Lead Article

Vitamin K – sources, physiological role, kinetics, deficiency, detection, therapeutic use, and toxicity

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Vitamin K is traditionally connected with blood coagulation, since it is needed for the posttranslational modification of 7 proteins involved in this cascade. However, it is also involved in the maturation of another 11 or 12 proteins that play different roles, encompassing in particular the modulation of the calcification of connective tissues. Since this process is physiologically needed in bones, but is pathological in arteries, a great deal of research has been devoted to finding a possible link between vitamin K and the prevention of osteoporosis and cardiovascular diseases. Unfortunately, the current knowledge does not allow us to make a decisive conclusion about such a link. One possible explanation for this is the diversity of the biological activity of vitamin K, which is not a single compound but a general term covering natural plant and animal forms of vitamin K (K_1 and K_2) as well as their synthetic congeners (K_3 and K_4). Vitamin K_1 (phylloquinone) is found in several vegetables. Menaquinones (MK_4–MK_{13}, a series of compounds known as vitamin K_2) are mostly of a bacterial origin and are introduced into the human diet mainly through fermented cheeses. Current knowledge about the kinetics of different forms of vitamin K, their detection, and their toxicity are discussed in this review.

INTRODUCTION

Vitamin K is well known as an essential factor in blood coagulation. Hence its name, vitamin K, which is derived from the German term for coagulation (Koagulation). Its importance was recognized by the Nobel Committee for Physiology or Medicine, since the Nobel Prize in Physiology or Medicine for 1943 was awarded to Henrik Carl Peter Dam and Edward

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Adelbert Doisy for their discoveries of vitamin K and its chemical nature, respectively.[1]

Vitamin K is not a single compound but is a term for many similar compounds that have the physiological function of this vitamin (Fig. S1 in the Supporting Information online). They share a common structure, the 2-methyl-1,4-naphthoquinone core, also known as menadione. The simplest form, which contains only this core, is known as vitamin K_3. In contrast to the natural forms, K_3 is hydrophilic and is not obtained through the diet. However, it acts as an intermediate in human metabolism.[2] Vitamin K obtained from the diet originates either from plant sources (in the form of vitamin K_1, known as phylloquinone [phytomenadione, phytonadione]) or more commonly from animal sources in the form of vitamin K_2 (menaquinone, generally abbreviated as MK, see Fig. S1 in the Supporting Information online). Vitamin K_4 also exists, and this term is associated with other synthetic forms of vitamin K. It may be a reduced form of vitamin K_3 (menadiol) or its ester forms (eg, diacetate vitamin K_3).

DIETARY SOURCES OF VITAMIN K

Vitamin K_1 is a single compound found in photosynthetic organisms like cyanobacteria, algae, and green plants.[3–5] Due to the high vitamin K_1 content in the green parts of plants, phylloquinone was originally thought to be present only in chloroplasts, but further research has confirmed that it is also present in peroxisomes and plasma membranes. It is even present in some non-photosynthetic parasitic plants. Its function in plants is best seen in the chloroplasts, where it is tightly bound to the thylakoid membrane. Here, it serves as an electron acceptor during photosynthesis, forming part of the electron transport chain of photosystem I. The function of vitamin K_1 in other cellular compartments is also associated with its redox properties.[5–7] In absolute amounts, it constitutes approximately 75%–90% of all vitamin K in our diet, but dietary K_1 has low bioavailability,[8–11] as will be discussed in more detail below. Green cruciferous vegetables (broccoli, brussels sprouts, cabbage, kale, kai-lan, etc.) are rich sources of vitamin K_1, but spinach, chard, parsley, and various types of lettuce also have considerable phylloquinone content .[8,12–14] In general, all edible green parts of plants can be considered an important source of vitamin K_1. Of course, this also applies to wild edible plants. Examples include stinging nettles (*Urtica dioica* L.), wild garlic (*Allium ursinum* L.), dandelion leaves (*Taraxacum campylodes* G.E.Haglund), and ground elder (*Aegopodium podagraria* L.), a relative of parsley whose vitamin content is comparable with that of cultivated vegetables.[15] Edible greens also include culinary herbs, of which marjoram (*Origanum majorana* L.), mint (*Mentha* × *piperita* L.), and savoury (*Satureja hortensis* L.) have the highest content of vitamin K_1, reaching values of up to 1250μg/100 g when fresh and over 3000 μg/100 g in dried herb samples. The seeds of some species of Apiaceae plants that are used as spices also contain significant amounts of the vitamin K_1.[16] Data on their vitamin K_1 content differ significantly from study to study because it can be affected by a number of factors, such as cultivar, cultivation method, cultivation site, climatic conditions, plant maturation, and the method of determination.[17–19] The richest vegetable sources of vitamin K_1, kale and spinach, are good examples of this. Its content in these vegetables appears to vary greatly: 250–1139 μg/100 g and 240–1220 μg/100 g, respectively.[12,15,20] In most cases, neither boiling nor microwaving affects the vitamin K_1 content of vegetables.[12] Certain vegetable oils are also important dietary sources of phylloquinone for humans. Of these, soybean oil is the most significant source, followed by rapeseed oil and olive oil, containing on average about 180, 130, and 55 μg/100 g of phylloquinone, respectively.[8,21,22] Since these oils are frequently used when cooking vegetables, they can enrich the food with additional vitamin K_1. Its content in vegetable oils is relatively stable when heated, reaching a maximum decrease of 15% after being heated for 40 minutes at 185–190°C. On the other hand, vitamin K_1 is extremely sensitive to daylight and fluorescent light, and these light sources decrease the vitamin content by 46% and 87% after only 2 days of exposure, respectively. Therefore, it is recommended to store oils in dark containers.[22]

Vitamin K_1 is the predominant form of vitamin K used in food supplements and drugs indicated in vitamin K deficiency, and therefore production of a large amount is required. Synthetic vitamin K_1 is manufactured by condensing naphthoquinone with isoprenoid precursors.[4,23] Demand for vitamins of a natural origin and their sustainable production is increasing, so research is underway seeking efficient production strategies. One promising option is the use of microalgae and cyanobacteria, which can be grown in bioreactors under highly controlled conditions and which produce a significant amount of vitamin K_1. This is true even when the algae are cultivated using conventional aquaculture techniques.[3,4,24,25]

Vitamin K_2 is produced by certain obligate and facultative anaerobic bacteria. The form of vitamin K_2 generated depends on the strain of bacteria. The various forms principally differ in the number of 5-carbon prenyl units, which ranges from 4 to 13 (Fig. S1B in the Supporting Information online). The number of these units is given as a suffix(-n), ie, they are named MK-4

to MK-13. It should be also pointed out that there can be differences between natural and synthetic vitamin K_2, as was demonstrated in the case of MK-7, for which the synthetic mono-*cis* form (2*Z*) was inactive or had little activity compared with the natural all-*E* form. Some bacteria also produce one or more saturated prenyl units, and this is indicated by an additional suffix, eg, MK-8(2H) in Fig. S1C in the Supporting Information online or MK-9(4H).[2,26–28] MK-4 (menatetrenone) is not produced by bacteria, but instead is formed in human and animal organisms from K_1. It can hence be both consumed in the diet or formed in the human body. The enzyme responsible for this conversion is UbiA prenyltransferase domain–containing protein-1 (also known as transitional epithelial response protein 1, TERE1). This enzyme can cleave the side chain to release vitamin K_3 and then prenylate it with geranylgeranyl pyrophosphate to produce MK-4. There is still some controversy as to whether or not vitamin K_3 is produced in the gastrointestinal tract (GIT), and whether the enzyme TERE1 catalyzes only prenylation. It is possible that both the production of K_3 in the GIT from human vitamin K by, eg, microflora, with subsequent metabolism to MK-4 by TERE1, and direct conversion of K_1 to MK-4 by TERE1 could be occurring.[2,8,29–32] Major sources of vitamin K_2 in the human diet include dairy products. Cheeses are a particularly rich source, due to the bacterial fermentation that occurs when they are produced. In fact, about one-half of all vitamin K_2 ingested by humans comes from cheese.[33,34] Other important sources of MKs are fermented vegetables such as sauerkraut, and in Japan, nattō.[27] The concentrations of MK-4 to MK-12 can reach high values (eg, 5–50 µg/100 g), with MK-8, MK-9, MK-10, and MK-11 being the major forms in many different cheeses (Fig. S2 in the Supporting Information online[8,27,35–37]). It should be stressed, however, that apparently discrepant data have been reported in relation to MK-10 content. Its levels can be even higher than that of MK-8 in some cheeses.[36,37] The type of vitamin K_2 is largely dependent on which bacterial species (eg, *Lactococcus, Propionibacterium*) is used in production. Ripening results in an increase in vitamin K_2 content.[37] Reduced-fat or fat-free cheeses contain less than one-quarter of the vitamin K_2 compared with their full-fat equivalents.[36] Sauerkraut is a less rich source of vitamin K_2 (total MKs content is approximately 5 µg/100 g), but it contains a substantial quantity of vitamin K_1,[8,27] while nattō is by far the richest source of vitamin K_2 (the content of MK-7 is approximately 900 µg/100 g). Nattō is produced from soybeans through fermentation with *Bacillus subtilis* var. *natto*, which is responsible for the production of MK-7. Other important dietary sources of vitamin K_2 are bovine and pork liver, whereas vitamin K_2 is barely present in most fish, with the important exception of eel.[27,37] Fermented beverages like beer and wine do not contain detectable amounts of MKs, because yeasts, in contrast to bacteria, do not produce MKs.[2,8,27] These theoretical nutritional data fit nicely into the assessment of human vitamin K_2 consumption in Europe. In a large epidemiological study, the intake of vitamin K_2 was more than 95% comprised of MK-4, MK-8, and MK-9; dietary MK-5 to MK-7 were detected in only low amounts.[10] It should, however, be pointed out that vitamin K consumption assessment based on a dietary questionnaire is not very precise.[38] Even though a large amount of research on vitamin K has been conducted, no precise daily requirement level for vitamin K (likely due to the wide range of vitamin K forms) has been established as yet. It has been suggested that the mean intake of vitamin K ranges from 70 µg/day to 300 µg/day,[10,11,31,39–41] and the current recommendation by the European Food Safety Authority indicates 1 µg/kg per day is an adequate intake of total vitamin K in both children and adults, including pregnant women.[31] Interestingly, the current average human intake of vitamin K in developed countries is in general above this recommendation, notwithstanding the low frequency of use of vitamin K supplements.[28]

An early study did not find substantial differences in the levels of vitamin K_1, MK-7, and MK-8 between young and older persons.[42] However, regarding what constitutes adequate vitamin K level and how usable vitamin K is in the elderly, current knowledge is unsatisfactory. Other research rather suggests a level of insufficient vitamin K status in the elderly.[29,43,44]

THE KINETICS OF VITAMIN K

Absorption

There are apparent differences in the absorption of the various forms of vitamin K. In general, bile salts enable the formation of mixed micelles, which allow the uptake of natural lipophilic forms of vitamin K into the enterocytes. The vitamin is further packaged inside the enterocytes into the chylomicrons, and it then enters systemic circulation directly via the lymphatic system[31] (Fig. 1A). The absorption of vitamin K_2, in particular long-chain MKs, is excellent and may even be complete due to the co-presence of fat, eg, in dairy products.[8,9] Vitamin K_1 is much less easily absorbed from the diet than dietary MK-7 or pure MK-4 are.[8,45] However, pure vitamin K_1 has better bioavailability than dietary vitamin K_1, pure MK-4, or pure MK-9, but lower than pure MK-7.[8,23,45,46] Dietary vitamin K_1 is tightly bound to plant

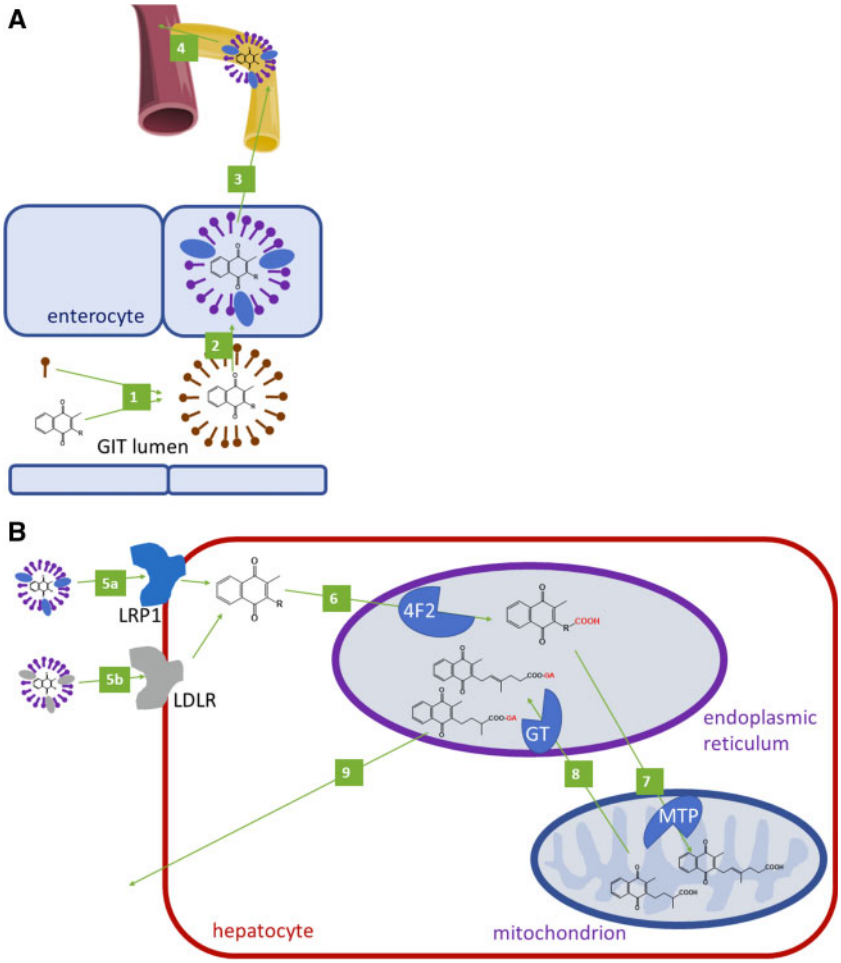

Figure 1 **Absorption and elimination of vitamin K. A:** Oral absorption of vitamin K. **1**: Formation of micelles from vitamin K and bile acids. **2**: Uptake of vitamin K from a micelle to an enterocyte. **3**: Formation and release of a chylomicron containing vitamin K. **4**: Through lymphatic circulation, vitamin K in the chylomicron enters systemic circulation (through the vena cava inferior). **B**: Metabolism of vitamin K in the hepatocyte. A chylomicron loaded with vitamin K binds to low density lipoprotein receptor-related protein 1 (LRP1, **5a**) or LDL particles loaded with vitamin K bind to LDL-receptors (LDLRs, **5 b**), and this results in the uptake of vitamin K into the hepatocyte. A similar process can also be observed with VLDL in the peripheral tissues (not shown). **6**: Vitamin K is ω-hydroxylated by cytochrome 4F2 in the endoplasmic reticulum. **7**: This metabolite is subsequently β-oxidized by mitochondrial trifunctional protein (MTP) to 5 C or 7 C or 10 C (not shown), which are subjected to glucuronidation by UDP-glucuronosyltransferase (GT, **8**) in the endoplasmic reticulum. **9**: Excretion of glucuronides in feces and urine

tissue, as mentioned, so pure vitamin K_1 is better absorbed than the dietary form. We note that there is no difference in the bioavailability of vitamin K_1 from various vegetables, and that cooking does not improve its absorption.[47] Fat, however, increases vitamin K_1 bioavailability from plant sources by about 3 times, but its bioavailability is still much lower than that of commercially solubilized vitamin K_1.[45] One likely reason is that fat stimulates bile secretion. There are also differences in vitamin K_1 bioavailability between people with different types of diet. Diet involving fast-food eating resulted surprisingly in better absorption of vitamin K_1 than other diets with the same amount of fat ingested. It is possible that vegetable oil offers more easily absorbable vitamin K_1 than whole vegetables.[48] Surprisingly, at least according to *in vitro* everted intestinal sac experiments, unsaturated fatty acids can decrease the extent of vitamin K_1 absorption.[49] There is also substantial interindividual variability in vitamin K_1 absorption.[45,48] Linear pharmacokinetics, ie, a linear relationship between dose and serum levels, were observed for both MK-7 and K_1.[23] Long-term administration of MK-7 results in a marked increase in its serum levels, in contrast to vitamin K_1, for which serum levels are only slightly above placebo.[23] This is because MK-7 has an elimination half-life of approximately 3 days, while that of vitamin K_1 is about 1–2 hours.[8,23] Further, MK-9 has been shown to have a long elimination half-life (about 60 hours).[46] This is, however, less than that of MK-7, which suggests that prolongation of the side chain is

not directly associated with increase in half-life. In contrast, MK-4 has a short half-life in systemic circulation, and, similarly to vitamin K_1, its level dropped virtually to zero 24 hours after administration.[45] In rats, the oral administration of various forms of vitamin K resulted in increased plasma and liver levels of vitamin K_3, and MK-4 to MK-11; MK-1 to MK-3 and MK-12 to MK-14, however, were apparently not absorbed. There was no correlation between plasma and liver levels since, surprisingly, the highest plasma levels were observed after the administration of vitamin K_3 and MK-8; MK-6 reached its highest levels in the liver.[50] Furthermore, vitamin K absorption can be decreased by certain drugs: cholestyramine can decrease vitamin K absorption likely due to the binding of bile acids; rifampicin can decrease vitamin K absorption due to the induction of metabolism; and orlistat forces the patient to decrease their intake of fat since it is associated with unpleasant GIT side effects.[51–54]

As mentioned, human microflora produces MKs. Many bacterial species are able to synthesize vitamin K_2, and the form synthesized is species specific: *Bacteroides* produce mainly MK-10 and MK-11, *Eubacterium lentum* MK-6, *Veillonella* MK-7, and *Enterobacter* and *Escherichia coli* MK-8.[9,27,55,56] There has been some discussion about the extent of absorption of MKs produced by human microflora.[9,27,31,57] One study demonstrated that they are able to be absorbed and reach systemic circulation, at least on a small scale.[58] Furthermore, it is known that large-spectrum antibiotics can inhibit the growth of some vitamin K–producing bacteria and increase the risk of vitamin K deficiency.[59,60] It is unlikely that the vitamin K_2 forms with large side chains are absorbed in the distal colon, but they can be absorbed in the terminal ileum, where microflora is still present, and the absorption is further supported by the bile salts there. These large MKs are tightly bound to bacterial inner cytoplasmic membranes, and bile salts are hence required for their solubilization.[27,61]

After intestinal absorption, vitamin K_1 appears to be taken up primarily by the liver. The reason for the predominant clearance of vitamin K_1 in the liver might lie in the fact that vitamin K_1 is transported mainly by chylomicrons, which are taken up by the liver, whereas vitamin K_2 forms are also present in the very-low-density lipoprotein/low-density lipoprotein (VLDL/LDL) particles that are transported from the liver to the extrahepatic tissue. However, vitamin K_2 is also apparently taken up by the liver, where it can be used for carboxylation of proteins.[2,31,46,62] Cellular uptake of vitamin K is managed via lipoprotein receptors.[2] In humans, plasma and serum levels of vitamin K_1, MK-4, MK-5, MK-6, and MK-8 are low, and are expressed in units of nM or even in lower concentrations.[42,46,48,63–68] The plasma concentrations of vitamin K_1 and MK-4 in mice and rats are apparently similar to those in humans.[32,69] There is, as expected, a large difference in plasma levels of MK-7 between European and certain Japanese populations due to the Japanese consumption of nattō. In Europe, the plasma levels of MK-7 are below 1 nM or in the low nM range,[42,65] while in one recent Japanese study, the mean plasma levels of MK-7 were 15.6 nM.[63] To enable a better comparison to be made, adjustments to triglyceride levels were recommended, but the results were essentially similar.[31,65] The total body pool of vitamin K_1 is roughly 0.55 μg/kg, but no such data are as yet available for vitamin K_2.[31]

DISTRIBUTION AND ELIMINATION

Experimental data has shown that vitamin K_1 and MK-4 are distributed differently. In rats, the same oral dose resulted in much higher levels in the liver of vitamin K_1 than of MK-4, while the opposite was found in the aorta.[70] This was also confirmed in human postmortem liver analyses, in which the amount of vitamin K_1 was always higher than of MK-4.[71] Surprisingly, the sum of the MK-7 to MK-11 levels in the human liver was mostly much higher than that of vitamin K_1.[71,72] One study found that there were no differences in the liver vitamin K_1 content and MK-7 to MK-9 content between non-cancerous liver samples and hepatitis or cirrhotic liver samples; however, a clear difference in the MK-10 to MK-13 content was observed. In that study, the subcellular localization of MK-10 and MK-11 was found to be mainly in the mitochondria.[72] The catabolism of vitamin K_1 and of vitamin K_2 shares common mechanisms, beginning with initial ω-hydroxylation mediated by CYP4F2, followed by shortening of the polyisoprenoic side chain via β-oxidation to carboxylic acids (in 5 C, 7 C or 10 C metabolites, Fig. 1B), which are glucuronidated and excreted in urine and bile.[2,31,73] Hence, the previously mentioned localization of vitamin K_2 forms in the mitochondria could be linked to their metabolism via β-oxidation. The urinary excretion of these metabolites can be used as a marker of vitamin K body status.[74]

THE PHYSIOLOGICAL FUNCTION OF VITAMIN K

γ-carboxylation process

The only well-known function of vitamin K in humans is its involvement in the γ-carboxylation of a number of proteins (Fig. 2). Vitamin K is a crucial coenzyme for the posttranslational γ-carboxylation of glutamic acid residues on the luminal side of the rough endoplasmic

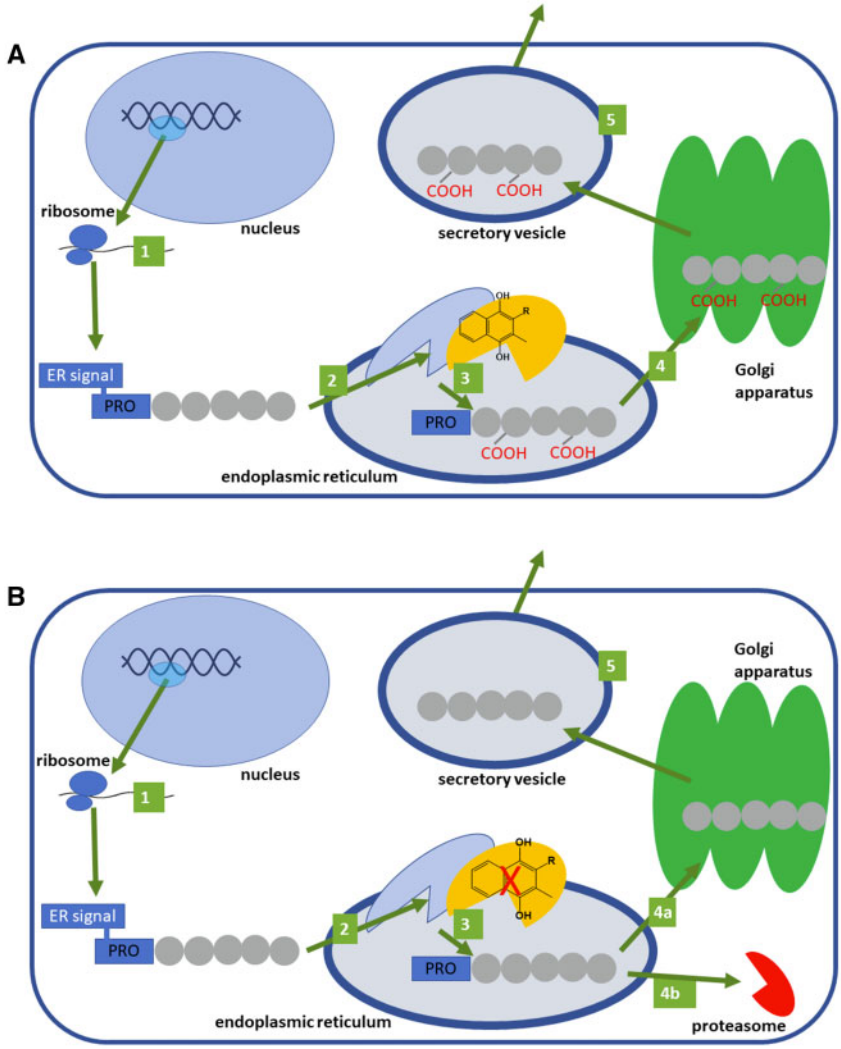

Figure 2 **The general fate of vitamin K–dependent proteins at the site of production. A**: Under normal conditions. **B**: Under vitamin K depletion. **1**: Preproprotein is synthesized from mRNA by ribosomes. **2**: Preproprotein is targeted to the endoplasmic reticulum (ER), where the ER signaling sequence is removed and the protein is processed by (vitamin K–dependent) γ-glutamyl carboxylase. **3**: Proprotein is carboxylated when vitamin K is present. **4**: Carboxylated or **4a**: uncarboxylated proprotein is transported to the Golgi apparatus, where the PRO-sequence is mostly cleaved (some proteins are also glycosylated there, not shown). **4b**: In some cases under vitamin K deficiency, the uncarboxylated protein is cleft by proteasome. **5**: Mature protein is extruded via a secretory vesicle into the extracellular space

reticulum (Fig. 3[75–78]). This process requires 2 enzymes [γ-glutamyl carboxylase (vitamin K–dependent carboxylase) and vitamin K epoxide reductase (vitamin K epoxide reductase complex subunit 1, VKORC1), which are likely located in close proximity to one another in the membrane of the endoplasmic reticulum[62,79–81]] as well as carbon dioxide and oxygen. Proteins that are undergoing γ-carboxylation contain a homologous sequence of about 18 amino acids long located immediately upstream of the carboxylated domain. This domain binds to the γ-glutamyl carboxylase and markedly facilitates the enzymatic reactions catalyzed by this enzyme. Interestingly, there are different affinities between the various vitamin K–dependent proproteins and the enzyme, which might explain some differences in their processing.[75,78,82] VKORC1 must first reduce vitamin K, which is found in food in its quinone form. The reduced form of vitamin K (hydroquinone) is modified by the carboxylase, by deprotonation, and by the subsequent incorporation of oxygen into the alkoxide. This form is a strong base that reacts with the targeted protein and forms a carbanion in the γ-position of glutamic acid. This enables carboxylation by γ-glutamyl carboxylase. The vitamin K epoxide must then be reduced again into quinone and subsequently hydroquinone by VKORC1, and the reaction continues.[75,76] Through this process, vitamin K is recycled, so the physiological requirements of vitamin K are relatively low.[83] Formed γ-carboxylated proteins are then likely transported via the classical secretory pathway through

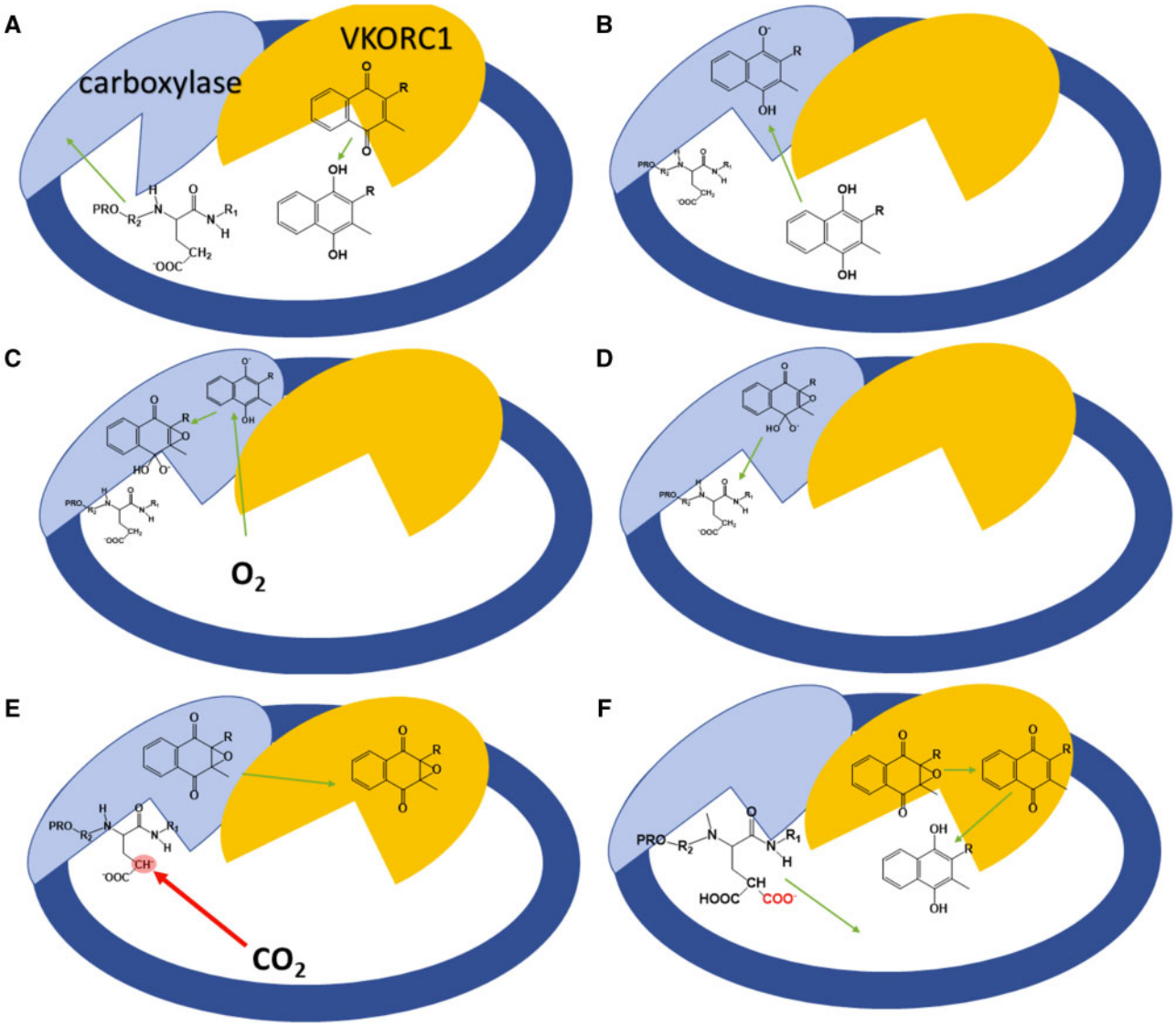

Figure 3 **Probable steps in the carboxylation process mediated by vitamin K in the endoplasmic reticulum**. A protein that contains a PRO-sequence is targeted and subsequently bound to the carboxylase in the first step (**A**). This binding markedly increases the enzymatic function of the carboxylase. The quinone form of vitamin K is reduced to hydroquinone by VKORC1. Hydroquinone is deprotonated by carboxylase in the next step (**B**). Oxygen reacts with deprotonated vitamin K hydroquinone to produce alkoxide (**C**). This strong base deprotonates the γ-carbon of glutamyl residue to form a carbanion, which reacts with carbon dioxide (**D–E**). At the same time, vitamin K epoxide is formed (**E**). γ-glutamyl carboxylation is accomplished and the formed protein is released from the enzyme and further transported to the Golgi apparatus (not shown), while vitamin K epoxide is converted first to vitamin K quinone and then to vitamin K hydroquinone (**F**) by VKORC1. Data for this figure were taken from Rishavy et al (2004),[75] Down et al (1995),[76] Ayombil et al (2020)[77] and Berkner (2000)[78]
Abbreviations: carboxylase, vitamin K–dependent γ-glutamyl carboxylase; VKORC1, vitamin K epoxide reductase

the Golgi apparatus, where the prosequence is mostly cleaved, as has been demonstrated for prothrombin by a Ca^{2+}-dependent serine proteinase.[84] There are, however, some differences: (1) matrix Gla-protein is exceptional, since the proprotein sequence is also maintained in the mature protein;[85] and (2) the impact of carboxylation on secretion is different (Factor II and Factor X can be secreted both in the uncarboxylated and carboxylated forms, while proteins C and Z are secreted only in the carboxylated forms).[86,87] In the case of protein C, the uncarboxylated form is decomposed by proteasome, which explains its inability to be excreted.[87] Although mutated γ-glutamic acid carboxylated proteins can be cleft intracellularly,[87] secreted γ-carboxyglutamated proteins cannot be reused and are excreted in urine.[61] As a result, they have attracted great interest as being potentially useful for diagnostic purposes, as will be discussed later in this article.

There are some differences in the carboxylation process related to the form of vitamin K. Vitamin K_3

(MK-0) is inactive and vitamin K_1 is less active than MK-4; with increasing numbers of isoprene units, from MK-4 to MK-10, the carboxylation activity decreases.[88] Also, MK-7 has been shown to be more active than vitamin K_1 in the recovery of vitamin K–dependent synthesis of coagulation factors.[23] In accordance with this, the best catalytic effects on carboxylation in rats were observed for MK-4 to MK-7. Another experiment showed MK-9 also to be active, but the lag time between administration and the effect was longer. Interestingly, vitamin K_3 is not significantly active, even after intravenous administration.[50] This information fits the reported trend in enzymatic vitamin K reduction, since vitamin K_3 was again here inactive. At a concentration of 10 μM, there was no difference between vitamin K_1 and MK-4 reduction, but at 100 μM, vitamin K_1 was a better substrate. MK-7 was much less reduced than either vitamin K_1 or MK-4.[89] Interestingly, there was little difference between the epoxide, hydroquinone, and quinone forms, suggesting that the reduction process is more rapid than carboxylation itself.[88]

In the absence of vitamin K, or with a blockade of VKORC-1 by coumarin anticoagulants, vitamin K quinone can be reduced to vitamin K hydroquinone by cytosolic NAD(P)H–dependent oxidoreductase (flavoprotein DT-diaphorase), but vitamin K epoxide is not a substrate for this enzyme, so vitamin K recycling is not enabled.[61] Moreover, at least in rats, DT-diaphorase has very low activity in the arterial wall compared with the liver.[90] This seems to explain why the effects of vitamin K anticoagulants on blood coagulation are easily reversed by the administration of vitamin K_1, which is rapidly taken up by the liver, whereas the extrahepatic inhibition of protein γ-carboxylation is not so easily reversed. Contrarily, MK-4 is able to normalize blood coagulation and reverse aortic calcification.[70] It should be mentioned that, in extrahepatic tissues, vitamin K epoxide reductase like 1 (VKORL1) can also be involved in vitamin K recovery; however, its importance under physiological conditions seems to be low.[2,81]

γ-carboxyglutamate proteins

Currently, there are at least 18 or 19 human physiological proteins and 1 pathological protein for which posttranslational maturation is enabled by vitamin K–dependent γ-glutamyl carboxylation[29,91] (Table 1). These proteins are known as Gla proteins (from γ-carboxyglutamic acid). They can be tentatively subclassified into a few categories according to their major (or more precisely their most well-known) effects. Many of these proteins have, however, more complex effects. There are 7 proteins involved in blood coagulation (coagulation factors II, VII, IX, and X and proteins C, S, and Z), 4 or 5 proteins primarily have effects on connective tissue mineralization or have a closely related role (4 well-documented Gla proteins: matrix Gla protein (MGP), osteocalcin, Gla-rich protein, and nephrocalcin; if the fifth, periostin, is a Gla protein is disputable), 4 are transmembrane receptors (proline-rich Gla proteins 1–4), 1 is a growth-factor-like signaling molecule, 1 binds to hyaluronic acid in the extracellular matrix, and the last is γ-glutamyl carboxylase itself.[92] Given the necessity of this enzyme for other Gla protein formation, it is not surprising that in contrast to other Gla proteins, it does not necessitate the proprotein activation of the enzyme. The individual vitamin K–dependent enzymes will be briefly characterized in Table 1 and the following text.

The first well-described proteins dependent on vitamin K were 7 players in the coagulation cascade, including (pro)coagulatory factors II (thrombin), VII, IX, and X, as well as anticoagulant proteins C, S, and Z.[93–97] Coagulation factors VII, IX, and X, and protein C have high structural homology both in gene and protein structure and organization.[94,97] In addition to γ-glutamyl carboxylation, they also undergo another specific posttranslational modification – the β-hydroxylation of aspartic acid or asparagine.[94] In general, we can classify them as proenzymes (the majority being zymogens) or co-factors (protein S and Z). Proenzymes act as serine proteases upon activation. In fact, they share several features with the digestive enzymes chymotrypsinogen/chymotrypsin and trypsinogen/trypsin. In contrast to these nonselective enzymes, the coagulation factors with enzymatic activity mentioned here have an additional polypeptide chain, which procures narrower substrate specificity.[94,97] All of these anticoagulation/coagulation factors, with the exception of protein S,[98] are synthesized mainly in the liver. The Gla content ranges between 9 and 13. Binding to calcium leads to modifications in these Gla proteins, and the resultant structural changes cause the exposure of the phospholipid-binding domain, which anchors them in membranes, exposing in particular phosphatidylserine.[61,94,97,99–101] This is apparently the crucial step, since in absence or inhibition of vitamin K regeneration, the coagulation process is inhibited. The roles of the vitamin K–dependent coagulation factors are shown in Fig. 4. Since protein Z is the newest member, it will be discussed here briefly. This anticoagulant protein shares similarities with other vitamin K–dependent proteins involved in the coagulation cascade, but lacks a functional serine protease activity.[100,102] Its complex with protein Z–dependent protease inhibitor (PZI) binds to factor Xa on the phospholipid surface and blocks it. It seems to be important during pregnancy, since low

Table 1 **Vitamin K–dependent Gla proteins and their available basic characteristics**

Protein	Year of discovery	Size of final protein	Number of Gla sequences	Function
Factor VII (proconvertin)	1951	50 kDa	10	Zymogen, procoagulant
Factor IX (Christmas factor)	1952	56 kDa	12	Zymogen, procoagulant
Factor X (Stuart–Prower factor)	1953	56 kDa	11	Zymogen, procoagulant
Gas6	1988 (murine fibroblasts)	75 kDa (678 AAs)	11	Likely a growth factor
γ-glutamyl carboxylase	1975	758 AA (94 kDa)		Integral membrane enzyme
Prothrombin	1894	72 kDa	10	Zymogen, procoagulant
Protein C (autoprothrombin II-a)	1960	56 kDa	9	Zymogen, anticoagulant
Protein S	1977	80 kDa (635 AAs)	11	Anticoagulant, cofactor of protein C, immune and vascular system regulation
Protein Z	1977 (bovine plasma), 1984 (human plasma)	62 kDa (360 AAs)	13	Anticoagulant, with PZI blocks factor Xa
Matrix Gla protein (MGP)	1983	11 kDa	5	Extracellular matrix protein
Nephrocalcin	1981	14 kDa (110 AAs)	2–3	Inhibitor of the formation of calcium renal stones
Osteocalcin (BGP, bone Gla protein)	1977	5.6 kDa (49 AAs)	3	Carboxylated – an extracellular matrix protein, uncarboxylated – a hormone
Periostin (osteoblastic-specific factor 2, OSF-2)	1993	90 kDa (836)	0 up to 24[b]	Extracellular matrix protein influencing the growth and repair of connective tissues
Proline-rich Gla proteins (PRGP1, PRGP2)	1997	PRGP1: 23 kDa (198 AAs) PRGP2: 17 KDa (153 AAs)	?	Transmembrane receptors likely involved in signaling pathways
Inter-alpha-trypsin inhibitor heavy chain H2 (ITIH2)	1988	72 kDa (648 AAs)	2	Heavy chain of inter-α-trypsin inhibitor
Transmembrane Gla proteins 3 and 4 (TGM3, TGM4)[a]	2000	TGM3: 23.7 kDa (212 AAs) TGM4: 19.9 kDa (177 AAs)	TGM3: 15 TGM4: 9	Transmembrane receptors
Gla-rich protein (GRP)	2008		15	Modulator of tissue calcification, anti-inflammatory activity
Transthyretin in Moyamoya disease	2008	27.5 kDa (dimer)	1	Unclear; not present in healthy patients

[a]Also known as PRGP3 and PRGP4.
[b]There is a report showing that periostin is not a Gla protein, and it is suggested that it can contain up 24 Gla residues (it has 28 glutamyl amino acid residues).
Abbreviations: AAs, amino acids ; PZI, Protein Z–dependent protease inhibitor

levels of protein Z are associated with several pregnancy complications.[103]

Another important γ-carboxyglutamic acid protein, osteocalcin (also known as BGP and bone Gla protein, Fig. 5), has been largely associated with its effect on bones. Its physiological role is apparently more complex and clearly not fully understood. Most research has been carried out in mice, which however, have 2 genes for osteocalcin, together with 1 osteocalcin-related gene. This is an important difference from rats and humans, which have only 1 osteocalcin gene. For this reason, caution should be taken when interpreting animal studies. Some, but not all, results have been confirmed in limited number of human studies.[104–109]

Osteocalcin is synthesized mainly in osteoblasts, with a lesser contribution from odontoblasts and hypertrophic chondrocytes. Three glutamic acid residues are γ-carboxylated,[104–106] and the signal sequence is removed by peptidases.[77] This carboxylated protein is secreted from the intracellular vesicles into the bone matrix. This form has, as is the case for coagulation factors, a high affinity for calcium ions, and hence it binds to these ions in hydroxyapatite. It should be mentioned that it represents about 15%–20% of the noncollagen proteins in the matrix, which renders it the most frequent noncollagen protein in bones.[108] It was once thought that carboxylated osteocalcin initiated hydroxyapatite formation, whereas the current data rather point out that

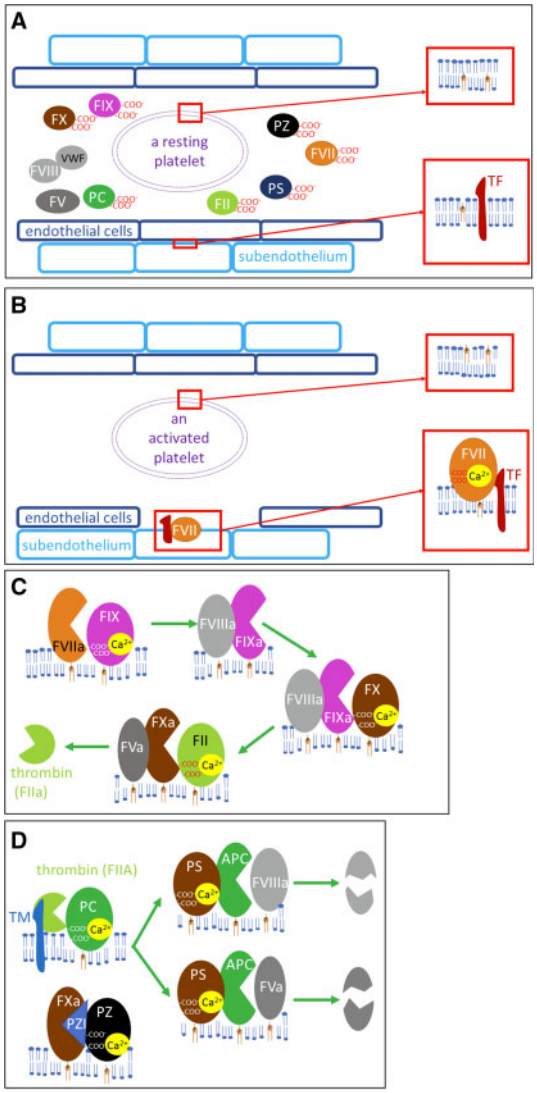

Figure 4 **Vitamin K (pro)coagulatory and anticoagulatory factors.** **A**: Resting state: coagulation factors are found in inactive forms in circulation, phosphatidylserine is not at the surface of the platelets (see the upper magnification), and tissue factor (TF) is not in direct contact with the blood (see the lower magnification). **B**: Activation of blood coagulation: in case of vascular damage, TF on subendothelial cells is now available for factor VII (FVII), which is activated. Platelet activation leads to modifications in the structure of the plasmatic membrane, with the exposure of phosphatidylserine on its surface. **C**: Activation of vitamin K–dependent coagulation factor: activated factor VII (FVIIa) cleaves factor IX (FIX) into an active enzyme (factor IXa, FIXa), which needs activated factor VIII (FVIIIa) for its activity. This complex activates factor X (FX) into an active enzyme (FXa), which needs activated factor V (FVa) for its activity. The whole FXa, FVa, calcium and phospholipid complex is also known as prothrombinase, and it activates thrombin (factor II, FII). Factor V (FV) or factor VIII (FVIII) are activated either by FXa or thrombin (not shown). **D**: Regulatory anticoagulant vitamin K–dependent factors: thrombin with thrombomodulin (TM) cleaves inactive plasma protein C (PC) into the active enzyme APC. For its enzymatic activity, APC also needs protein S (PS). PS does not require proteolysis in order to be active in PC-catalyzed lysis. This complex cleaves both FVa and FVIIIa. Protein Z (PZ) is a cofactor of PZ-dependent protease inhibitor (PZI), which blocks the enzymatic activity of FXa

it regulates the hydroxyapatite size and is crucial for the alignment of apatite crystals to collagen fibers. This ability is considered to be the reason for osteocalcin's effect on bone strength and its protective properties against bone fractures.[104,107] The data on other systemic effects of osteocalcin are less conclusive. Regardless, during bone resorption, the pH in bones decreases, which leads to osteocalcin decarboxylation and its release into the circulation.[105,106] Un(der)carboxylated osteocalcin is considered by most authors to be a hormone.[104,105,107,108] It has been shown, at least in animal studies, to affect glucose and lipid metabolism and to modulate fertility; it even has central nervous system effects, and could be involved in some types of cancer.[104–106,108] The largest data are on the cross-link between osteocalcin and insulin, and un(der)carboxylated osteocalcin has been shown to improve insulin sensitivity and glucose tolerance. It causes the release of insulin, mainly via incretin glucagon-like peptide-1. Insulin, on the other hand, stimulates bone resorption and promotes the decarboxylation of osteocalcin, enabling its systemic effects. However, these data have been only partly confirmed in human studies; apparently, various factors such as gender, concomitant disease, and age influence the final effect.[105–109] In addition, the information available on un(der)carboxylated osteocalcin receptors is not fully conclusive. Un(der)carboxylated osteocalcin has been shown to bind to GPRC6A, a G-protein-coupled receptor family C group 6-member A, in most tissues, and many of the above-mentioned effects are likely mediated by its binding to this receptor.[108] At least in mice, another receptor mediates its effects in the brain.[104,105] One candidate, Gpr158, has recently been suggested.[110] In addition, un(der)carboxylated osteocalcin is considered to be a marker of bone quality, since its levels correlate with bone mineral density and are increased in bone fractures.[111,112] Supplementation with vitamins K_1 or K_2 decreases the serum un(der)carboxylated osteocalcin level or the ratio of un(der)carbocylated to total osteocalcin[68,109,113–116]; hence, un(der)carboxylated osteocalcin is considered one of the markers of vitamin K deficiency.[74]

As carboxylated osteocalcin modulates extracellular matrix mineralization in bones, another protein that is dependent on vitamin K, MGP, blocks this process in other tissues where it would be pathological. In fact, it is synthesized in vascular smooth muscles and chondrocytes. It protects arteries and cartilage against mineralization.[85,117] Apparently, both carboxylated osteocalcin and activated MGP act at the local level, ie, at the site where they are produced.[85,118] In contrast to uncarboxylated osteocalcin, it is not known whether uncarboxylated MGP has any systemic effects. Moreover, MGP is not only carboxylated in 5 of 9 glutamate

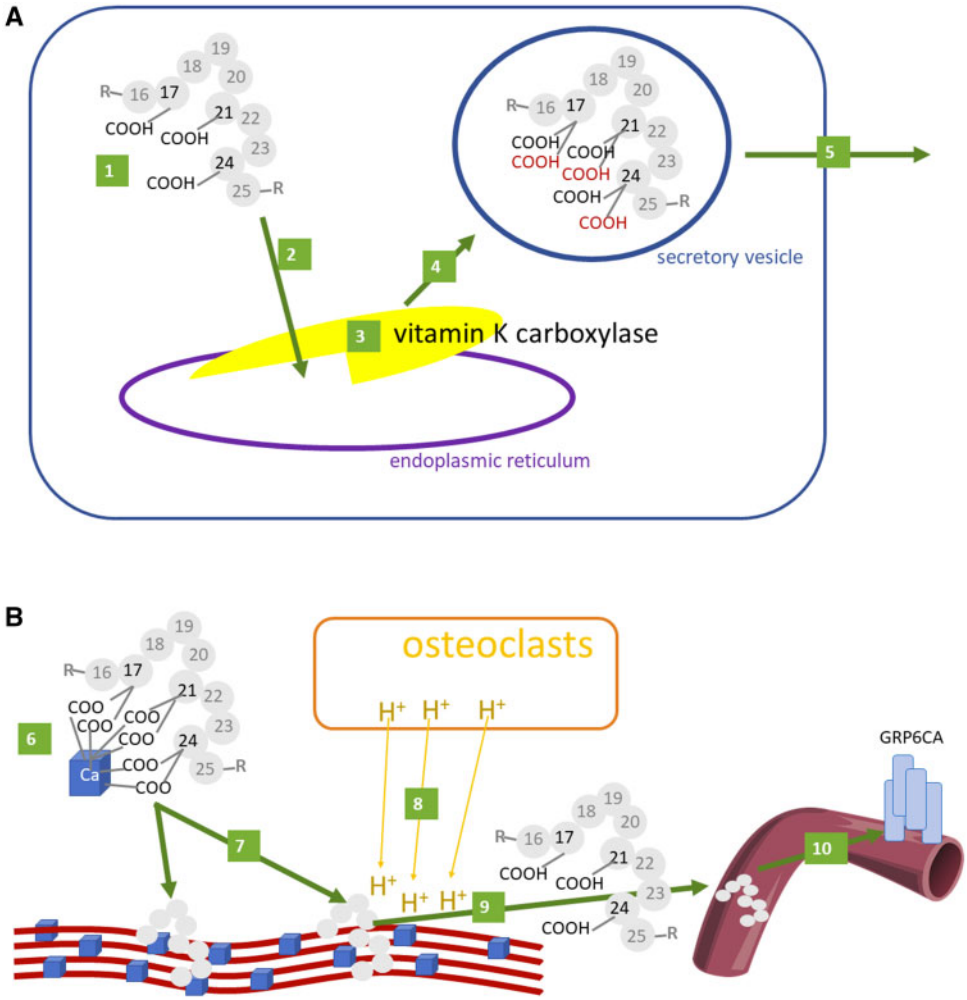

Figure 5 Osteocalcin. **A**: Synthesis and release from the osteoblast. **B**: Effect on bones and systemic release. Osteocalcin (**1**) is synthesized as preproprotein, then travels to the endoplasmic reticulum (**2**), where 3 glutamic acid residues at positions 17, 21 and 24 are γ-carboxylated by γ-glutamyl carboxylase (vitamin K carboxylase) (**3**). The carboxylated osteocalcin is further processed (eg, see Figure 4), transported in the vesicles (**4**), and released into the bone matrix (**5**), where it binds calcium ions in hydroxyapatite (**6**). This is a crucial step in bone formation – the correct alignment of collagen fibers with hydroxyapatite (**7**). When osteoresorption takes place, osteoclasts decrease the pH level in the bones (**8**); this can lead to the decarboxylation of osteocalcin to form un(der)carboxylated osteocalcin, which is released into the systemic circulation (**9**), where it could exert its hormonal effect, in particular by binding to the GRP6CA receptor (**10**)

residues, it also undergoes serine phosphorylation in 3 of 5 serine residues (Fig. S3 in the Supporting Information online[85]). Carboxylation is again carried out by γ-glutamyl carboxylase, while phosphorylation occurs by the Golgi casein kinase.[85,119] The function of phosphorylation is currently unknown, but it might be responsible for regulating trafficking. The inhibition of soft tissue calcification might be accomplished by its binding both to calcium and to bone morphogenic protein-2 (BMP-2).[85,117] Solid evidence of the importance of MGP as an inhibitor of soft tissue calcification can be derived from MGP-deficient animals, which generally die within 2 months due to the calcification and subsequent rupture of the thoracic or abdominal aorta.[120] Extensive arterial calcification is also observed in patients with Keutel syndrome, in which mutation of the *MGP* gene is present.[85,121] Since the MGP can be presented as uncarboxylated-unphosphorylated, or uncarboxylated-phosphorylated and vice versa, or carboxylated-phosphorylated, it has been tested for diagnostic purposes. The results are ambiguous, but high levels of uncarboxylated-unphosphorylated MGP is considered to be a marker of vitamin K deficiency. It decreases after administration of vitamin K, but is dependent on age .[68,83,85,91,117,122]

Periostin: Periostin is another suggested γ-carboxyglutamyl protein, or more precisely glycoprotein, with an effect on bones. It was initially discovered in a mouse osteoblastic cell line and was formerly named osteoblastic-specific factor 2 (OSF-2).[123] The current name derives from its presence in the periosteum of long bones. It is, however, widely expressed both in the fetus

and in adults, and its effects are clearly not limited to the bones. It plays an important role in the growth and repair of damaged connective tissues (eg, bones, teeth, skin, tendons, and valves).[124–127] It stimulates inflammatory reactions (eg, by the activation of NF-κB),[125,126,128] which is likely linked to the healing process. On the other hand, it is also associated with fibrosis.[125,127,128] On the molecular level, it is secreted into the extracellular matrix, where it can bind to several integrins and initiate signaling cascades associated with protein kinases.[124,126] Interestingly, the γ-carboxylation of the possible 28 glutamyl residues has not been widely investigated, and there is a report indicating that gamma-glutamyl residues were not detected in periostin, either in lungs from patients suffering from idiopathic fibrosis or in a cell culture.[129] It is, hence, not clear whether it is a Gla protein. Periostin has been suggested to be a marker of type II immune reactions in chronic inflammatory diseases such as asthma and atopic dermatitis, as well as a predictor of the effect of biologic drugs acting on this level. It is also thought to be a useful marker when making risk assessments of developing fractures and when investigating left ventricular function after myocardial infarction and chronic kidney disease.[124–127,130] Given the diversity of these processes, it cannot be considered a selective marker. Moreover, it is highly expressed in youth, most likely due to its impact on bone growth.[125] Contrarily, smoking decreases its expression.[126] There have been some attempts to develop drugs that influence periostin or its receptors.[125,127,131] Periostin has 4 isoforms designated 1–4, which differ in the presence/absence of particular exons, leading to varied (sometimes opposite) effects.[91] This can complicate the deciphering of its physiological function and its possible use as a therapeutic target.

Nephrocalcin: Nephrocalcin is another acidic glycoprotein containing a Gla sequence. It is produced in the human kidneys. Nephrocalcin has 4 similar forms that differ in Gla content. Normal form A contains 3 Gla residues, while B and C only contain 2, and D none. In particular, normal form A binds 4 atoms of calcium and has a high affinity to calcium oxalate. It inhibits calcium oxalate crystallization. Interestingly, uncarboxylated nephrocalcin has been isolated from the urine and even from the kidney stones of patients suffering from them. Increased nephrocalcin has been detected in the urine of patients with renal cell carcinoma.[132–136]

Gas6: Gas6, a product of growth arrest–specific gene 6, shares 43% homology with protein S and has the same protein organization.[137,138] In contrast to protein S, plasma Gas6 levels are very low, in subnanomolar concentrations, and it is not produced by the liver.[98,138] Its target is the so-called TMA, three tyrosine kinase receptors that gave name to this family—Tyro3 (Sky), MerTK (c-mer), and Axl. Gla residues bind to calcium ions, and this causes a structural modification, which likely enables the binding of Gas6/protein S to the TMA receptors. Protein S binds only to the former 2 receptors and contains a thrombin-sensitive cleavage site, in contrast to Gas6.[98,138] In fact, Gas6 is likely not involved in coagulation cascade-like protein S, but on the other hand it is involved in platelet aggregation.[139,140] The pathophysiological function of Gas6 is extensive.[138,139,141,142] It inhibits inflammatory reactions and plays a role as a growth factor, which may reflect its impact on cancer growth. Gas6 overexpression is observed in several cancers, and in general its expression predicts a poor prognosis.[142] In addition to the aforementioned receptor binding, the Gla residues (via calcium and phosphatidylserine) are attached to damaged tissues in line with its role as a growth factor.[138,139] It is also involved in tissue fibrosis.[141] Gas6 has also been suggested to affect vascular calcification. However, this has recently been refuted.[143] Since protein S binds to the same 2 receptors as Gas6, there may be an overlap between some of their functions, but decisive data on this are missing. The crucial involvement of protein S in blood coagulation is undisputable.[98] One good example is the clear risk of venous thromboembolism in humans with rare cases of protein S deficiency.[144] Interestingly, no other apparent important symptoms in these patients are described, which belies the other physiological role(s) of protein S. In any case, protein S is involved in immune and vascular system regulation. It should be emphasized that approximately 60% of protein S circulates with complement component C4b. It has been speculated that the C4b–protein S complex can bind via its Gla domain to the surface of certain membranes and in this way regulate the complement activity.[98]

Heavy chain 2 of inter-α-trypsin inhibitors: Two γ-glutamic acid carboxylated residues have also been discovered in heavy chain 2 of inter-α-trypsin inhibitors (gene *ITIH2*). The function of heavy chains of the inter-α-trypsin inhibitor family is interaction with hyaluronic acid, with a possible impact on maintaining the integrity of the extracellular matrix. Originally, the inter-α-trypsin inhibitor was designated a protein inhibitor. Its enzyme inhibition properties are rather weak, especially when compared with other physiological inhibitors. The structure is specific. It is composed of a common light chain (known as bikunin) to which 1 or 2 heavy chains are attached by a unique ester bond between the carboxyl group of the aspartic acid terminal residue and a C6-hydroxy group of the N-acetyl galactosamine residue of the chondroitin sulphate of the light chain. The 6 heavy chains that are currently known are structurally related. The function of the Gla residues of heavy chain

2 is unknown, since 3 other characterized heavy chains (1, 3, and 4) do not contain them .[145–149]

Proline-rich Gla proteins and transmembrane Gla proteins: Targeted amino acid sequence research resulted in the discovery of other Gla proteins. First, 2 proteins with specific proline-rich cytoplasmic regions were found. They were, hence, named proline-rich Gla proteins 1 and 2 (PRGP1 and PRGP2). They are widely distributed, with the highest expression of PRGP1 being in the spinal cord and of PRGP2 being in the thyroid gland.[150] PRGP1 expression is limited to some tissues, whereas PRGP2 expression is more extensive.[151] Further research showed that carboxylated PRGP2 is localized on the cell surface, and it was suggested that it has an intracellular binding partner YAP (Yes-associated protein).[152] Later, other structurally similar members of the vitamin K protein family were discovered. They were initially named transmembrane Gla proteins 3 and 4 (TMG3 and TMG4). They are also known as PRGP3 and PRGP4. In fact, TMG3 is similar to PRGP1 and TMG4 to PRGP2. Both TMG3 and TMG4 are widely expressed in both fetal and adult human tissue.[151]

Gla-rich protein: The last-discovered member of the vitamin K–dependent proteins is Gla-rich protein (GRP). In comparison with other Gla proteins, it contains the highest number of γ-glutamic acid moieties, which span over the entire protein. It was initially discovered in the calcified cartilage of Adriatic sturgeon. Its ortholog is found in many species, including humans.[153] In humans, GRP is widely expressed. Carboxylated GRP apparently acts as an inhibitor of tissue calcification, in particular of vascular and chondrocyte calcification, possibly by direct interaction with calcium.[154–158] Its anti-inflammatory properties have also been documented.[157] Given its similar function to osteocalcin and MGP, it is not surprising that it has a similar expression pattern, at least in some pathologies.[154,157] Higher GRP accumulation has been observed in calcified aortic valves and atheromatous regions. Interestingly, foam cells contain lower levels of carboxylated and higher levels of uncarboxylated GRP.[154] Similarly, higher *GRP* gene expression but only uncarboxylated GRP were detected in fibroblast-like synoviocytes from osteoarthritic patients. At the same time, lower levels of γ-glutamyl carboxylase and VKOR1 were found in these samples.[157]

Transthyretin: There is one other Gla protein that is contrarily considered to be pathological. Physiologically, transthyretin, a protein involved in the transport of the thyroid hormone thyroxine and retinol, is not a Gla protein. But in rare cerebral steno-occlusive Moyamoya disease, its γ-glutamic acid carboxylated counterpart was observed in cerebrospinal fluid. It has been observed in 71% of patients suffering from this disease.[159–161]

Some researchers have suggested that vitamin K might play an additional role(s) in the human body, such as directly binding to intracellular receptors, interfering with enzymes, or having antioxidant and/or anti-inflammatory activities.[2,44,162]

VITAMIN K DEFICIENCY

The symptoms of vitamin K deficiency are spontaneous cutaneous purpura, epistaxis, gastrointestinal, genitourinary, gingival, or other bleeding. Although vitamin K deficiency is not common in adults, it can occur for several reasons: (a) poor vitamin K dietary content, (b) many pathological states (eg, liver disease, cholestasis, cystic fibrosis, alcoholism, malabsorption states (including inflammatory bowel disease), and bariatric surgical intervention), and (c) pharmacotherapy with several drugs (mainly coumarin-based anticoagulants, but also the drugs previously mentioned that interfere with the absorption, metabolism, and synthesis of vitamin K, such as rifampicin and antibiotics).[2,29,54,111,163,164] In new-born babies, there is also a risk of hypovitaminosis, since (a) they have poor vitamin K stores, (b) vitamin K levels are low in breast milk, and (c) immature GIT microflora is likely not a source of the vitamin. In line with these findings, exclusively breast-fed infants are more susceptible to the development of vitamin K hypovitaminosis than formula-fed children.[31,165,166]

DETECTION OF VITAMIN K AND OTHER MARKERS OF VITAMIN K STATUS

Making an accurate bioanalysis of vitamin K deficiency is not an easy task. Because it is among the most lipophilic and least abundant fat-soluble vitamins, it is extremely difficult to determine in human biological samples, even when using the most modern instrumentation. It can be measured directly in plasma, but there are many forms of vitamin K, and an abnormal lipid profile may also affect the result.[74]

Chromatographic methods have become dominant for directly measuring vitamin K. An example of such analysis is shown in Fig. S4 in the Supporting Information online.[167] Unfortunately, the complex preparation of the samples also relates to the detection methods, such as mass spectrometry (MS), which has become the detection method of choice in vitamin K determination. There are many methods used for measuring systemic vitamin K levels.[167–187] Some of them are briefly characterized in Table 2.[167–187] Vitamin K deficiency will lead to a state in which Gla proteins cannot be carboxylated and are hence mostly released in

Table 2 **Advantages and disadvantages of various analytical techniques for the determination of vitamin K in human biological matrices**

Technique	Sensitivity (nM if not specified otherwise)	Advantages	Disadvantages	References
LC-PDA/UV	K_1: 67–89[a] MK-4: 742[a]	Commonly available technique	Complicated sample treatment Low sensitivity	Zhang et al (2019)[169]; Chatzimichalakis et al (2004)[168]
LC-FLD	K_1: 0.07[b]–0.22[a] MK-4: 0.09[b]–0.45[a] MK-7: 0.05[b]–0.26[a]	Commonly available technique Sensitivity	Pre- or post-column reduction Complicated sample treatment	Ahmed et al (2015)[172]; Kishikawa and Kuroda (2014)[170]; Klapkova et al (2018)[171]; Zhang et al (2019)[169]
LC-EC	K_1: 0.11[a] MK-4: 0.22[a] MK-7: 0.16[a] Metabolites in urine: 3.5 fM[a]	Low limits of detection	Long analysis times (up to 80 min) Complicated sample treatment	Zhang et al (2019)[169]; Harrington et al (2005)[173]; Kamao et al (2005)[174]
LC-CL	K_1: 0.07[a] MK-4: 0.09[a] MK-7: 0.16[a] K_2: 6.4[a]	Wide working range Sensitivity	Reaction with CL reagents Long analysis times (up to 50 min) Complicated sample treatment	Ahmed et al (2011)[177]; Zhang et al (2019)[169]
LC-MS	K_1: 0.07[c]–0.44 or 1 pmol/g[b] MK-4: 0.07[c]–0.47 MK-7: 0.05[c]–1.55 MK4-13: 1–30 pmol/g[b]	Selectivity Sensitivity	Financial investment Highly skilled personnel Matrix effects Complicated sample treatment	Gentili et al (2014)[176]; Dunovska et al (2019)[179]; Abro et al (2014)[183]; Kamao et al (2007)[175]; Karl et al (2014)[167]; Levêque et al (2019)[182]; Hu et al (2018)[180]
SFC-MS	K_1: 0.22[a,b]	Short analysis time (4 min) Selectivity	Matrix effects Not commonly used Complicated sample treatment	Górská (2019)[184]; Sandvik et al (2017)[178]
Immunochemical assays	K_1: 0.11–44[a,b] K_2: 0.56–17.98[a,b]	Commonly available technique Low operational cost Small amount of sample and solvents	Cross-reactions Impossible to differentiate individual forms of vitamin K_2	MyBioSource[185–187]
Electrochemical sensors	K_2: 9 pg/mL[a] (0.02)[d]	Simplicity, cost effectiveness, reproducibility, easy handling, miniaturization and high sensitivity	Not commonly used Application till now only in microbiological, food and pharmaceutical samples Impossible to differentiate individual forms of vitamin K_2	Jedlinska et al (2018)[181]

[a]LOD (limit of detection).
[b]LOQ (limit of quantification).
[c]LLOQ (lower limit of quantification).
[d]Recalculated for MK-4.
Abbreviations:K_1, Vitamin K_1 (phylloquinone); K_2, Vitamin K_2 (either the methodology does not allow differentiation between individual K_2 forms or not specified); LC-PDA/UV, liquid chromatography with photodiode/ultraviolet detection; LC-FLD, liquid chromatography with fluorescence detection; LC-EC, liquid chromatography with electrochemical detection; LC-CL, liquid chromatography with chemiluminescence detection; CL, chemiluminescence; LC-MS, liquid chromatography with mass spectrometry detection; SFC-MS, supercritical fluid chromatography with mass spectrometry detection

their uncarboxylated form.[61] Uncarboxylated vitamin K proteins can thus be alternative targets for quantitation, but these assays are not yet standardized, and some suffer from important drawbacks [74,163] or there are other aspects to consider. These proteins were formerly known as PIVKA (proteins induced by vitamin K absence). However, not all uncarboxylated proteins are secreted.[86,87] Furthermore, elevated expression of proteins can overwhelm the intrinsic carboxylation system and lead to the production of uncarboxylated proteins, as was documented with PRGP2.[152] Additionally, in contrast to coagulation Gla proteins, other Gla proteins

are apparently not fully carboxylated, even in healthy organisms.[28,109,156] The time aspect should also be considered, since some coagulation factors have shorter or longer half-lives.[188] PIVKA II (des-carboxyprothrombin) is an earlier-known marker of vitamin K status with low sensitivity.[68] It is (1) increased in many infants, and (2) its normalization takes months. In adults, it is clearly diagnostically better than prothrombin time or partial thromboplastin time, which are also affected by liver diseases, hematological diseases, and some other diseases, and their sensitivity in mild vitamin K deficiency is also low.[31,74,163] Factor VII has also been tested, due to its short half-life, but it is not a sensitive marker.[31] Another tested marker of poor vitamin K content in the body, and in particular in the bones, is uncarboxylated osteocalcin. The levels of total osteocalcin vary, so the percentage of uncarboxylated osteocalcin seems to be a more suitable marker. However, the available data do not fully support this, and estrogen levels might influence it.[31,163] Uncarboxylated-unphosphorylated MGP as a marker of vitamin K deficiency was mentioned above. A sensitive marker of vitamin K_1 dietary depletion is the urinary concentration of Gla residues. However, data on vitamin K_2 deficiency in relation to this marker are not available, no parameter cut-off for vitamin K deficiency has yet been established, and the measurement usually requires 24 h urinary collection.[31,68,74] Moreover, supplementation of vitamin K did not modify urinary Gla residues, in contrast to the clear increment in the carboxylated:total osteocalcin ratio.[64] Another possibility is the measurement of urinary menadione (vitamin K_3) excretion, which also changes in response to vitamin K depletion or repletion.[68]

THE PROPHYLACTIC AND THERAPEUTIC POTENTIAL OF VITAMIN K

Approved indications of vitamin K administration are not extensive. Vitamin K is indicated for the prevention of hemorrhagic disease in newborns or as an antidote to correct vitamin K antagonist overshooting or poisoning, since vitamin K antagonists are also used as rodenticides. Oral administration is preferred in most cases. Prophylaxis with vitamin K markedly decreased the vitamin K deficiency in new-born babies due to their above-mentioned poor vitamin K stores.[31,165,166] It has been suggested that preterm infants have an even greater risk of vitamin K deficiency, but there are currently insufficient data in relation to the prevention of this deficiency.[189] Importantly, the effect of vitamin K administration on coagulation normalization is very rapid.[166]

Analyzing the 18 or 19 Gla proteins formed by presence of vitamin K, it is apparent that the effects of vitamin K are not limited to blood coagulation. First, their effects on bone and vascular calcification are becoming more apparent. It is, hence, not surprising that a large amount of research has been dedicated to investigating whether increased intake and/or supplementation with vitamin K can positively affect osteoporosis, fractures, and cardiovascular diseases.[29,31,163,165] An effect on cancer has also been suggested, and there are reports on a possible relationship between vitamin K and cognitive function, highlighting its role in brain physiology.[190]

There are solid background data justifying testing of the possible use of vitamin K in the treatment of osteoporosis and the prevention of bone fractures: (1) there is the above-described positive effect of osteocalcin on bones, (2) a number of studies have reported that low serum vitamin K_1 levels or low vitamin K_1 intake and high un(der)carboxylated osteocalcin levels are associated with an increased risk of fractures, (3) the pharmacological inhibition of vitamin K reduction due to binding to VKORC1 by coumarin anticoagulants such as warfarin may affect bone quality, (4) similarly, the absence of VKORC1 is associated with calcification abnormalities, and (5) vitamin K was a successful treatment in many animal studies (eg, in the tail-suspension rat model of bone volume loss, or in a sciatic neurectomized rat model of osteoporosis).[2,111,163,191–195] Most epidemiological as well as clinical studies have demonstrated that the intake of vitamin K can have a positive effect on the bones. On the other hand, this was not found in all studies. Additionally, 2 of the positive effect trials were retracted , so the real effect of vitamin K on osteoporosis remains rather elusive.[196] A meta-analysis of 4 prospective cohort studies and 1 nested control study, including a total of almost 81 000 subjects, suggested that the population with the highest intake of dietary vitamin K_1 had a 22% lower risk of fractures. A subgroup analysis documented that the effect was observed after 10 years. The authors also observed that an increase of daily vitamin K_1 intake by 50 µg was associated with a 3% lower risk of fracture.[197] There are also meta-analyses reporting the effect of vitamin K supplementation. The first meta-analysis of 17 studies showed that vitamin supplementation had a positive effect on bone mineral density in the lumbar spine, but not at the femoral neck. Moreover, the net effect on the lumbar spine was questioned by the authors, since it was observed only in Asian and not in Western studies. It was also limited to women and to vitamin K_2.[198] The latter is not so surprising, given the observed differences in the pharmacokinetics and pharmacodynamics between these forms. The authors speculated that a greater effect might be observed in secondary bone loss caused,

eg, by pharmacotherapy with glucocorticoids.[198] Additionally, in another meta-analysis, it was shown that the effect of vitamin K_2 is observed mainly in postmenopausal women with osteoporosis.[113] This investigation of 19 randomized controlled trials with 6759 participants reported the improvement of lumbar and forearm bone mineral density, in particular, after long-term use by osteoporotic women. There was no significant improvement in non-osteoporotic women. The doses in the analyzed studies were mostly 45–90 mg/day of MK-4 or 100–180 µg/day of MK-7 . The overall effect on fractures was numerically high (a decrease in risk by 36%), but not significant. However, a significant decrease of 53% was reported after rejecting 1 study that induced heterogeneity. Interestingly, an older meta-analysis, which included mainly trials with MK-4, showed a massive and even more potent reduction of risk of hip, vertebral, and nonvertebral fractures.[199] This analysis was, however, apparently influenced by 2 retracted trials.[196] Also, the newest meta-analyses did not clarify the issue, since when clinical trials with different vitamin K forms were assessed, vitamin K supplementation decreased the risk of clinical fractures but not vertebral fractures, although, when assessing the effect of randomized studies with solely MK-4, there was no significant effect on any fracture incidence. Both meta-analyses, however, confirmed a significant effect of vitamin K supplementation on lumbar bone mineral density.[116,200] The net effect could also have been influenced by different concomitantly used drugs and comparators (eg, vitamin D, bisphosphonates). Summing up the available information, some positive effect of vitamin K on osteoporosis and decreased frequency of bone fractures seems to be possible, but apparently a long period of treatment is needed to achieve this effect. In addition, vitamin K may not be able to stop a reduction in bone mineral density or content in some parts of the skeleton, but may slow down its progression. Again, this effect may require long-term treatment. The effect of MK-7 supplementation on femoral bone is one example. It was not present after 1 or 2 years of treatment; 3 years were needed to document its positive influence.[115] Based on this information, long-term supplementation with vitamin K in postmenopausal women with osteoporosis might have some potential utility, in particular, given its negligible risk of serious side effects. This is also supported by the fact that MK-4 in relatively high doses is registered in Japan for postmenopausal osteoporosis.[61,111]

The main reason for speculating that vitamin K has positive cardiovascular effects stems from its involvement in the synthesis of MGP. As has been described here in detail, MGP blocks the calcification of arteries. It need not be emphasized that the calcification of arteries is an independent risk factor for the development of coronary heart disease and coronary events.[201,202] In addition, several clinical trials have reported that vitamin K antagonists promote atherosclerotic calcification.[29] There has been a lot of discussion about whether there is subclinical hypovitaminosis in certain population groups. A substantial under-γ-carboxylation of vitamin K–dependent proteins has been found in postmenopausal women,[64,114] and about one-quarter of a cohort ranging from 42 years to 89 years had very low plasma vitamin K_1 levels.[66] If this theory is correct, then vitamin K supplementation should reduce the risk of arterial calcification and coronary artery disease. Epidemiological studies from the Netherlands have found that vitamin K_2, but not vitamin K_1, limits coronary calcification and decreases the incidence of and mortality due to coronary artery disease.[10,11,203] Also, a very recent Norwegian study has found that higher intake of vitamin K_2 decreased the incidence of coronary artery disease, but vitamin K_1 had no effect, notwithstanding the fact that higher quartiles of vitamin K_1 intake, compared with vitamin K_2 intake, were associated with higher physical activity.[204] The lack of effectiveness of vitamin K_1 was confirmed by the extension of the Dutch EPIC study with a median follow-up of 16.8 years. The same study, however, also reported only a tendency of vitamin K_2, particularly its long chain forms, to reduce cardiovascular mortality.[205] Considering the differences between the pharmacokinetic and pharmacodynamic data on vitamins K_1 and K_2 encompassing: the forms of vitamin K_2 having longer half-lives, a more potent effect, and higher concentrations in extrahepatic tissues, a stronger effect of vitamin K_2 over vitamin K_1 would be expected. On the other hand, a similarly designed Spanish epidemiological study found a different outcome. Both forms of vitamin K decreased cardiovascular mortality, but the decreases were insignificant. However, an increased intake of vitamin K_1, but not vitamin K_2, during the study significantly attenuated cardiovascular mortality.[206] Explaining this difference is not easy. One should emphasize that the intake of vitamin K_1 was apparently much higher in the Spanish study, which is quite logical, given the difference in vegetable consumption between these two countries. Although the populations were different, this was likely not the reason: on the one hand, a higher consumption of vitamin K_1 was linked with a healthier lifestyle in the Spanish study, but in the Dutch EPIC study, it was linked with hypertension, hypercholesterolemia, and diabetes. On the other hand, higher vitamin K_2 consumers in the Rotterdam Study had a rather healthier lifestyle. There is also an American epidemiological study in which the average consumption of vitamin K_1 was even lower. This study reported that vitamin K_1 decreased the incidence of

coronary heart disease, but had no effect on mortality due to this disease or on the risk of stroke.[39] Interestingly, the authors themselves reported that a positive impact was seen in connection with dietary patterns associated with a high phylloquinone diet. Indeed, higher intake of vitamin K_1 generally reflects healthier diets and lifestyles.[68]

Another study found that patients suffering from chronic kidney disease with inadequate total vitamin K intake ($K_1 + K_2$) had higher cardiovascular and all-cause mortality than those with adequate intake.[40] A recent meta-analysis of 21 observational studies found that higher intake of either vitamin K_1 or vitamin K_2 was associated with a decrease in incidence of coronary artery diseases, with the impact of vitamin K_2 being numerically higher. Regardless, neither form of the vitamin impacted the cardiovascular fatalities.[207] Another meta-analysis confirmed that neither form was found to have an impact on cardiovascular mortality.[208] It should also be emphasized that all of these studies were epidemiological studies using questionnaires to assess dietary habits with its known limits[38,68]; for a definite conclusion on the effect of vitamin K on cardiovascular diseases, prospective clinical studies are needed. The current clinical studies have not brought decisive data: supplementation with vitamin K_1 slightly reduced coronary artery calcification in older persons, while supplementation with MK-7 did not influence femoral arterial calcification in diabetic patients.[67,209] A meta-analysis of 3 controlled trials also found that vitamin K supplementation decreased vascular calcification. It should, however, be mentioned that this effect was largely driven by one single study.[122] The most recent systematic review was not conclusive about the possible impact of vitamin K on calcification of major vessels.[210] On the other hand, higher levels of uncarboxylated dephosphorylated MGP, as the above-mentioned marker of vitamin K deficiency, were found to be associated with higher incidence of coronary artery disease and even cardiovascular mortality.[207] This was, however, not fully confirmed in another meta-analysis.[122] It should also be mentioned that higher levels of uncarboxylated dephosphorylated MGP can be a consequence of administration of vitamin K antagonists and can, therefore, simply reflect that these patients are more ill.[207,211,212] Hence, positive cardiovascular effects are not clearly documented for either form of vitamin K, and clinical long-term studies are definitely needed. Based on their negligible toxicity, vitamin K_1 and/or vitamin K_2 might be potentially administered to patients suffering from coronary artery disease in cases where there is an assumption of low vitamin K levels. They should not, however, be treated with vitamin K anticoagulants.[28]

There are additional data concerning the effects of vitamin K on all-cause mortality, cancer, and diabetes. Higher levels of uncarboxylated dephosphorylated MGP are associated with all-cause mortality, and this supports the possible impact of vitamin K.[207,208,212] The Spanish PREDIMED epidemiological study found that a higher intake of vitamin K_1 or vitamin K_2 decreased both all-cause mortality and cancer mortality.[206] The effect of vitamin K_1 on all-cause mortality was contrarily not observed in the most recent study data obtained from the extension of the Dutch EPIC study, which also reported a positive influence of long-chain vitamin K_2 forms only, and then only in 1 of 3 models.[205] Also, a recent meta-analysis did not find an impact from either form of vitamin K on all-cause mortality.[208] The EPIC-Heidelberg study did not find any relationship between vitamin K_1 and cancer incidence or mortality, but it did document that vitamin K_2 decreased mortality due to cancer. This finding was likely driven by the effect of vitamin K_2 on lung cancer. Vitamin K_2 also decreased the risk of developing lung and prostate cancer, but had no effect on colorectal or breast cancer.[33] The existing literature has some data on the anticancer effects of vitamin K. Most data relate to synthetic vitamin K_3, which (on a molecular basis) is apparently a much more active anticancer drug than either vitamin K_1 or vitamin K_2. Vitamin K_3 can redox-cycle, and this represents the major difference from the natural forms of vitamin K, which have a side chain blocking this pro-oxidation mechanism. Interestingly, although the natural forms of vitamin K are less active, a few clinical phase I/II studies have reported some promising data for both.[213]

Higher vitamin K_2 has been suggested to slightly decrease the risk of developing diabetes mellitus type II, while vitamin K_1 had only a tendency in this respect. Vitamin K_2 also very slightly increased HDL cholesterol and decreased systemic inflammation.[34] Since the scientific evidence is limited in such cases, no recommendations can be given at the moment.

An emerging issue is the impact of vitamin K on age-related diseases. Low vitamin K levels are suggested to be associated with functional decline, osteoarthrosis, sarcopenia, mobility limitation and disability, and frailty in older persons. These conditions might be at least partly related to low-grade inflammation and pathological calcification. Both of these processes can be related. Vitamin K can, hence, bring some benefit when they occur. The mechanism has not been elucidated, but it might be associated with inhibition of soft tissue mineralization, anti-inflammatory effects, and impact on the mitochondria.[29,44]

Very recent research on vitamin K has brought a surprising discovery: vitamin K_2 (form not specified) was found in a docking study to be the best ligand for

free fatty acid binding pockets in the SARS-CoV-2 spike protein, and hence could potentially impact its binding to human cells via the ACE2 receptor.[214]

TOXICITY OF VITAMIN K

There have been almost no reported cases of natural vitamin K systemic toxicity, either in animals or in humans. There has been some concern that a high intake of vitamin K could result in overcoagulation. However, this has not been observed, possibly due to the limited sites for γ-carboxylation. Contrarily, very high doses of vitamin K can paradoxically cause hypoprothrombinemia, as has been documented in rare human case reports. In animals, massive doses have led to hemorrhages and anemia.[215] Available data in humans show that 10 mg/day of vitamin K_1 given for 1 month is not associated with any adverse effects. This is in line with the findings from animal experiments, in which even 2 g/kg for the same period of time was safe.[31] In general, the reported side effects of vitamin K have only been local, eg, minor gastrointestinal complaints and skin rashes after vitamin K_2, which disappear after discontinuing administration.[9,28,113,116] Clinically, intramuscular administration of vitamin K_1 is not very convenient due to pain at the site of injection and skin bruising. Therefore, more modern approaches such as microneedles are being examined.[29] It should be mentioned, however, that vitamin K_3 in high doses can lead, in a dose-dependent manner, to a toxic reaction, including hemolytic anemia, particularly in new-born infants. This reaction is likely associated with the redox-cycling of this vitamin, which does not occur in natural vitamins K_1 and K_2 due to the absence of unsubstituted position 3, which is highly reactive and binds thiol groups to form thioethers.[2,216] Furthermore, preparations of vitamin K_1 available for intravenous administration are sometimes associated with anaphylactoid reactions. Because of the lipid solubility of vitamin K, the preparations are aqueous colloidal suspensions that can induce anaphylactoid reactions. However, liposomal preparations can avoid this adverse reaction.[217]

CONCLUSIONS

Vitamin K has been traditionally linked with coagulation. Although this connection is correct, vitamin K has many other roles in human physiology. Vitamin K is necessary for the posttranslational modification of 18 or 19 proteins, among which 11 or 12 are not related to coagulation. In particular, its involvement in connective tissue calcification has stimulated intense research, although the data are still inconclusive. Vitamin K is not a single compound. Indeed, the diversity and the varied biological properties of the various forms of vitamin K can potentially explain the failure of research to date to clearly establish the role vitamin K plays in human beings. Apparently, additional (and particularly prospective clinical) studies are needed in order to clarify its non–coagulation-related effects in humans. Regardless, supplementation with natural forms of vitamin K is largely safe and hence is acceptable under specific conditions, such as in postmenopausal osteoporosis.

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SUPPORTING INFORMATION

The following Supporting Information is available through the online version of this article at the publisher's website.

Figure S1 **Chemical structures of vitamin K**
Figure S2 **Vitamin K content in selected food**
Figure S3 **Structure of matrix Gla protein (MGP)**
Figure S4 **An HPLC analysis of vitamin K standards and a spiked human fecal sample**

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