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REVIEW

Vitamin K and bone metabolism: the myth and the truth

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ABSTRACT

Several natural/syntheticproducts, containing different metabolites of vitamin K, are freely available and their use is suggested for non-specific 'bone health'. It is therefore necessary and useful to address this specific aspect of their use, describing the state of the art in basic, translational, and clinical studies, reported in the literature. In fact, we do not have any certainty about a real effectiveness of vitamin K in preventing fragility fractures. To date, published clinical trials have been conducted on different ethnicities, with different dietary habits, different supplementations and doses of vitamin K, and with different fracture risk factors. This review aims to provide basic cultural tools to improve the knowledge of readers concerning the use of vitamin K for bone health, helping them to develop an independent critical spirit.

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Vitamin K and bone: a relatively recent history

Under the term vitamin K (VK), it is represented a group of structurally similar, fat-soluble vitamins identified less than 100 years ago.

VK has been discovered in 1935 by the Danish biochemist Henrik Dam, who observed that the chicks nourished with sterols free, low-fat containing, diet developed major subcutaneous and intramuscular bleeding [1]; hence, the name of antihemorrhagic 'vitamin K' (K for 'Koagulation') [2]. He never could think that such a discovery would have, in the future, a potential impact also on human bone health.

Lately, the existence and the importance of VK have been confirmed [3,4]. This review aims to treat the possible and hypothetical pathophysiological roles that VK molecules may have in bone health and metabolism, with the purposes of trying to trace, with intellectual honesty, a distinction between what it has been already assessed from what it is still to be better defined, or known, in this complex field, before translating in the clinical use the prescription of VK for bone health, without the awareness of how this is really truth.

It is not the aim of these authors to go into the deep of such a difficult issue, but the real intention consists of eliciting interests, in particular in not skilled readers, on one or more aspects on the relationship between VK and bone, so that each one may have the opportunity to realize which could be the issue that best fits to his/her own need.

A link between VK and bone metabolism

In 1974, the role of VK in the process of carboxylation of prothrombin residues was identified, opening the possibility that VK could have a pathophysiological role also in metabolic pathways other than the blood clotting, in particular the bone metabolism.

In fact, the identification of amino acid-carboxyl-glutamic acid (Gla) in the prothrombin as the product of action of VK [5] unequivocally showed that VK acts as a cofactor for posttranslational carboxylation of the Gla residues [6].

Currently, several bone proteins have been demonstrated to host Gla residues that represent Ca^{2+} binding sites. Among them, we mention at least six VK-dependent bone proteins, described in Table 1.

The pathophysiology of VK

Different forms of VK have been described in nature, representing unique fat-soluble vitamins with specific function of coenzyme (posttranslational carboxylation): VK1, VK2, and VK3 (Figure 1). They are structurally related compounds, essentially differentiating in the number of repetition of isoprene units.

VK1 or phylloquinone or phytonadione

It is present in the chloroplasts in plants/vegetables, found in highest amounts in green leafy vegetables, and directly involved in photosynthesis. Animals may also convert it to VK2.

VK2 or menaquinones

They are produced at the intestinal level by bacterial synthesis and reported as MK-*n*, where *n* indicates the number of repetitions of 5 units of carbon (synthesized by the bacteria) (Figure 1).

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Table 1. VK-dependent bone proteins known up to date.

- Osteocalcin (or bone Gla protein, BGP)
- Matrix Gla protein (MGP)
- Protein-S
- Gla-rich protein (GRP)
- Periostin
- Periostin-like factor (PLF)

Principal alimentary MK-*n*s are MK-4 (menatetrenone) and MK-10, mostly in foods containing fat (e.g. fermented cheese) that will improve absorption and bioavailability versus VK1.

It has been suggested that MK-4 is produced by endogenous conversion of food VK1, MK-7, 8, and 9 [7]. Most of the production of MK-*n* takes place in the colon (see below) where the bile salts are deficient.

VK3 or menadione

It represents a provitamin molecule and is a synthetic analog, sometimes used, but not in economically developed countries for its potential toxicity, as a nutritional supplement because of its VK activity.

All the above cited various forms of VK differ for plasma half-life ($T^{1/2}$) and bioavailability, with K2/MK-7 having higher $T^{1/2}$ and bioavailability when compared to phylloquinone (VK1) and VK2/MK-4 [8].

VK metabolism: from source to destination

Differently from other fat-soluble vitamins, dietary VK is rapidly lost to the body resulting in comparatively low tissue stores. Deficiency is made difficult by the ubiquity of VK in the diet, synthesis by gut microflora in some species (see below), and relatively low VK cofactor requirements for γ-glutamyl carboxylation.

Alimentary VK is absorbed in the small intestine, through bile salts action. No specific transport proteins are currently known for VK and after intestinal absorption, VK1 and VK2, with the exception of VK3 menadione, are transported in plasma by triglycerides-rich lipoproteins (chylomicrons) from lymphatic circulation to the liver and other tissues, such as the blood and bone.

Relatively to bone tissue, few studies focused on the mechanisms of cellular uptake of VK, with the only direct investigations being carried out on cultured bone cells [9–11]. Newman et al. [9] showed the dependence for the human osteoblastic uptake of VK1 on heparan sulfate proteoglycans on the cell surface and apoE in lipoprotein particles.

Absorption

VK1 absorption from food, mainly leafy greens, is extremely low. Only 10% is absorbed in our body, while VK2 absorption from food, mainly cheese, is almost complete and is higher than VK1.

Excretion

The excretion occurs mainly with bile and less with the urine [12].

Natural VK1 and VK2 in foods and their advised daily intake

Table 2 summarizes the main alimentary sources for VK1 and VK2 and their related quantity for 100 g of food.

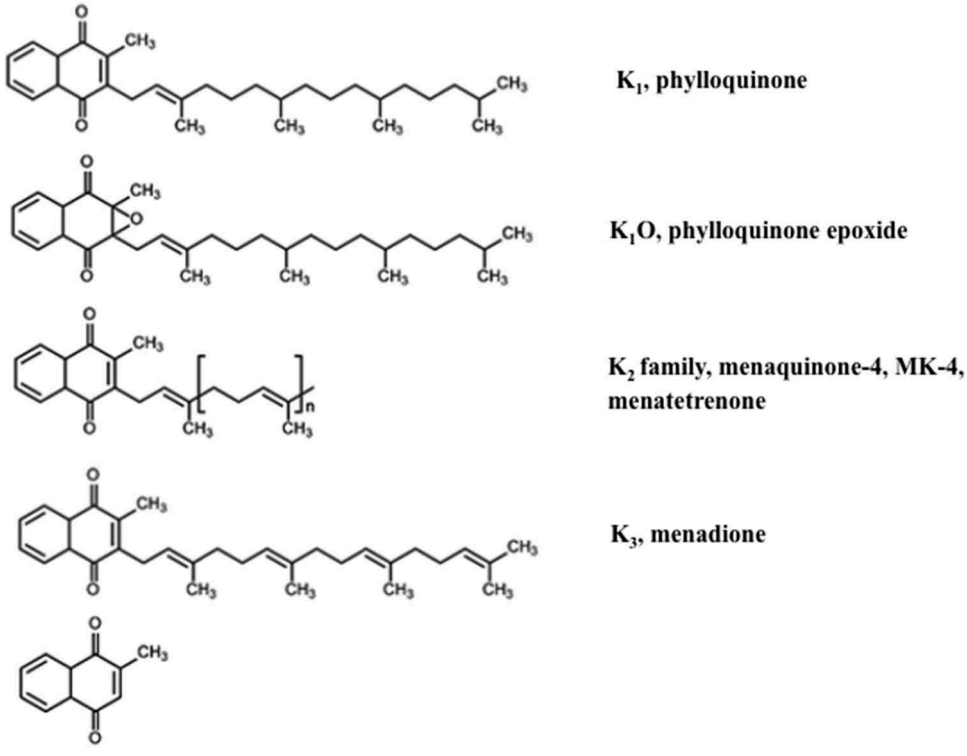

Figure 1. Different forms of VK (modified from http://oregonstate.edu).

Table 2. Foods type and related VK1 and VK2 contents (mcg) for 100 g.

Meat	0.5–5.0	1–30
Fish	0.1–1.0.	0.2–4.0
Fruits	0.1–3.0	–
Green vegetables	100–700	–
Cereals	0.5–3.0	–
Natto	20–40	900–1200
Cheese	0.5–10	40–90
Other dairy products	0.5–15	0.2–50
Eggs	0.5–2.5	10–25
Margarine and vegetable oils	50–200	–

Greater amounts of VK2 (1100 mcg/100 g) are in the traditional Japanese dish called natto, mentioned in many Japanese studies on VK.

European diet consists of various cheeses fermented and contains large amounts of VK2/MK-7 (62 mcg/100 g).

Few side effects have been reported for dietary assumption of VK, such as the increase of hot flashes and night abdominal pain. There was no evidence of toxicity associated with intake of menaquinone (VK2), even at a daily dose of 45 mg.

However, it has been reported that the dose of VK in food needed to reduce bone loss and fracture risk is much higher, from 200 to 500 mg/day, or more, for an optimal carboxylation of osteocalcin (OCN) [13].

The adequate daily intakes of VK, related to different life phases and sex, can be summarized as it follows: (1) infants 0–6 months of age 2.0 mcg/day, 7–12 months of age 2.5 mcg/day; (2) children 1–3 years of age 30 mcg/day, 4–8 years of age 55 mcg/day, 9–13 years of age 60 mcg/day; (3) adolescents 14–18 years of age 75 mcg/day. All the above intakes are the same in both sexes; (4) Adult ≥19 years of age, 120 mcg/day for males and 90 mcg/day for females; (5) during pregnancy and nursing the daily intake is 75 mcg/day and 90 mcg/day for ≤18 and ≥19 years of age, respectively [14,15].

Specifically, cheese and fermented 'Japanese' soya beans called 'natto' are food rich of VK2, also present at lower measure in eggs, meat, and fish.

The role of gut microbiota in VK homeostasis

Similarly to food bacteria, the intestinal bacteria represent the main non-dietary source of VK2 menaquinones. Human gut microbiota (GM) is made of 10–100 trillion of microbes, distributed along the gastrointestinal tract with a relative major abundance in the distal gut [16]. Although approximately 70 divisions of bacteria hosts have been described, only two of these can regarded as the predominant ones: the Bacteroides and the Firmicutes [17]. Specifically, only Bacteroides synthesize menaquinones [18–20], used as substrates for electron transport and oxidative phosphorylation. In fact, Bacteroides can synthesize MK-10 and MK-11, the very long chain forms (with higher number of prenyl units) of menaquinones. MK-7, MK-8, MK-9, and MK-12 molecules represent the minority [18–21].

Moreover, Bacteroides produce VK2 isoprenologues, in which one or more of the prenyl units are saturated [19,20,22,23]. An adult human liver may store about 200–300 nmol of VK, even if a large interindividual variation has been reported [24,25].

VK2, especially MK-7 to MK-13 menaquinones, predominate (90%), while VK1 phylloquinone represents the minority (10%) [24–26]. In particular, MK-10, MK-11, MK-12, and their isoprenologues are the main fractions of liver menaquinones, mainly produced by Bacteroides, with only a small amount introduced with diet [19,21–23]. Thus, GM is mainly responsible for VK2 storage in the liver.

However, it is not currently demonstrated if this liver storage may supply the necessary VK2 for an adequate Gla–proteins carboxylation in physiologic conditions or when dietary intake is insufficient.

Animal studies on rat suffer of the existence of coprophagy among the investigated strains, introducing a major bias in the dietary VK intake assessment.

In human VK deficiency-induced states, the hepatic VK1 phylloquinone stores exhibit a faster reduction than long-chain VK2 menaquinones, due to the existence of different lipid content between them [25,27]. When the VK intake is nearly to or equal to zero, it has been observed an initial buffering linked to liver stores, followed by an evident deficiency with a consequent Gla–proteins decarboxylation and alteration in coagulation pathways [28,29]. However, such studies considered only the restriction of dietary VK1 phylloquinone and, probably, GM alone cannot sufficiently produce adequate amounts of MKs for the maintenance of the related physiological roles when an external source is lacking, but what exactly could happen when they are in a reduced, not completely absent, intake is still undetermined and the specific contribution of GM in maintaining VK2 stores in conditions of severe and mild deficiency needs to be further investigated.

Some authors hypothesized that MK-4 can be produced from intestinal conversion of dietary VK1, but conflicting results also on this issue still exist [30–32].

Moreover, it is necessary to specifically and adequately investigate what could happen in subjects undergoing antibiotic treatment, known to potentially alter GM composition and function.

Finally, since the bacterial colonization begins immediately after birth, through direct contact with maternal microbiota during lactation, any potential quantitative and qualitative changes on GM by maternal feeding may contribute to predispose the offspring to several human diseases, including those affecting bone pathophysiology and health.

Functions of VK

The main known, established, functions of VK can be summarized. VK is essential for

(1) blood clotting;
(2) conversion in active forms of either the clotting factors synthesized in the liver as inactive precursors or prothrombin to thrombin;
(3) formation of fibrinogen into fibrin, which leads to the formation of a clot; and
(4) stimulation of bone formation and reduction of bone resorption.

Assessment of biochemical values of VK

It is noteworthy to take into consideration that VK2 intake can be easily evaluated by specific Food Frequency Questionnaires (FFQ), or measuring plasmatic levels, a method affected by blood lipid alteration. However, the functional evaluation of VK2 activity seems the most useful to assess the VK2 intake in the clinical practice; it can be tested by quantification of undercarboxylated VK2-dependent proteins.

MK-7 is often detectable in Japanese people who regularly eat natto. In fact, MK-7 has a long plasma half-life and at the same equimolar daily intake produces plasma concentrations approximately 5-fold higher than those for VK1 [33].

Effects of VK deficiency and excess

Deficiency

VK deficiency increases the risk for bleeding and hemorrhage. It is less frequent than estimated for action of antibiotics that kill VK2-producing intestinal bacteria.

In the last 50 years, an occurrence of a VK2 deficiency has been suggested, maybe due to modifications of dietary habits, population aging (the absorption of liposoluble vitamins is reduced in elders), and to other factors related to intestinal metabolism.

The role of VK1 in maintaining homeostasis of blood coagulation is mandatory for survival, whereas the deficiency of VK2 has almost long-term effects on individual health.

In particular, VK2 deficiency could be secondary to a sort of preferential 'choice' that favors the VK1 when reduced dietary intake and/or intestinal absorption are present [34].

Excess

K hypervitaminosis may manifest with thrombosis, hemolytic anemia, and jaundice, especially in children. Toxic effects are due to an increased hemolysis.

VK supplements

VK1 and VK2/MK-4 and MK-7 are also available in pharmaceutical forms.

VK1 is the most common commercially available form. VK2 (MK-4 and MK-7) is an approved supplement in Japan for the prevention and treatment of osteoporosis [35].

VK3, or menadione, is the synthetic form of the VK, soluble in water that can be converted into VK2 in the body.

US FDA does not allow sale of menadione as a food supplement for humans for its potential harmful side effects.

Safety/adverse effects of VK supplements

On the basis of the physiopathology background, a long-term dietary supplementation with VK2 in subjects with a proven deficiency, may contribute to reduce cardiovascular events and osteoporotic bone fractures, improving also osteoarticular dynamic in old patients.

However, a real VK2 deficiency is restricted to patients who received a previous renal transplant and those undergoing dialysis [36–38].

It is important to stress that most of VK supplements are well tolerated and safe, even if, sometimes, VK1 supplements may affect the lipid profile, sensitivity to insulin, and the glycemic state.

Rare adverse effects with VK2/MK-4 (menatetrenone) have been described, such as skin lesions and minor gastrointestinal side effects.

It is well known that VK reduces effect of anticoagulants such as warfarin and for those who take warfarin has been suggested to avoid: (1) consuming VK; (2) consuming foods with high content of VK (see Table 1); and (3) keep inconstant the current intake of VK.

Drug interactions have been reported for antilipemic or antidiabetic molecules [39].

VK cycle and its genetic regulation

The relatively low requirements of VK are strictly dependent on the ability of animals, including humans, to regenerate VK from its epoxide metabolite via the VK cycle (Figure 2).

VK cycle consists of the metabolic pathway describing the cellular recycling of the metabolite VK 2,3-epoxide (K > O) produced as a byproduct during the synthesis of VK-dependent proteins at the endoplasmic reticulum (ER) level. As mentioned above, the VK cycle is intimately linked to the gamma-glutamic carboxylase enzyme (GGCX) present in all cells synthesizing Gla-containing proteins (Figure 2). The two major enzymatic activities involved in VK cycle are the (1) VKOR activity that enables conversion from the epoxide metabolite to the native quinone form; and (2) VK reductase activity enabling the reduction from the K quinone to the K hydroquinone (KH 2) form.

VKORC1 gene

The gene *VKORC1*, on chromosome 16p11.2, encodes the catalytic subunit 1 of the VK epoxide reductase complex, responsible for the reduction of inactive VK 2,3-epoxide to active VK in the endoplasmic reticulum membrane.

It has to be also considered that since different genetic variants account for different genotypes of *VKORC*, appearing to modulate warfarin dose requirements, presumably by affecting VK availability [40], such variants could also to have a role in influencing the susceptibility to develop pathological condition other than coagulopathies.

MicroRNA: miR-133a

Recently, it has been reported that a microRNA, namely miR-133a, expressed in human liver tissue, would play a regulatory effect directly on expression of *VKORC1* in humans. In fact, it has been demonstrated that miR-133a interacts with the 3′UTR of *VKORC1* and that miR-133a levels correlated inversely with VKORC1 mRNA levels in liver samples from healthy subjects. This *VKORC1* gene regulation by miR-133a may have potential

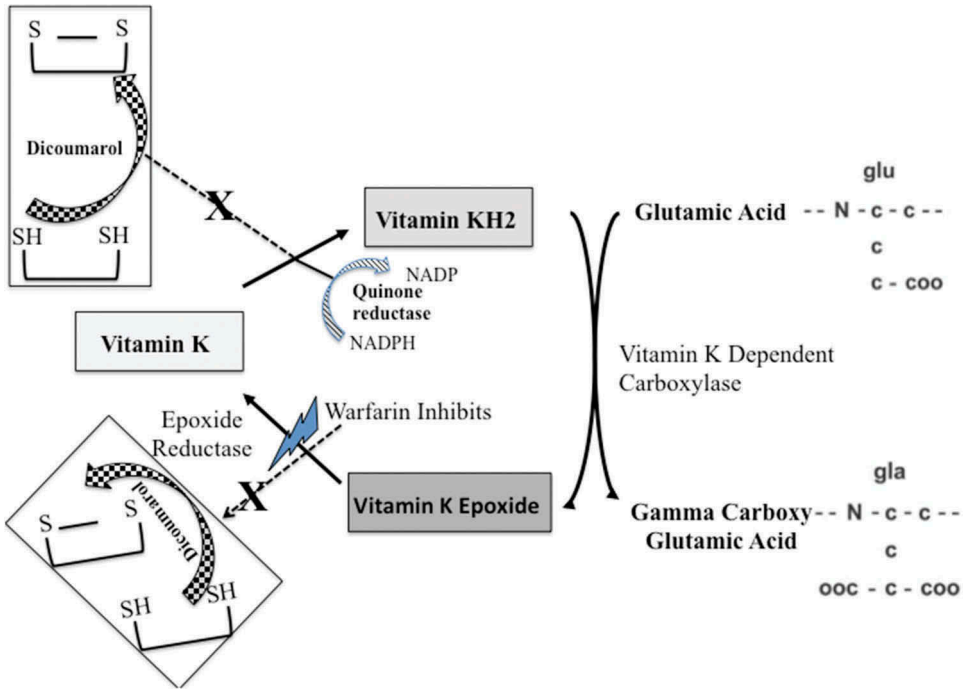

Figure 2. Vitamin K cycle.

importance for the pathophysiological role of VK in humans [41].

UbiA prenyltransferase-containing domain 1 (UBIAD1) enzyme

This novel human enzyme participates in the cellular conversion of VK1 phylloquinone to menaquinone (MK)-4 [42].

Recent studies suggest that side-chain cleavage of oral phylloquinone occurs in the intestine, and that menadione is a circulating precursor of tissue MK-4.

However, a clear role of this enzyme in bone health is still waiting to be demonstrated.

VK and bone metabolism

A low dietary VK intake and high proportion of uc-OCN have been reported as independent risk factors for hip fractures [43–46].

As reported above, VK acts as a cofactor for GGCX that converts glutamate residue (Glu) protein bound to residues of γ-carboxyglutammate (GLA) in the VK-dependent proteins, activating them.

There are evidences on VK (in particular, VK2) to direct the Ca^{2+} into the skeleton, avoiding to be deposited in organs, joints, and arteries [47].

Synergy between vitamin D and VK in the transcription/activation of OCN

OCN is a protein containing Gla acid residues and amino acids important in connection with calcium. It is also identifiable as bone matrix Gla protein (BGP).

It is produced by osteoblasts (OBLs) and involved, presumably, in the synthesis of bone tissue, through the growing and elongation of the crystals of hydroxyapatite, by interacting, via calcium ions, with the latter with the carboxylated Gla residues, at the level of the amino-terminal portion of the protein [48].

It is noteworthy to remember that 99% of the calcium in the human body is stored in the bone tissue in the form of hydroxyapatite.

It has been described that the transcription of OCN is stimulated by vitamin D and the expression of the OCN, in cultured cells, becomes apparent only when the mineralization process is started.

Endocrine regulation of glucose homeostasis through OCN secretion could be very effective, because it would favor the β-cell proliferation and secretion insulin. The OCN active could exist in decarboxylated/undercarboxylated form, or with absence/reduction of carboxy Gla residues, and it is able to promote the secretion of insulin.

As above reported, the process of γ-decarboxylation of OCN is a VK-dependent mechanism. The reduced form of VK is an essential cofactor, which is then oxidized to VK epoxide to be, then, again converted to reduced VK form (*VKORC1*), allowing a new reaction of γ-carboxylation [49].

Maturation and activation steps of OCN

OCN undergoes posttranslation modifications at three Gla residues (E13, E17, and E21) by the enzyme GGCX in OBL endoplasmic reticulum and the γ-carboxylation increased the affinity of OCN for the mineral component of bone [49].

Although a direct role for OCN in the process of mineralization of the extracellular matrix has not been demonstrated *in vivo* [50], it seems to be involved in bone mineral accrual [51] and glucose metabolism [52].

OCN has been recently reported to influence cell function, insulin sensitivity, the production of adiponectin, energy expenditure, and obesity [52,53], thus assuming the typical features of a hormone:

(1) It is a cell-specific molecule, 'secreted' by OBL.
(2) It is produced first as a prepropeptide that goes, successively, meeting in rifts by peptidases.
(3) Its mature form, decarboxylated/undercarboxylated, is evaluable in serum (100–1000 ng/ml in mice).
(4) Several cells possess the specific receptor for the OCN, GPRC6A, a membrane protein coupled to the G protein [48].

It can be reasonably suggested that either bone repair or remodeling may require VK supplementation.

The association between VK levels and the OCN carboxylation state brought to the suggestion that the primary mechanism underlying the protective influence of VK on bone may involve carboxylation of OCN [54], but some clinical data contrast with this issue and suggest that VK treatment, at doses capable of correcting under-carboxylated OCN in healthy postmenopausal women did not correlate with altered bone turnover, density, or geometry [55].

Other VK-associated bone proteins

Apart the OCN, as above cited, the identification of other BGPs boosted research on VK and bone metabolism. In fact, along with coagulation factors (II, VII, IX, X, and prothrombin), protein C and protein S, matrix Gla protein (MGP), Gla-rich protein (GRP), periostin, Gas6, periostin-like factor (PLF), and other VK-dependent proteins may have a role in calcium homeostasis, inhibition of vessel wall calcification, endothelial integrity, facilitation of bone mineralization, tissue renewal and cell growth control, and probably numerous other effects [56–61]. In particular, BGP and MGP have low affinity with clotting factors in the blood, although the two types of molecules are believed to have departed from a common ancestor [60,61].

MGP, with its five VK-dependent Gla residues, conferring high affinity for calcium, phosphate, and hydroxyapatite, is a potent inhibitor of calcification, of recognized importance for the vascular health [61,62].

However, VK also seem to act directly on these parameters, as masterfully reviewed by Pimm PM in 2010 [63] who specifically reported that VK2 could be an important support for cardiovascular and blood vessel wall integrity. In fact, an observational study, on 16,057 postmenopausal Dutch women considered their VK intakes, collected through a FFQ, and were followed for over 8 years for the occurrence of coronary heart disease (CHD), and VK2 intake, but not VK1, was associated with decreased CHD risk [64].

MGP

It is regarded as the gatekeeper of vascular 'ossification,' inhibiting the precipitation of calcium, as hydroxyapatite crystals form, at the site of elastic lamellae, which blocks the removal of the initial foci of calcification.

Gla residues are critical to the function of MGP, and undercarboxylated, inactive MGP is formed under a state of inadequate VK or VK antagonism. Consequently, it has not to be considered inappropriate that an alteration of protein carboxylation, to its site of tissue expression, may be associated with development and progression of cardiovascular disease (CVD). Since both osteoclasts and OBL synthesize MGP, that accumulate in bones in the carboxylated form with fetuin [61,65], the bone itself can be an important source of complexes of calcium-phosphate–fetuin-MGP with a consequent effect on vascular calcification [61,66].

GRP

It is a small Gla protein, locates in chondrocytes of sturgeon and rats and is also expressed in rats bone cells, thus suggesting an important role during the skeletal formation. In fact, both OBL and osteocytes give a clear positive signal for the GRP-mRNA at the trabecular bone. The most remarkable feature of GRP is the large number of Gla residues in its mature form and it may enlarge the spectrum of bone VK-dependent carboxylation/decarboxylation functional process for calcium homeostasis.

Such a huge potential of calcium binding, by Gla residues, suggests its physiological role of modulator of calcium in the extracellular domain [58,61].

In fact, a system of partition without contributions of bone remodeling system controls both the basal level and the minute by minute correction of Ca^{2+} in plasma by flows of Ca^{2+} to the outside and toward the inside by a ion-exchangeable pool in the bone endocanalicular network [61,67].

Periostin and PLF

Both periostin [59] and PLF [60] are carboxylated proteins recently identified.

Periostin, produced by mesenchymal stromal cells from which derive all non-hematopoietic bone cell lines (stromal fibroblasts, osteocytes, chondrocytes, and adipocytes), is highly expressed in the bone extracellular matrix and interacts through its fasciclina domains with integrins on the cell surface and extracellular matrix proteins [59,61,68].

PLF, one of the splice variants from the periostin locus, is expressed mainly in the periosteum during the early adaptive stage of remodeling [60].

Protein-S

The role of protein-S in the bone remains partially undefined [57].

VK-dependent proteins, bone health, and metabolism

Although the roles and exact biomolecular mechanisms underlying fine bone calcium homeostatic tuning are still unknown, it has been suggested that the syncytium formed by bone lining cells–osteocytes may modulate it through several mechanisms. One of these could be represented by the production of non-collagenous Gla proteins that complex large amounts of calcium and physically link to hydroxyapatite

crystals, thus regulating appropriate free calcium concentration in the extracellular fluid [61,69].

However, according to the bony specific features of OCN, MGP, GRP, periostin, and PLF, it could be suggested that tissue- and cell-specific VK-dependent carboxylation/decarboxylation processes, through evolutionary adaptive steps, contributed to either create regulatory pathways in bone or to the development of bone metabolism, energy metabolism, calcification, serum calcium homeostasis, and angiogenesis [61].

Pleiotropic functions of VK on bone

VK acts on (1) carboxylation/decarboxylation processes of tissue- and cell-specific Gla proteins with structural and regulatory functions in the bone (and non-mineralized tissues); (2) new signaling pathways involving transcription factor, steroid-xenobiotic receptor (SXR), class 1 of intracellular receptors, which also belongs the vitamin D receptor family [70,71].

VK2 and the 'calcium paradox'

VK2 deficiency could act as a one of the key factors involved in the 'calcium paradox,' suggesting an explanation for the suggested evidence of increased CVD risk and mortality related to the high dose of calcium supplementations [61,72,73] associated or not with vitamin D [74]. However, the nurse's health study suggested that subjects taking their vitamin D supplementation reduce risk of CVD [75]. Results from the ongoing VITamin D and OmegA-3 TriaL (VITAL) [76] could be helpful to address this issue. Up to date, it has to be demonstrated if VK2 deficiency could be, or not, the connection between calcific atherosclerosis and OP, opening new intriguing therapeutic scenarios for both diseases.

In particular, two trials, one in postmenopausal women and the other in patients with CAC, are still ongoing [77,78] and they could helpful in providing a still lacking evidence to support a direct connection on it.

VK and bone cells

VK and OBL

Several protein targets of VK are osteoblastic proteins and components of the bone matrix.

As written above, VK is the principal regulator of the molecular maturation of the OCN and interacts with MGP (calcification inhibitor matrix Gla protein) [79]. Moreover, it promotes and stimulates mineralization [80]. VK stimulates the differentiation, training, activity, and metabolism of OBLs [81], stimulating the expression and production of markers such as type 1 collagen, osteopontin, and MGP as also the lamellar bone formation [82].

It is has been reported that dicoumarol, a naturally occurring anticoagulant acting as a functional VK depleter (similarly to warfarin drug), not only influence the formation of bone callus, but also reduces the total amount of newly synthesized bone [83].

In particular, warfarin seems not have influence on the VK-dependent effects on (1) OBLs–osteocytes transition; (2) expression of E11 osteocyte marker; and (3) moving of receptor activator of nuclear factor κB ligand (RANKL)/OPG ratio toward an anti-osteoclastogenic environment. The 'resistance' to warfarin may suggest pointing to a specific path of VK in the bone, carboxylation-independent, linked to its genomics action, but it still remains to be addressed [70].

Recently, VK2 action on OBL formation and activity is accomplished by down-regulating basal and cytokine-induced nuclear factor kB (NF-κB) activation, by increasing the inhibitor of NF-kappa-B (IκB) mRNA, in a γ-carboxylation-independent manner. Moreover, VK2 demonstrated to prevent the repression by tumor necrosis factor·α (TNFα) of the Small Mother Against Decapentaplegic intracellular proteins that transduce extracellular signals from transforming growth factor beta (TGFβ) ligands to the nucleus where they activate downstream gene transcription signaling induced by either TGFβ or bone morphogenetic protein-2. Such findings suggest, at least *in vitro*, a hypothetical pro-anabolic action on skeleton that need of stronger evidences before to prescribe VK2 for this purpose [82].

Differently from what described for VK2, it seems that VK1 may require a much higher dosage or may act via different mechanisms and should not be considered equivalent or interchangeable with VK2 [81].

VK and osteoclasts and osteocytes

In pre- and mature osteoclasts, it has been described that VK blocks: (1) circulating osteoclast precursors [84]; (2) osteoclastogenesis disabling NF-kB, by suppressing bone resorption [81,85,86]; and (3) apoptosis of mature osteoclasts in a completed resorption cycle [87].

It exhibits ability to promote carboxylase-dependent and -independent mechanisms (through the SXR-related pathway?) with (1) collagen accumulation; (2) cell–matrix interactions; (3) matrix mineralization; (4) mineral maturation; and (5) osteocytes differentiation, all determinants of bone quality.

As reported with respect to OBL, VK2 acts also on osteoclast formation and activity by down-regulating basal and cytokine-induced NF-κB activation, by increasing IκB mRNA, in a γ-carboxylation-independent manner, thus suppressing osteoclastogenesis [81], as also suggested by the evidence that VK2 ameliorates bone destruction in rheumatoid arthritis by downregulating RANKL production.

Summary of actions of VK on cells bone

The molecular mechanisms of VK action on bone cells are still poorly defined.

Expanding the functional range of VK in the bone

In 2009, Atkins et al. provide convincing evidence that VK synthetic counterparts induce cellular events both with bone-carboxylation and -independent mechanisms. In their well-designed study, Atkins and colleagues showed that VK homologues induce mineralization of bone matrix, promoting

the transition to OBLs into osteocytes and inhibit the expression of RANKL in the osteocyte-like cell line MLO-Y4, thus potentially hindering osteoclastogenesis [70]. The balance between RANKL and osteoprotegerin, potentially achieved under the stimulus of VK, could result in a positive outcome of bone mass by reducing the relative phase of the resorption of bone remodeling sequence activation. This inhibitory effect of VK on the production of RANKL merits further discussion, however, being in agreement with observed results *in vivo* [88], but in disagreement with those observed *in vitro* in a more immature phenotype of OBL [89].

The positive effect of the exposure to VK on the matrix mineralization is partly due to carboxylation; in fact, warfarin, a binder known of the VK-epoxide reductase, inhibits recycling of VK and interferes with these positive effects.

VK2/MK-4 and MK-7 exhibit similar ability in the carboxylation of OCN. Supplementation with VK2/MK-7 seems to be more effective versus phylloquinone (VK1) in the OCN carboxylation [7].

VK influences the transcription of genes necessary for the expression of osteoblastic markers and those involved in the assembly of the collagen [90].

In vitro and animals' studies suggest that VK2/MK-4 can be involved in the regulation of inflammation, oxidative stress, and apoptosis (all, in turn, may reduce bone resorption) [91].

VK and oxidative stress of bone cells: a new hypothetical role for VK molecules?

It has been already demonstrated oxidative stress conditions antagonizes the Wnt-signaling in mice OBL precursors by diverting β-catenin from T-cell factor to Forkhead box O-mediated transcription. In fact, oxidative stress represents a major pivotal pathogenetic factor of age-related bone loss and strength in mice, leading to a decrease in OBL number and bone formation.

Almeida et al. revealed that the expression of Fox O-target genes increases, whereas the expression of Wnt-target genes decreases, with increasing age in C57BL/6mice. Moreover, we show that in OBL cell models, oxidative stress (exemplified by H_2O_2) promotes the association of FoxOs with β-catenin, β-catenin is required for the stimulation of Fox-O target genes by H_2O_2, and H_2O_2 promotes FoxO-mediated transcription at the expense of Wnt-/T-cell factor-mediated transcription and OBL differentiation [92].

These changes are temporally associated with increased OBL and osteocyte apoptosis, decreased OBL number and bone formation rate, increased levels of reactive oxygen species (ROS), and a corresponding increase in the phosphorylation of p53 and p66shc, two key components of a signaling cascade that influences apoptosis and life span in invertebrates and mammals. In agreement with our findings, others have shown that H_2O_2 suppresses OBL differentiation of bone marrow progenitors and promotes OBL apoptosis in an ERK-dependent manner *in vitro*, and that an increase in ROS leads to osteopenia in other murine models and, perhaps, in humans [93–95].

Recently, a possible antioxidant role of VKORL1 has been suggested. The reduced form of VK, KH2, is known to be a potent radical-scavenging species [96]. It has been suggested, through the suppression of lipid peroxidation in rat liver microsomes that the VK epoxide cycle could provide a localized defense against free radicals in its endoplasmic reticulum membrane environment [97]. In fact, experiments showed that hydroquinones of K and MK-4, but not their quinones, were effectively able to suppress lipid peroxidation reactions induced by pro-oxidants in rat liver microsomes. The antioxidant response was completely abolished both by 2-chlorophylloquinone (chloro-K 1) and warfarin, known inhibitors of GGCX and VKOR, respectively, suggesting either the need for a functional VK-epoxide cycle or that the radical-scavenging VK species is being continually regenerated [98].

Finally, the expression level of VKORL1 positively correlated with the reduction in intracellular by chemical agents-induced ROS and inversely correlated with oxidative protein damage. Based on this data, it has been proposed that the primary role of VKORL1 is to reduce VK and thus to provide a VK-dependent mechanism for a global tissue protection against oxidative injuries [98].

Animal studies on VK, GM, and bone health

Up to date, the studies on GM–bone axis are limited and the real effect of the gut-microbial agents or their synthesized products cannot be adequately addressed for bone health [99]. It has been recently reported that GM may play as a pivot in the sex-steroid-deficiency-induced bone loss. In fact, it has been postulated that postmenopausal osteoporosis could result, at least in part, from the sex-steroid-deficiency-induced chronic inflammatory state trough and the increasing production of TNFα by activated T cells as one of the possible molecular mechanisms, even if it is still unknown the real nature of the antigens bringing to the T-cell activation.

In animal studies, intestinal microbiota has been reported of fundamental importance in inducing, training, and functioning of the host immunity, both by contributing to inflammatory processes and regulating the bone mass accrual.

Interestingly, the treatment with two probiotics, *Lactobacillus rhamnosus* GG or VSL#3TM preparations to the gut flora of sex-steroid-deficient mice was able to reduce gut permeability, to amortize the inflammatory responses, and to protect against bone loss [100].

Clinical studies on VK and bone

Comparison of VK1 and VK2 supplementations on bone health

A study on 173 women, randomized into three intervention groups and one control group has been recently performed. More specifically, the three intervention groups included (a) subjects supplemented with 800 mg/day of calcium and 10 mcg/day of vitamin D3 (CaD, $n = 38$); (b) subjects supplemented with 800 mg/day of calcium, 10 mcg/day of vitamin D3, and 100 mcg/day of phylloquinone (0.221 mcmol; CaDK1, $n = 38$); (c) subjects supplemented with 800 mg/day of calcium, 10 mcg/day of vitamin D3, and 100 mcg/day of MK-7 (0.154 mcmol; CaDK2, $n = 39$) with fortified milk and yogurt.

No dietary intervention was delivered to the control group, which continued with the usual diet during the period of 12 months.

The beneficial effect of the intervention in the two study groups with VK reflects primarily the suppression of bone remodeling process and positive changes in LS-BMD (lumbar spine-bone mineral density). As also suggested by authors, the holistic nature of the intervention and probably the synergistic effect of supplemented nutrients essential for bone health (i.e. milk protein, vitamin D, VK, calcium, magnesium, and phosphorus), together with the increase levels of physical activity and improving eating habits, could probably provide an explanation for these favorable changes [101].

VK, biomarkers, and vitamin D

The total level of serum OCN may represent a sensitive marker of bone formation, although it can increase also in bone resorption, and, consequently, the degree of γ-carboxylation of OCN seems to be sensitive to nutritional interventions with VK and therefore is a relative measure of the state of the VK [102].

Circulating levels of undercarboxylated-OCN (uc-OCN) are higher in postmenopausal versus premenopausal women and clearly in over 70-year old females. Although not definitively assessed, high uc-OCN/OCN ratio could be predictive of risk of hip fracture in older women [43,103].

Despite that the VK deficiency seems the most likely cause of high uc-OCN/OCN ratio, an inverse relationship between vitamin D status and uc-OCN levels, as well as a significant reduction of uc-OCN/OCN with vitamin D supplementation have been described [104].

A higher uc-OCN/OCN ratio reflects also a poor overall nutritional status with inadequate vitamin D, which would explain these observations [105].

However, a randomized intervention, controlled versus placebo, study on young and in postmenopausal women suggests that (1) supplementation of vitamin D does not reduce uc-OCN/OCN; or (2) no any additive effect with VK supplements [101,105,106].

VK levels, bone mineral density (BMD) and risk fracture

A prospective study of 944 Japanese women (age ranging 20–79 years) revealed a positive association between baseline values of DXA assessed total hip (TH) BMD and intake of natto in the postmenopausal women. During the 3-year follow-up, the BMD loss rate, at femoral neck (FN) level, was significantly lower in natto consumers (more than 200 mcg/day of MK-7) versus nonconsumers [107].

Both TH- and FN-BMD result significantly higher in approximately 2000 Japanese men aged ≥65 years who regularly consume at least 1 pack/day of natto (corresponding to ≥350 mcg/day of MK-7) versus less than 1 pack/week users (<50 mcg/day of MK-7).

However, it has to be also considered that together with natto also other compounds (e.g. soy isoflavones) with potential bone benefits are present [108].

Conflicting results in Japanese, European, and USA studies

According to the review by Iwamoto et al., considering several randomized clinical studies (RCTs), from 2000 to 2009, ambiguous results on the effects of menatetrenone (a synthetic VK2) on BMD, as also on fracture incidence, in osteoporotic postmenopausal women exist [109].

However, the considered RCTs were extremely inhomogeneous in several parameters, such as the design and the size of the study, the types of control groups (placebo, untreated or treated some with alfa-calcidiol or estrogens or etidronate).

Ambiguous results have been reported even in the European and USA studies. In 3 years RCT on 325 healthy postmenopausal women, VK2/MK-4, at a dose of 45 mg/day for 3 years, improves measures of bone strength. However, this dose is about 500 times more than the advised daily intake of VK [110].

In a 1-year US-RCT, double-blind and controlled versus placebo, on 365 healthy postmenopausal women with inadequacy of VK, defined by uc-OC values ≥4%, neither high doses of VK1 (1000 mcg/day), nor of VK2/MK-4 (45 mg/day) had an effect on bone turnover markers (BTMs) or on LS/FN-BMD. All the participating subjects received calcium (630 mg/day) and vitamin D3 (400 IU/day) [57]. In another double blind versus placebo RCT, on 334 Norwegian healthy postmenopausal women (from 1 to 5 years), no effect of 360 mcg/day of VK2/MK-7 (capsules of natto) was observed on BMD at various sites after 1 year versus baseline [13]. Another RCT, versus placebo, on 244 Dutch postmenopausal women, 180 mcg/day of VK2/MK7 for 3 years revealed a significant reduction of bone loss at the FN, but not in other sites [111].

At present, the potential of supplementations VK2 on bone health has yet to be established in larger RCTs.

The Nurses' Health Study (72,327 subjects included) demonstrated that females, aged 30–88 years, with an intake of phylloquinone, VK1, <109 mg/day have higher risk of hip fracture at 10 years versus higher intake of VK1 [44].

The Framingham Heart Study included males and females with an average age of 75 years and median VK1 intake of 56 mg/day exhibiting a higher risk of fractures of the proximal femur in 7 years versus average intake of 254 mg/day.

Reduced values of circulating VK1, reduced intake of VK1, low intake of VK2 (MK-7), and high serum levels of uc-OC have been all associated with increased risk of fracture in most observational studies [47,54].

However, observational studies did not reveal any association between intake of VK1 and BMD [112].

Recently, it has been reported, in a Norwegian elderly population that both VK1 and 25OHD levels are lower in patients suffering for hip fracture and that they can be independently and synergistically associated with hip fracture risk after adjustment for confounding factors [113]. In particular, a significant interaction between 25OHD and VK1 and a significant correlation between total-OC and VK1 and 25OHD have been described. Thus, according also to these Authors, future intervention studies should include both vitamins.

Associations between VK1, BMD, and fractures: observational studies

A cross-sectional study on a cohort of 3199 middle-aged Scottish women demonstrated that subjects in the highest quartile of dietary intake of VK1 (162 mcg/day) have a significantly higher BMS-LS and FN-BMD versus lowest quartile (59 mcg/day) [114]. Recent cross-sectional and case-control studies revealed associations between high intake of VK1 and reduction of the incidence of hip fracture [113,115]. Other studies confirmed an association between reduced intake of VK1 and low BMD in women, but, in general, there is less evidence for the association between intake of VK1 and increase of BMD [47].

These studies all suggest that an adequate intake of VK may be necessary to reduce the bone mass, or the requirements of VK to maintain bone health may be greater than the current alimentary intake. Once the need to VK for bone health is satisfied, an extra intake may not add benefits.

However, major limitation of these studies is represented by the fact that a high intake of VK1 is indicator of a healthy diet, high in vegetables, containing also other protective nutrients for the bone.

Studies that measured plasmatic VK1 showed that its higher levels associated with reduced risk of fracture [84,116].

The incidence of vertebral fractures has reported to be inversely correlated with LS-BMD and VK levels in the study to 4 years on 379 Japanese women of 30–88 years [116].

Therefore, the results obtained by only observational studies, in the absence of RCTs, do not conclude that the VK may exert an independent protective effect on bone health.

Up to date, observational studies do not give unequivocal association between levels of VK2 (MK-7 and MK-4) and fracture risk. Few association studies on menaquinone (K2) and health bone exist, with limited food sources of MK-4, the main form of VK2 in Western diets.

Supplementation of VK, BMD, and fractures: clinical trials and their meta-analysis

Clinical trials in various populations examined the effects of VK on BMD. Two systematic reviews and meta-analysis synthesized the results [117,118].

In the latest one [118], data are reported from 17 trials with VK in healthy populations and in patients, aged 18 years of age and older, with primary and secondary osteoporosis. Including 10 studies of VK2 (8 studies with MK-4, at doses of 15–45 mg/day and 2 studies with MK-7, at doses of 0.2–3.6 mg/day) and 7 studies on VK1 (0.2–10 mg/day).

Supplementation with VK had no effect on the FN-BMD, but increased the LS-BMD on the average of 1.3% after 6–36 months of supplementation. Subgroup analyzes, based on the type of VK supported, showed that VK2 increased significantly LS-BMD of 1.8%, while VK1 had no effect.

The treatment effect on LS-BMD revealed higher increases in Asian than in Western populations.

However, after the exclusion of studies with high risk of bias, no significant effect of VK on LS-BMD has been reported.

The reported estimations are probably distorted by large differences in study populations, in methodology, process quality selected, and publication bias [119].

A recent Korean survey on health and nutrition, from 2010 to 2011, analyzed raw data from the fifth Korea National Health and Nutrition Examination Survey for adults (2785 men, 4307 women) aged over 19 years. After cross-sectional analyses, only positive association between VK intake and FN-BMD in men, after adjustment for bone-related factors, has been found. In particular, women in high tertiles of VK intake had a significantly higher LS- and FN-BMD versus women in lowest tertiles. Moreover, the higher VK intake the lower risk for osteoporosis in women, but such finding was lost after adjusting factors. Thus, in this survey, a low dietary VK intake was associated with low BMD, suggesting the need to increase the dietary VK intakes for maintaining BMD, even if it has to be proven [120].

Effects of VK1 supplementation on fractures

Effects of VK1 supplementation on fractures are limited, and based on a single study (Integration Trial with VK in postmenopausal women with osteopenia) because most studies of VK1 were not designed to examine the fracture.

A double-blind, placebo-controlled, clinical trial of supplementation with VK for 440 postmenopausal osteopenic Canadian women found a statistically significant effect of VK1, 5 mg/day, on all fractures after 2–4 years of supplementation (9 women with 11 fractures in the VK1 versus 20 women with 21 fractures in the placebo group; hazard ratio 0.48, 95% CI: 12:20–0.98), but the fracture was a secondary product of the process [35].

Effects of VK2 supplementation on fractures

They are based on the results of eight studies on Japanese patients with primary or secondary osteoporosis. Systematic review and meta-analysis of seven Japanese clinical trials assessed that supplementation with MK-4, doses 15–45 mg/day in 12–24 months, significantly reduced fractures of hip, vertebral, and nonvertebral [117].

However, the eighth trial (the largest) published in 2009 has a different conclusion [121].

A large open Phase IV study on 4378 Japanese osteoporotic women, with/without prevalent vertebral fractures, with intervention with MK-4 and calcium to 3- and 1-year follow-up, in which there was no restriction on the use of drugs for osteoporosis, demonstrated that a combined treatment, with MK-4, 45 mg/day (in 3 doses of 15 mg/day) and calcium versus calcium alone, was not associated with any change in the incidence of vertebral fractures at 3 years (5.9% vs. 5.7%), and no changes in the incidence of all clinical fractures at 4 years (2.5% vs. 2.1%) [121].

In 1/11 *post-hoc* analysis of subgroups, without correction, found a statistically significant reduction in new vertebral fractures in a subgroup of women with more than five prevalent vertebral fractures (20.3% vs. 33.2%). A double-blind, randomized, clinical study of comparison with 0.36 mg/day of

MK-7 versus placebo for 3 years in 244 Dutch postmenopausal women without osteoporosis found 1 new vertebral fracture in women in MK-7 group (n = 120) and 6 in the placebo group (n = 124), but there were few statistical differences between fractures of the 2 groups [110].

Up to date, in literature, it has been published that VK2 supplementation can protect against fractures, but the data are not consistent.

Methodological limits of the current evidences

Several limitations can be reported, such as: (1) various issues methodology reported in Japanese trials with VK2/MK-4; (2) selection bias due to lack of 'blinding' and consequent bias of assessment; (3) high rates of abandonment; (4) extreme inhomogeneity of participant populations, such as elderly with primary or secondary osteoporosis; (5) inadequate report concerning the sufficiency/insufficiency of vitamin D and calcium intake; (6) possible existence of different baseline risk of fractures within the populations; (7) generalization of the results with VK2/MK4 in healthy postmenopausal women, replete with calcium and vitamin D; (8) lack of studies on VK supplementation and fractures as the primary outcome; and (9) unclear overall effect of VK supplementation on preventing fractures.

Furthermore, many differences among the several international studies concerning VK status BMD and risk of fractures exist, such as (1) differences in the form of VK used; (2) baseline dietary intake of VK not always computed; (3) differences in sufficiency/insufficiency of vitamin D and calcium intake reports or the lack of these data; (4) differences in study populations; and (5) Japanese studies are with VK2 in the form of MK4, European studies with VK2/MK7, and North-American studies mainly with VK1.

Combination therapy studies with menatetrenone (K2) and amino-bisphosphonates

Concurrent therapy with bisphosphonates and VK could be promising since bisphosphonates possibly interfere with VK activation.

An RCT for the benefit of combination therapy with VK2 and alendronate (ALN) in osteoporotic postmenopausal described increase of FN-BMD and reduction of uc-OCN serum levels higher in the VK2 plus ALN group versus ALN [121].

Recently, effects of risedronate, alone or combined with VK2, on serum uc-OCN and carboxylate-OCN levels in an osteoporotic Japanese population of postmenopausal women have been investigated. Although no significant difference was observed for uc-OCN decrease rate and incidence of vertebral fractures between treatments, uc-OCN levels in patients with incident vertebral fractures were significantly greater in subjects treated with combination therapy than in patients on risedronate alone [122].

In Japanese studies, the threshold values for increased fracture risk associated with serum concentrations of uc-OCN are set at 4.5 ng/ml for treatment-naïve of postmenopausal women and 2.6 ng/ml for postmenopausal women treated with a-BPs.

VK could play an important role in preventing fractures in postmenopausal women with osteoporosis [123,124].

VK antagonists and bone health

Oral anticoagulants, such as warfarin, are known antagonists of VK. Unfortunately, there are only few studies on the chronic use of warfarin and risk of fractures in older women. An association between long-term treatment and risk of fracture (in one study) has not been shown [125]. Another study revealed a significantly higher risk for rib and vertebral fractures in consumers of warfarin compared with nonusers [126]. In elderly patients with atrial fibrillation, the long-term treatment was associated with significant increased risk of osteoporotic fractures in men but not in women [127]. A meta-analysis of 11 studies revealed that oral anticoagulation associated with modest reductions in BMD at the wrist, but no change at the hip or spine levels [126].

An altered nutritional status of the VK or warfarin use, with high concentrations of serum uc-OCN, resulted in increased risk of fractures.

Recently, a study on 70 children, affected by chronic diseases and at increased risk of developing thrombosis, exposed to long-term warfarin treatment. These subjects are more likely to have complex underlying medical conditions and a low body mass index (BMI) percentile as also other deficiency such as a decreased growth hormone (GH) production. This study has revealed that BMD could be negatively influenced by the BMI and GH deficiency as risk factors, suggesting both an early detection and intervention in these patients [128].

A Japanese study on 42 atrial fibrillation patients at high risk for atherosclerosis with one or more coronary risk factors. Twenty-four of these patients had been treated with warfarin for at least 12 months, and 18 were without warfarin. In the whole sample, bone alkaline phosphatase, uc-OCN and RANKL were measured as BTMs. Both the uc-OCN and RANKL levels resulted significantly higher in the subjects treated with warfarin, suggesting that long-term warfarin therapy may be associated with bone mineral loss in older patients [129].

A recent study revealed that an 'early-trigger' intravenous VK may optimizing target-driven care in warfarinized patients with hip fracture [129].

Future development of new anticoagulants that do not block the recycling of VK may represent a safer alternative to the use of antagonists of the VK [130].

Expert commentary

There is still a limited evidence for VK on preventing fractures and this does not recommend the routine use of VK supplements for the prevention of osteoporosis and fractures in postmenopausal women and men. It has been suggested that vitamin D and VK may have a synergistic action, but it is not currently known if it occurs in an independently manner.

Several information on the relationship between vitamin D and bone health can be regarded as miliary stones in bone metabolism. Up to date, similar acquisitions have not been clearly addressed concerning VK: (1) relationship between levels 25(OH)D and reduction of femoral BMD (old men) [131,132]; (2) importance of vitamin D repletion and response

to the antiresorption therapy [133]; (3) low levels of vitamin D and increased risk of proximal femoral fracture [134]; (4) duration of the persistence of vitamin D low values and risk, at 10 years, of proximal femoral fracture [135]; (5) vitamin D plus Calcium supplements: small reduction in the risk of hip fractures (9 trials, 49,853 participants); (6) vitamin D plus Calcium supplements: reduction of the risk of any type of fracture (10 trials, 49,976 participants) [136,137].

Currently, we have several limitations before prescribing consciously a VK-enriched diet or VK supplements for a better bone. Moreover, we do not have any clear information concerning which biological markers could be the more sensitive and accurate to assess both positive and negative effects of VK intake on the skeleton.

The evaluation of the carboxylation state of OCN could potentially represent one of these potential biomarkers, but we still have problems concerning the sensitivity and specificity of OCN assessment, such as (1) the incomparability of the tests used in different laboratories; (2) the existence of physiological fluctuations in circulating levels (10–15%); and (3) the sharing of the variable undercarboxylated component. The uc-OCN exhibits a reduced binding affinity to the mineral, but the clinical trials with VK supplements did not address if circulating levels of uc-OCN could improve both bone mineralization and BMD and it is not currently possible to establish if an adequate/maximum carboxylation of OCN, through VK supplementation, may always result in increases of BMD and/or if it may exert greater effects on BMD in patients with primary or secondary osteoporosis.

However, effect of VK on BMD seems to be more evident in osteoporosis, or in subjects with vitamin D-insufficiency for the possible interactions between VK and vitamin D.

Five-year view

Very few studies report the total intake of VK at baseline. More studies will be necessary for understanding the role VK on bone health in subgroups at greater risk for suboptimal intake. Future studies will have to further examine the effects of VK1 and K2 on bone parameters, taking also into account that the effect of VK supplementation on BMD could be 'masked' in those with sufficient intake of VK [35].

VK seems to be important for bone health. In fact, low intake of VK, low circulating levels of VK, and high levels uc-OCN are all associated with increased risk of hip fractures in observational studies [47].

However, the results of clinical studies are still inconclusive and it is uncertain whether supplementation with VK1 or K2 decreases the risk of fractures, vertebral or non-vertebral, due to the methodological limitations of the trials assessing these results.

The effectiveness of VK on fractures and bone-quality needs to be confirmed in the future with large RCTs with sufficient statistical power to detect true and clinically significant differences between the groups compared.

Future research and open questions

It must still be considered and understood which type of VK (K1, K2, K3?) should be used in the daily clinical practice, as a supplement/drug in osteoporosis for a better bone health. Future intervention studies should include either vitamin D or VK to prove/confirm that they are independently and synergistically associated with the risk of fracture (protection). Currently, the following open questions need to be addressed: (1) despite a minimal effect on BMD, VK may have a protective effect on fractures?; (2) apart from the role of VK in γ-carboxylation, could be other VK-dependent bone routes present in influencing the risk of fractures?; (3) the effect of VK on fracture could be mediated through its effects on the quality, geometry, or strength of bone?

Perspectives on GM and bone health in humans

Preclinical studies suggest that GM may positively impact BMD and strength bone parameters, with administration of probiotics associated with higher bone mineralization and greater bone strength. The preferential bacterial host showing such beneficial effects on bone health is the Lactobacillus that represents the best candidate for future clinical intervention trials. However, at the moment, we do not have sufficient data, from the few human *in vivo* studies, on the existence of a relationship between efficacy and stage of bone development, also considering that early phases of life importantly impact bone health, perhaps via modulation of the GM. In addition, data on eventual gender-specificity and/or -differences on the efficacy on bone health, the effective dose of probiotics, the timing and the duration of treatment with probiotics are also lacking. A balance between pathogenic and beneficial microbiota throughout childhood and adolescence could be relevant to gastrointestinal health in general and to a favorable synthesis and maintenance of VK, specifically [138]. Thus, in example, it could be intriguing to adequately design large population studies investigating the relationship and/or the association between lactation from women developing osteoporosis lately in life, the occurrence of this bone abnormal condition in their lactated progeny, and VK metabolism.

Although more animal and human studies to correlate composition of GM, VK metabolism, and bone health are needed, it can be suggested that GM may play a significant role in inducing bone loss and increasing bone turnover, at least in sex-steroid-deficient mice, opening to speculate that the GM may be involved also in regulating the magnitude of bone loss experienced by postmenopausal women [100].

Finally, in recent years, the concept has grown that oxidative stress is linked with aging and many other chronic degenerative diseases, including osteoporosis and since oxidative stress is generated by an excessive production of free radicals and reactive ROS; it may provoke oxidative damage to different biomolecular systems. Such damages can be counteracted by antioxidant systems, enzymatic or nonenzymatic, and administration of antioxidant vitamins, as VK, may strongly contribute in decreasing levels of oxidative stress, with potentially beneficial effects on bone quality, as demonstrated in various experimental models.

Several evidences suggest that estrogens may represent one of the mechanisms contrasting the deleterious action of ROS against bone health, also suggesting a potential role of reactive species in uncoupling bone turnover. However, the definitive consensus on the involvement of oxidative stress in the derangement of bone

homeostasis is still lacking, due to the controversial results of the few human *in vivo* studies so far conducted.

In particular, bone healing in patients with long-bone fractures may accelerate following multivitamins administration able to decrease the levels of both oxidative stress and free radicals. Moreover, VK, C, or B deficiencies are modifiable risk factors for OP. Therefore, improving antioxidant vitamins status could help in the treatment and prevention of OP especially in elderly people.

In conclusion, in next 5 years, either preclinical or clinical human studies will have to produce evidence-based data to support a role for VK supplementation in metabolic bone diseases, such as osteoporosis, their prevention among healthy, postmenopausal women, and elder men receiving, or less, vitamin D and calcium supplementation. Interventional studies investigating the isolated role of VK molecules on bone parameters are required. Further studies on the best probiotic strains, their dose, and algorithm of administration to be used are needed, since their administration seems to have a great potential for bone health that could recommend economic investments to implement the research. We hope also that additional studies will clearer establish a causal relationship between oxidative stress and bone loss in postmenopausal women and aging men through the administration of antioxidant protective agents, and specifically VK molecules, through diet or supplementations.

Key issues

- Potential benefits of the VK (studied > 30 years), in postmenopausal women on health bone/risk of fractures/bone forming and resorption markers;
- Intervention studies have shown that the VK provides significantly improved levels of uc-OCN in post-menopausal women with normal BMD;
- Inconsistent results in women with low BMD;
- None study exhibits any improvement of bone resorption/formation markers, 25OHD levels. Not sufficient EBM data for supplementation of VK in the prevention of osteoporosis in healthy post-menopausal women receiving vitamin D plus calcium.
- No improvement on BMD demonstrated in most studies.
- No interventional study on the assessment of fracture risk.
- Studies on the isolated effects of menatetrenone (VK2/MK4) showed a significantly improved of OCN, but inconsistent results on bone alkaline phosphatase, N-terminal telopeptide of collagen 1 (NTX,) and urinary markers.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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