the starting material is simpler and more environmental friendly than with traditional organic solvents. In fact CO_2 can easily evaporate into the air or be recovered by condensation. Besides not showing residues of solvents, vitaMK7® contains neither additives

nor preservatives, is 99% pure by HPLC and allergen free (EP 1803820; US 7,718,407 and JP 5043425).

The complete analytical characterization was carried out according to USP Method for the evaluation of purity assay of MK7 and published in the current USP monograph of MK7.

In this method, instead of conventional liquid chromatographic analysis, involving the use of C18 reverse phase stationary phases, a C30 functionalized silica has been used to test the presence of conformational and positional isomers of MK7.

VitaMK7® contains the highest purity of MK7, more than 99% of *all-trans* MK7 assay, preserved during all phases of production through controlled, reproducible steps and a gentle and effective purification process that allows avoiding unpleasant possible degradation and isomerization processes, visible in poor fermentation-derived products.

Lowest content of *cis* isomer, lowest content of MK6 and no trace of unknown and unidentified chemical impurities define the unquestionable evidence of high purity (Fig. 17).

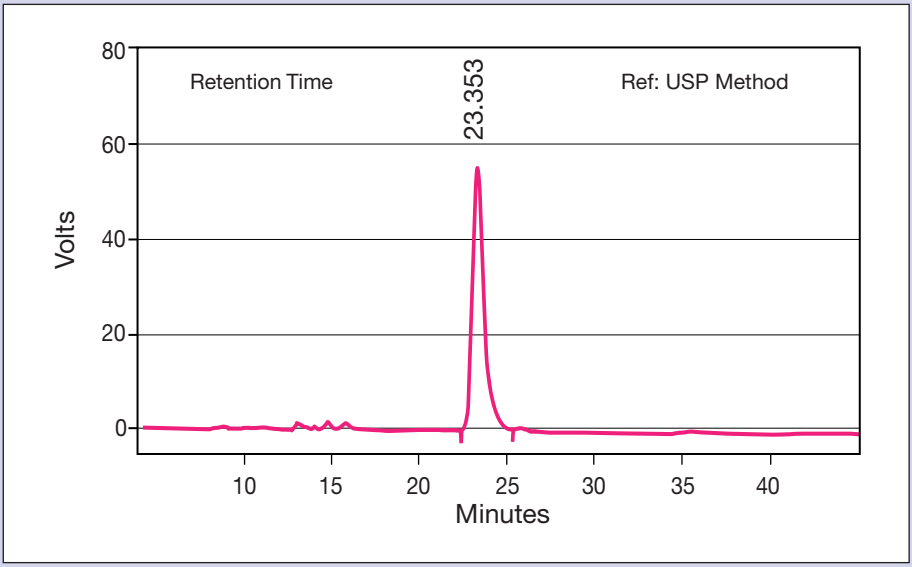


Figure 17. Isomeric purity identification of vitaMK7® by HCPLC analysis - USP Method.

Figure 18. Free-statements of vitaMK7®.

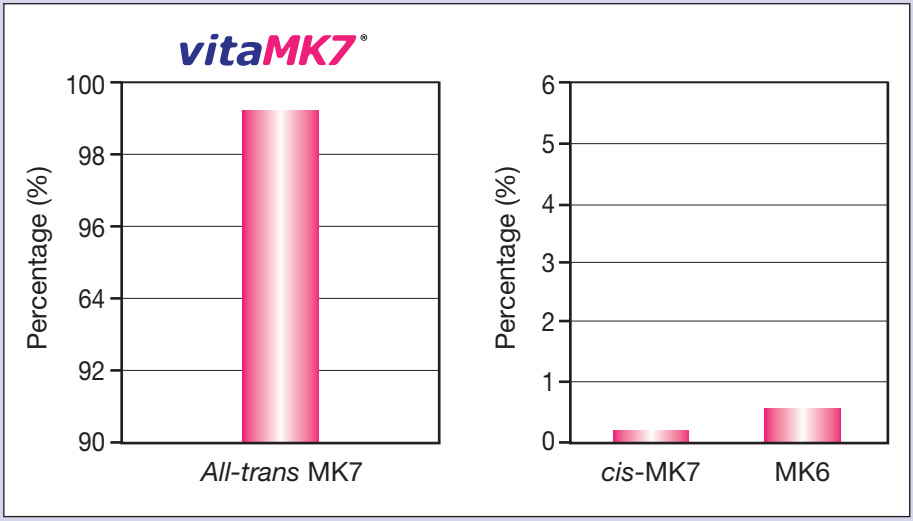


Figure 19. Typical content profile of branded high pure fermentation-derived MK7, vitaMK7®.

During more than ten years of production, the company has been able to optimize the process maintaining the natural features of MK7 whereas organoleptic qualities have been improved through advanced fermentation technologies. VitaMK7® is a white, odorless, neutral taste and flowing powder without the bad smell often present in other types of MK7.
The manufacturing process does not include starch-containing plant powder like soya or rice flour and is stated as SOY- and GLUTEN-FREE. No solvents are used during the manufacturing process and for the purification vitaMK7® is free from all known allergens, metals and organic solvents and “Free From” can be applied (Fig. 18,19).

5.2.1 Stability of vitaMK7®

Thanks to the clean profile, vitaMK7® shows excellent stability data - more than 3 years at room temperature - and remarkable assay recovery to short time light exposure (48h), the real stability issue, of more than 90% (Tab. 5).

		Recovery
vitaMK7®	48 h	>90%

Table 5. VitaMK7® assay recovery to short time light exposure (48 hours).

Gnosis consistently performs stability tests on its raw materials and tests potential harsh environment to support their applications in finished products, and vitaMK7® is not an exception.
Calcium formulations seem to be a potential issue that has created a palpable concern among the vast majority of manufacturers of dietary supplements, who want to be sure about the real content of vitamin K2. Therefore, a deep evaluation of the behavior of vitaMK7® with the most common calcium salts has been carried out, also in stress conditions.
VitaMK7® shows excellent stability data, a recovery rate of MK7 equal to 99% and 100% in formulations with calcium carbonate and calcium citrate, respectively. Even when subjected to stressed conditions such as 40°C/75% RH (Fig. 20).

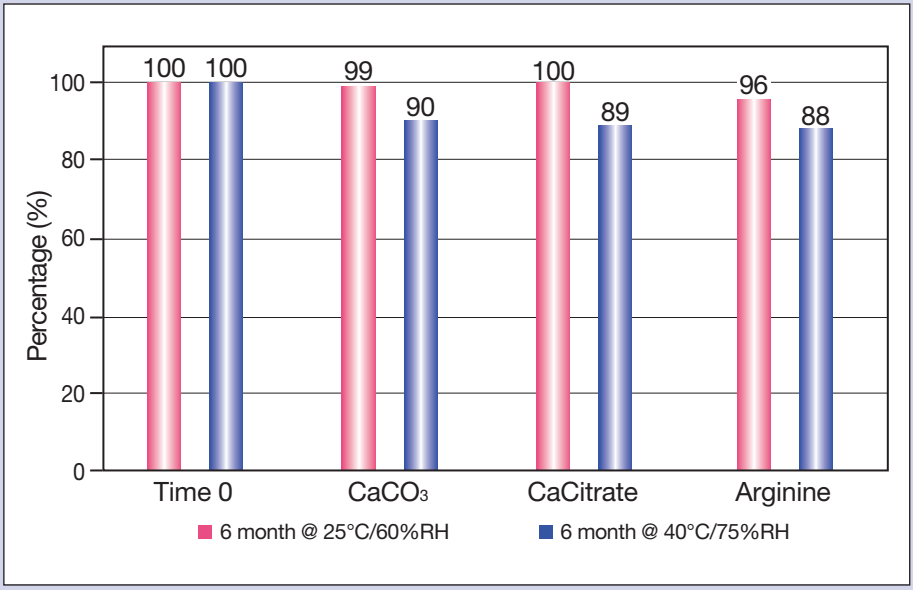


Figure 20. VitaMK7® stability profile in different formulations at 6 months, 25°C and 40°C.

VitaMK7® is stable in several formulations included liquid form (water, yogurt, oil) and milk powder preparations and is suitable to be combined with different calcium salts without any coating, which increases non-natural substances.

5.2.2 USP requirements

VitaMK7® is the only MK7 in compliance with USP Standard Reference, which defines purity, methodology and requirements of the high-quality standard for an optimal vitamin K2.

Gnosis held a role in the definition of these standards proposing the most detailed and accurate specification of MK7 for the establishment of a qualified MK7. A successful exchange of information between Gnosis and the Reference Commission at USP allowed to adapt the draft monograph as a model for the most advanced quality standards of vitamin K2 with the relative issue of the monograph.

The Australian Health Authority - TGA (Therapeutic Goods Administration) has recently recognized the USP monograph as a guidance of the quality expected for vitamin K2 products (Tab. 6).

Test	Value	Method
MK7 Identification (HPLC)	Positive	USP <621>
Purity		
MK6	≤1.0%	USP <621>
Cis MK7	≤1.0%	
Single Unknown Impurity	≤1.0%	
Total Impurities	≤2.0%	

Table 6. VitaMK7® compliance to USP monograph.

5.3 VitaMK7® - *in vitro* studies

VitaMK7® has been studied in published pre-clinical and clinical studies, as described in ref. 34 and 80. When tested in a single dose experiment in healthy volunteers (1 mg with a fatty breakfast) vitaMK7® behaves with a bioavailability pattern typical of MK7, in accordance with data described for natto-derived MK7 (Fig. 21) (66).

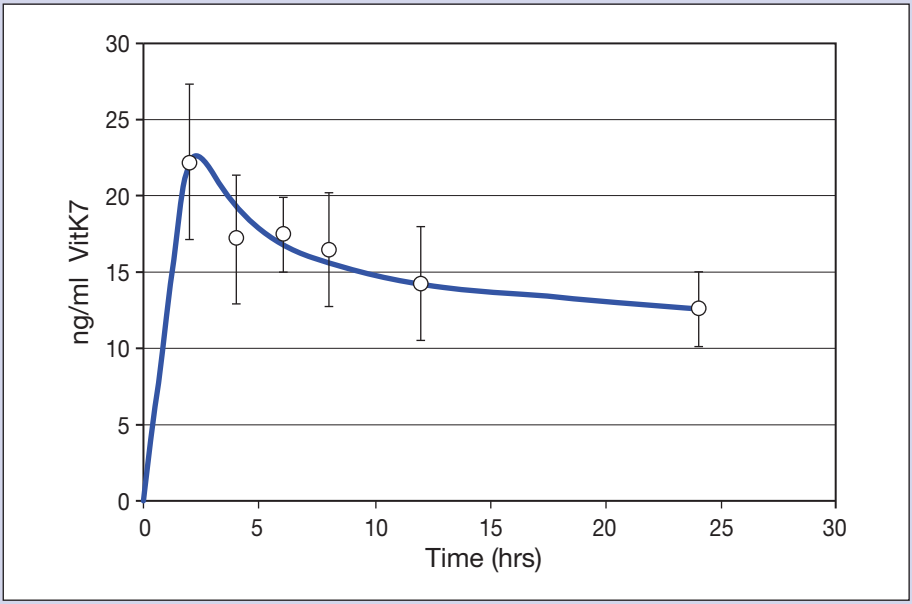


Figure 21. Plasma levels of MK7 after a single dose (1 mg) of vitaMK7®.

When administered at a dose of 45 or 90 µg/day, dissolved in olive oil, for a two-week period (34) vitaMK7® yielded plasma levels of 1.28 (SD 0.24) ng/ml, and of 2.47 (SD 0.23) ng/ml, and the high dose was accompanied with a significant improvement of osteocalcin carboxylation. These data are more extensively described in the following pages.
A summary of the published studies of these cellular and clinical effects is reported and discussed below.

5.3.1 MK7 and osteogenesis

Clinical orthopedic surgery constantly deals with fracture repair and reconstructive procedures where bone healing ability is essential. In fact, complications during fracture healing, including delayed union and non union, can arise as a result of a multitude of factors. Building of new bone relies on the capability of undifferentiated mesenchymal cells to differentiate along an osteogenic pathway which leads to the formation of osteoprogenitor cells, capable of forming new bone. This phenomenon is known as osteoinduction, and any material capable of stimulating could have remarkable benefits in clinical practice. Vitamin D3 is synthetized in the skin from its precursor, 7 dehydro cholesterol, through the action of U.V. light. It is well known to be involved in calcium and phosphorus metabolism and to promote their absorption in the gut; it also has a direct effect on cartilage and bone to promote normal skeletal development and turnover. Vitamins D and K have indeed been shown to have osteoinductive properties. Already in 1997 it has been shown that the addition of vitamin K2 to a human osteoblast cell line treated with vitamin D3 increased the accumulation of Gla containing osteocalcin in the extracelluar matrix (81).
In 2008, vitamin D3 and K1 association was shown to modulate *in vitro* the differentiation towards osteoblastic phenotype of hMSCs derived from fracture sites (82).

5.3.2 Effect on gene induction

More recently the same authors, in collaboration with our group, tested the effect of vitamin D3 and MK7 on the differentiation of human mesenchymal stem cells (hMSCs) toward the osteoblastic lineage (80). Bone marrow (BM) from male patients undergoing surgical treatment of pelvic injury was extracted from the upper

superior iliac crest. BM mononuclear cells were isolated and plated in culture flasks. The cells were supplemented once a week with vitamin D3, vitamin MK7 or a combination of both, while cell cultures in unsupplemented medium, collected at the same time points, were used as controls. The expression of 84 genes related to osteogenic differentiation was tested by PCR array technology. MK7 (vitaMK7®) alone upregulated two of the 84 genes considered, namely amelogenin Y linked (AMELY) and growth differentiation factor-10 (GDF10). GDF10 is a peculiar type of BMP (Bone Morphogenetic Protein) which has been shown to promote osteoblastic differentiation and augment bone formation (83). In our hMSCs cultures this gene was remarkably induced in the early stage of differentiation by single-vitamin exposure with either vitamin D3 or MK7, showing a clear additive and time-dependent effect following co-treatment of up to 17 days. More recently GDF10 has been shown to act as a signal for axonal sprouting in rodent and primate models of stroke. These new neuronal connections are causally associated with functional recovery after stroke (84).
Amelogenin plays a fundamental role in the organization of enamel rods during tooth growth, and in the development of cementum, which is essential for the attachment of teeth to the alveolar bone. Among the genes considered, those involved in bone formation and mineralization that were mostly affected by vitamin D3 and MK7 included BMP, OC and ALP. In fact, these genes are known to be major osteoblast differentiation-related markers. Vitamin D3 is a known inducer of OC expression thus promoting OC deposition in the ECM and consequent bone mineralization. Our data show that OC was already upregulated after 10 days of treatment with vitamin D3, alone or in combination with MK7, by approximately 85-fold. This induction was further increased after 17 days by D3 (172 fold up-regulation). The association D3/MK7 brought the up-regulation to 235 fold; therefore MK7 enhanced D3 induction of OC by 1.36-fold.

5.3.3 Cellular levels of osteocalcin and osteogenesis-related proteins

Also flow cytometry and immune-enzymatic measurements showed that vitamin D3 treatment produced an increase of the intracellular protein content, together with a rise in cOC/ucOC ratio. MK7 treated cells showed a decrease in

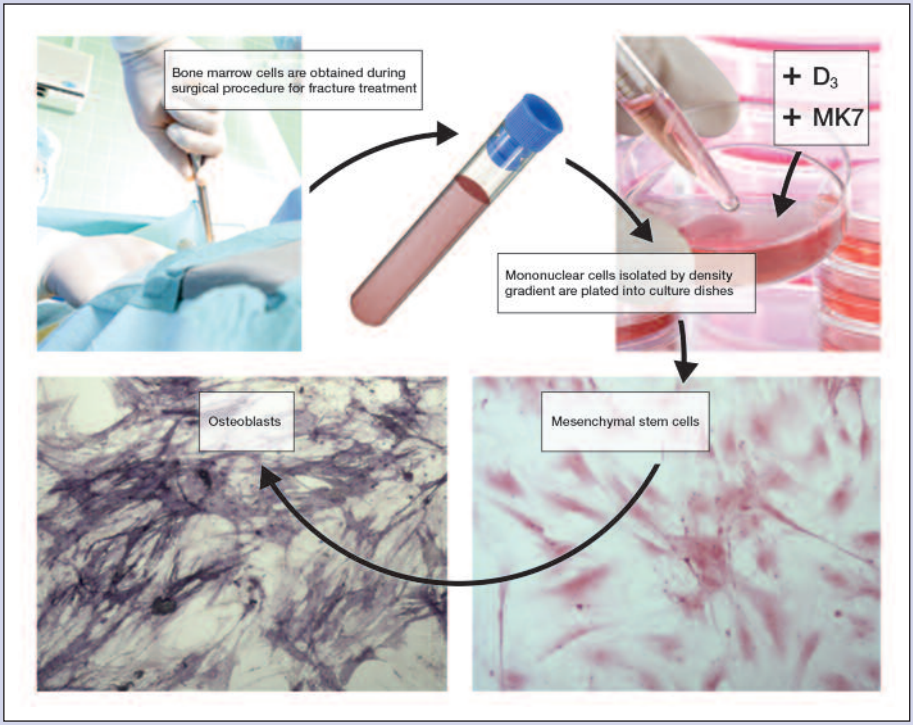


Figure 22. Schematic representation of in vitro effects of vitaMK7®. MK7 specifically improved gene expression of Amelogenin 4.7 times, and 5 times the expression of GDF10 (Gigante, 2015).

intracellular osteocalcin; in fact the carboxylative effect of MK7 plays a major role in the secretion of the active form of the protein and its consequent accumulation in the extracellular matrix.

Vitamin cosupplementation also remarkably affected both VEGFA (Vascular Endothelialn Growth Factor A) and its receptor FLT1, a key player in both angiogenic and osteogenic process. Angiogenesis has been shown to be of vital importance during bone healing vascular invasion being a prerequisite for endochondral bone formation and fracture healing.

In conclusion, MK7 was able to potentiate vitamin D3 effect on osteogenic differentiation and to enhance deposition of osteocalcin in the extracellular matrix. Moreover, a specific effect of MK7 was observed on the induction of genes

involved in codifying proteins which are essential for the formation of the periodontal ligament (Amelogenin) or for bone morphogenesis (GDF10). A newly discovered role for GDF10 concerns its capability to act as growth promoting signal after stroke (Fig. 22) (80).

5.4 VitaMK7® - clinical studies

5.4.1 VitaMK7® into action: molecular effects of MK7-supplemented olive oil

Among menaquinones, menaquinone-7 is very hydrophobic, due to its long isoprenoid chain. As pointed out earlier in this book, the chemico-physical properties of this molecule make it transportable by plasma lipoproteins, increase the extrahepatic availability and produce the longest half life (66). All these features lead to remarkably higher levels (7- to 8-fold) of MK7 content, during prolonged intake, compared with K1.

Extra-virgin olive oil is an essential component of the Mediterranean diet, endowed with undisputable beneficial effects, and a good solvent for lypophilic vitamins. Our group conducted a study aimed at verifying whether supplementing a diet with extra-virgin olive oil enriched with MK7 would result in a significant increase of this molecule in plasma levels and whether this was associated with biological effects (34).

5.4.2 MK7 absorption

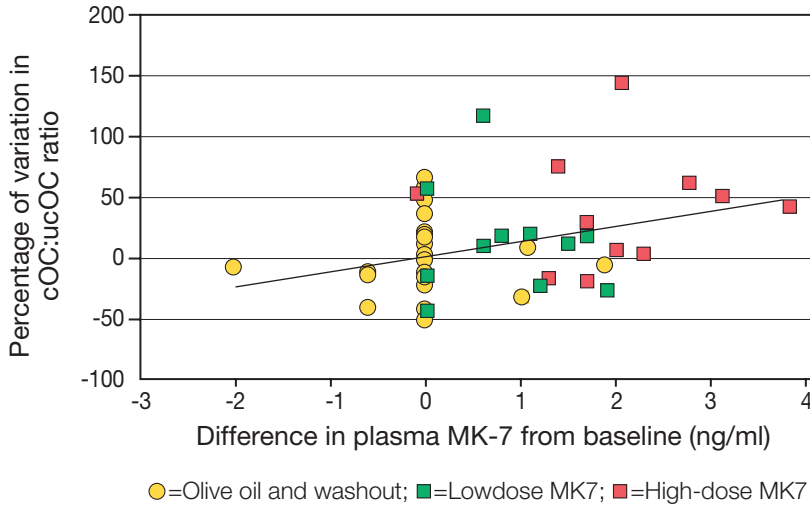
The trial was conducted on twelve healthy subjects and lasted 56 d. From day 0 to day 14, the volunteers supplemented their diet only with 20 ml extra-virgin olive oil not enriched with MK7, taken in two daily doses with the main meals. From day 15 to day 28, volunteers supplemented their diet with extra-virgin olive oil enriched with 45 mg (low dose) MK7. The vitamin MK7 formulation was vitaMK7® (Gnosis, Desio, Italy), and the oil formulation for this trial was prepared by Costa D'oro (Spoleto, Italy).

Following 2 weeks of washout (from day 43 to day 56), the volunteers were invited to supplement their diet with oil containing 90 mg MK7 (high dose). Throughout the study oil was only used as a dressing, but not for cooking. Oil also contained vitamin E from Roche (Milan, Italy) (1 mg/20 ml), vitamin B6 from Carlo Erba (Milan, Italy) (0.5 mg/20 ml) and CoQ10 from Kaneka (Osaka, Japan)

(20 mg/20 ml low dose; 40 mg/20 ml high dose). The biological effects of CoQ10 coadministered with MK7 were described in a companion paper (85). Basal plasma levels of MK7 were very low, showing nondetectable levels in half of the tested subjects and an average value, for the remaining volunteers, of 0.42 (SD 0.17) ng/ml. Supplementation of the diet with extra-virgin olive oil alone did not produce any significant variation of MK7 plasma levels. On the contrary, supplementation with MK7-enriched extra-virgin olive oil resulted in a significant and dose-dependent increase in plasma levels (low dose, 1.28 (SD 0.24) ng/ml, P<0.001; high dose, 2.47 (SD 0.23) ng/ml, P<0.001). A period of 2 weeks of washout was sufficient to restore basal plasma levels.

5.4.3 MK7 and osteocalcin carboxylation

After supplementation of the diet with the high-dose MK7-supplemented olive oil, a significant increase in carboxylated osteocalcin (cOC) was also



(overall correlation R^2 0·11 (n 48; P=0.02); low dose, olive oil, washout R^2 0.009 (n 36; P=0.59); high dose, olive oil, washout R^2 0.197 (n 36; P=0.007)

Figure 23. The ratio carboxylated osteocalcin/undercarboxylated osteocalcin (cOC/ucOC) shows a significant correlation with MK7 levels (Brugé, 2011).

found both compared with study entry and with supplementation (with extra-virgin olive oil alone), and a decrease in undercarboxylated osteocalcin (uOC) levels.

These data are highlighted by the cOC:ucOC ratio, a well-recognized index of the functionality of osteocalcin. Following 2 weeks of supplementation with the high dose of MK7-enriched olive oil, a highly significant increase in the cOC:ucOC index was observed compared with olive oil alone ($P<0.01$). An interesting correlation was found between percentage of variations of plasma cOC:ucOC ratio and differences in MK7 plasma levels compared with study entry for each subject at different experimental points (Fig. 23) (34).

Plasma variations in MK7 levels capable of significantly activating osteocalcin were practically found only after the daily dose of olive oil fortified with 90 mg MK7. In fact, after this dose a significant increase of the cOC:ucOC ratio, which is known to correlate with bone mineralization status, was observed.

We may reasonably hypothesize that older volunteers, or postmenopausal women, could have shown more consistent increases, also due to a higher requirement of vitamin K. The observed improvements would have likely been even more significant after a longer treatment.

Conclusions: established functions and emerging roles

In the early decades following the discovery of vitamin K, its role beyond coagulation was progressively elucidated. Vitamin K, particularly menaquinones, have always attracted research attention, due to the potential in reducing both osteoporosis and cardiovascular diseases. MK7 is the superior form of K2 because is more bio-efficient and shows additional health functions.

Vitamin K2 is crucial for bone development from conception to senior years and, taken in adequate nutritional doses, it is important for cardiovascular health (by preventing calcium deposit in arteries) with important implications for large population groups.

In western population vitamin K2 deficiency seems to be common, by increasing the regular intake of vitamin K2 in the general population we may reduce worldwide healthcare spending relative to bone and cardiovascular health.

In this scenario it is important to look for products containing high quality vitamin K2. First of all they must have a pure vitamin K2 as MK7, with the highest percentage of *all-trans* stereochemical structure (ideally more than 99%), the only active form of the vitamin. As a matter of fact, *cis*-analogs of vitamin K2 are biological inactive and only the fermentation-based manufacturing process guarantees the absence of unnatural impurities. Isomeric purity can establish the origin of the sample containing MK7, natural (fermentative) or synthetic, and must be clearly identified.

MK7 extracted from *Bacillus subtilis* natto is also characterized by the presence of a marker of process, MK6, a byproduct of the biofermentative process.

Today, the fermentation and the chemical production of MK7 coexist, but -excluding guesswork and marketing speculation- a fermentation-derived method of production based on *Bacillus subtilis* natto, used for hundreds of years by the Japanese people in natto food, guarantees a MK7 production identical to the one used for ages as key nutrition element.

In order to keep a satisfactory vitamin K status we must have a sufficient vitamin K2 intake, together with a proper functioning of the gamma-carboxylase enzy-

matic system, which allows the formation of the Gla residues in the vitamin K-dependent proteins. The vitamin K oxidoreductase (VKOR) then brings back oxidized vitamin K to its reduced, active form KH2, as described in the initial chapters of this book and has a strategic role in ensuring the proper functioning and biochemical activity of MK7.

While promising results have been reported for MK7, more trials need to be conducted to determine vitamin K status and new health outcomes. In addition, comparative trials are required to address the question of relative contribution of individual forms of vitamin K to human health.

Clearly, significant gaps in the current knowledge on menaquinones exist. However, there is merit for considering menaquinones, namely MK7, as a key element when developing future recommendations for vitamin K intake.

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Glossary

AF	Atrial Fibrillation
ALP	Alkaline Phosphatase
AMELY	Amelogenin Y linked
BAC	Breast Arterial Calcification
BM	Bone Marrow
BMD	Bone Mineral Density
BMP	Bone Morphogenetic Protein
CAC	Coronary Artery Calcium
CHD	Coronary Heart Disease
CKD	Chronic Kidney Disease
DOACs	Direct Oral Anticoagulants
dp-ucMGP	dephosphorylated-undercarboxylated MGP
ECM	Extracellular Matrix
GDF10	Growth Differentiation Factor-10
GGCX	Gamma-Glutamyl Carboxylase
Gla	Gamma-carboxyglutamic residue
Glu	Glutamic acid residue
GRA	Gla Rich Protein
HMSCs	Human Mesenchymal Stem Cells
INR	International Normalized Ratio
KO	vitamin K 2,3-epoxide
MGP	Matrix Gla Protein
MK4	menaquinone-4
MK7	menaquinone-7
MV	Matrix Vesicles
NPC1L1	Niemann-Pick C1 Like 1 transporter
OC	Osteocalcin
uOC	undercarboxylated Osteocalcin
ucOC	undercaboxylated Osteocalcin

PIVKA	Proteins Induced by Vitamin K Absence or antagonists
PIVKA-II	undercarboxylated prothrombin
PAD	Peripheral Arterial Disease
PT	Prothrombin Time
VEGFA	Vascular Endothelial Growth Factor A
VKA	Vitamin K Antagonists
VKOR	Vitamin K epoxide reductase
VKORC1	the gene encoding for VKOR complex subunit 1
VSMC	Vascular Smooth Muscle Cells

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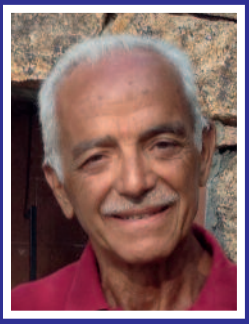

Gian Paolo Littarru originally graduated as MD (Doctor of Medicine) and then specialized and worked in medical biochemistry throughout his scientific career. After his first years as a researcher at the University of Texas, he became a Biochemistry Professor at the Catholic University in Rome and then at the Ancona Medical School, Polytechnic University of the Marche, Italy. His main research field has been focused on coenzyme Q and lipophilic antioxidants. He is the founder of the International Coenzyme Q10 Association, where he served as chairman until a few years ago, and cooperated on this field with various, renowned research groups. Within his involvement on isoprenoid compounds his research interest was extended to vitamin K, namely to MK7. The peculiar properties of this molecule were investigated by Prof. Littarru's group in vitro and ex vivo, with a multidisciplinary approach involving the participation of biochemists, molecular biologists, nutritionists, orthopedic surgeons, also in cooperation with the biotechnology and nutrition industry. The main results of this research effort and the potential impact on some medical issues are discussed in this book.