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The Role of Vitamin K in Bone Remodeling and Osteoporosis

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Abstract

Vitamin K is an essential fat soluble vitamin involved in the regulation of normal coagulation. However, growing evidence highlights that this molecule appears to be also implicated in the regulation of other important biological functions such as bone mineralization, calcium homeostasis, apoptosis, cell growth and signal transduction. In particular, many studies have focused their attention on the protective effects of vitamin K on bone tissue in the outlook of its use in the prevention and treatment of osteoporosis in post-menopausal women. The objective of the present paper is to review data of the literature regarding the metabolic effects of Vitamin K in bone tissue and its clinical role in the prevention and treatment of osteoporosis.

Keywords: vitamin K, bone metabolism, glutamic acid, osteocalcin, osteoporosis

Abbreviations

OC, osteocalcin; GLA, matrix γ-carboxyglutamic acid; MGP, matrix γ-carboxyglutamic protein; PK, phylloquinone; MKS, menaquinones; MK-n, menaquinone-n; VKD; Vitamin K-dependent; Glus, glutamic acid residues; Glas, carboxylated Glus; KH2, K quinol; uoOC, carboxylated form of OC; COC, carboxylated OC; TOC; total carboxylated OC; DPD deoxypyridinoline crosslink; BMD bone mineral density; GLY, glycine (GLY); ARG, arginine; BAP, alkaline phosphatise; sNTX, N-telopeptides of type I collagen; Moabs, monoclonal antibodies.

1. Introduction

Vitamin K belongs to a family of structurally related fat-soluble compounds, which have a common 2-methyl-1, 4-naphthoquinone nucleus but differ in the structures of a side chain at the 3-position. To date two naturally-occurring forms of vitamin K have been identified and designated as vitamins K1 and K2. Vitamin K1, also known as phylloquinone (PK), is a 2-methyl-3-fitil-1,4-naphthoquinone. It is synthesized by plants and algae (Booth 2012). PK is the predominant form present in the diet and the only natural vitamin K available for therapeutic purposes (Figure 1). Vitamin K2 comes from animal sources. It may be synthesized by intestinal bacteria or may be formed following the metabolism of dietary phylloquinone in the liver or extrahepatic tissues. Vitamin K2 comprises several forms of vitamin K called menaquinones (MKS). These forms are designated as menaquinone-n (MK-n) according to the number (n) of prenyl units present in the molecule, which may vary in lenght between 1 to 14 units (MK-1 to MK-14) (Majerus & Tollefsen, 2006). PK is mainly present in green leafy vegetables and vegetable oils, while MKS can be found mainly in animal products such as meat, eggs, butter, cheese and fermented soy products (Kaneki, Hosoi, Ouchi, & Orimo, 2006). It is to mention that there is also an another synthetic form vitamin K, namely, vitamin K3 or menadione (Meganathan & Bentley, 1982) (Figure 1). However, this molecule is not even a vitamin but rather a precursor form that is converted in active vitamin K2 in the body. In mice, PK is converted into MK-4 following the removal of an integral side chain (Nakagawa, 2010; Shearer & Newman, 2008; Thijssen & Drittij-Reijnders, 1994; Yamamoto, Komai, Kojima, Furukawa, & Kimura, 1997; Okano et al., 2008). In this form MK-4 accumulated in the brain. Two paths by which PK can be converted to MK have been described so far (Nakagawa, 2010). One of these pathways involves the formation of an intermediate compound, the so called intestinal K3-d7, from PK (PK-d7) followed by its prenylation to form MK-4 (MK4-d7). The reaction of prenylation consists in a modification of the covalent binding of isoprenoid units of 15-20 carbon atoms to the C-terminal residues. The second pathway involves the release and prenylation of K3-d7. Both these reactions occur in the brain (Okano et al., 2008; Nakagawa, 2010). It has been shown that,

in humans, menadione is a catabolic product of different forms of vitamin K (Thijssen, Vervoort, Schurgers, & Shearer, 2006).

2. Mechanism of Action of Vitamin K

2.1 The γ-carboxylation Reaction

Vitamin K is as a essential cofactor for the post-translational modifications induced during the biosynthesis of vitamin K-dependent proteins. These modifications consist in the carboxylation of glutamate to γ-carboxyglutamic acid (Gla) (Figure 1) in order to confers calcium-binding properties to vitamin K-dependent proteins (La Guardia, Giammanco & Giammanco, 2010; Berkner, 2008).

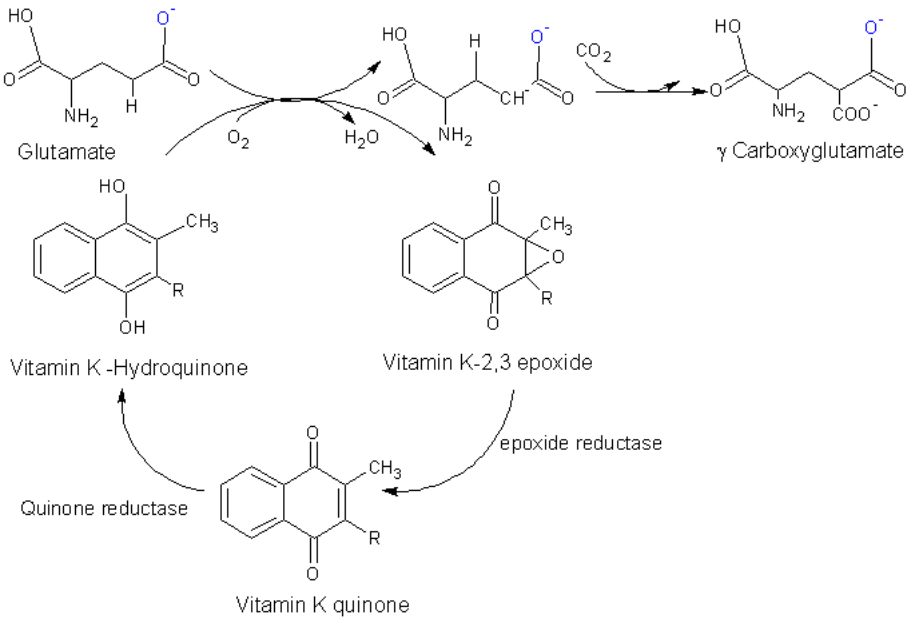

Figure 1. γ-carboxylation reaction

To date, two distinct groups of Gla-containing proteins have been identified, i.e., those involved in blood coagulation such as prothrombin, factors II, VII, IX and X (Suttie, 1985), the γ-carboxy-glutamic form of the anticoagulant proteins C and S, and those found in calcified tissue such as bone protein osteocalcin (OC), matrix GLA protein (MGP), growth arrest-specific 6 protein (Gas6) and four proteins endowed with a single transmembrane domain whose functions have still not fully understood (Brown, Stenberg, Persson, & Stenflo, 2000; Kulman, Harris, Xie, & Davie, 2001). The identification of proteins with carboxylated glutamic acid residues involves the production of monoclonal antibodies (Moabs) which selectively recognize the γ-carboxy-glutamic residues present in specific proteins and peptides. These antibodies, as demonstrated by western blot and immunofluorescence assays, are specific for most of the proteins containing glutamic acid residues including those mentioned above. In addition, these Moabs may also recognize other proteins such as conantokin G, a protein present in the cone snails, and a protein similar to the X factor present in the snake venoms (Brown, Stenberg, Persson, & Stenflo, 2000). The carboxylation of Vitamin K-dependent (VKD) proteins uses vitamin K epoxidation to convert glutamic acid residues (Glus) into carboxylated Glus (Glas) thus allowing VKD proteins. This modification make these proteins able to actively modulate several important physiological processes including hemostasis, apoptosis, bone mineralization, calcium homeostasis, cell growth, and signal transduction (Booth, 2012). The physiologically active form of vitamin K involved in triggering γ-glutamyl carboxylation is the reduced-form vitamin K quinol (KH2). The enzyme, in presence of O_2, CO_2, and glutamate-containing substrate, catalyses the γ- carboxylation of glutamic acid residues on vitamin K-dependent protein (Gla protein) and, at the same time, the formation of vitamin K 2,3-epoxide (Rishavy et al., 2004) (Figure 1). The reaction of γ-carboxylation is facilitated by the so called “GLA domain”, that contains post-translational modifications of glutamate residues induced by vitamin K-dependent carboxylations to form gamma-carboxyglutamate (Gla). (Booth, 2012). The "Gla" domain present in the coagulation factors contains 45

amino acids and it is located near the the N-terminal amino acid of proteins. The carboxylation reaction is triggered by the interaction between a region of the adjacent protein generally constituted by 18 to 28 amino acids and the enzyme carboxylase. In the case of coagulation proteins, the number of residues of glutamate that undergo γ-carboxylation are comprised between 9 and 13 (Majerus & Tollefsen, 2006). Vitamin K hydroquinone is then regenerated by a coumarin-dependent 2,3-epoxide reductase which, similarly to γ-glutamyl carboxylase, is an integral membrane protein of the endoplasmic reticulum (Wajih, Sane, Hutson, & Wallin, 2004). During the process of reduction of vitamin K from epoxide to hydroquinone, the active sites of the thiol 2,3-epoxide reductase are oxidized. This results in an inactivation of the enzyme whose activity is regenerated by a unknown reductase (Rishavy, Usubalieva, Hallgren, & Berkner, 2011). The synthesis of reduced vitamin K is a rate limiting factor of the carboxylation reaction in cells, while the overexpression of the 2,3-epoxide reductase appears to facilitate this process. However, because of the saturation of the reductase responsible for its activation, the increased activity of the enzyme is negligible (Berkner, 2008). Both γ-glutamyl carboxylase and 2,3-epoxide reductase act as a multifactorial system which also includes the protein "chaperone" calumenin. Experimental observations show that, in an in vitro system of γ-carboxylation, which comprises γ-glutamyl carboxylase and 2,3-epoxide reductase, calumenin is associated with the γ-glutamyl carboxylase and inhibits the activity of this latter enzyme. In fact, the silencing of the gene encoding calumenin by siRNA results in, at least, a 5 fold increase in the activity of γ-glutamyl carboxylase (Wajih et al., 2004).

2.2 Vitamin K γ-carboxylation-independent Effects

It is well established that one of the main characteristic of vitamin K is its antioxidant properties (Vervoort et al., 1997). These observations have lead to utilize MKS to protect neuronal cells from apoptosis. (Li et al., 2003) Both PK and MK-4, at 30 nM and 2nM concentrations respectively, have been reported to inhibit oxidative stress induced cell death in oligodentrocyte precursors (Li et al., 2000). These findings suggest that the antioxidant activity of MK-4 is at least 15 times more potent than that of PK (Li et al., 2003). In vitro studies show that PK is mainly distributed at microsomial level, where γ-carboxylation occurs, while MKS are mainly present in mitochondria where redox reactions of the electron transport chain take place (Reedstrom et al., 1995; Georgellis, Known, & Lin, 2001). An additional biological effect of MKS is that to bind the enzyme 17β-hydroxysteroid dehydrogenase type 4, thereby modulating estrogen metabolism (Otsuka et al., 2005). These observations indicates that, unlike PK, MKS, in addition to the γ-carboxylation, may be also endowed with many "γ-carboxylation independent" functions which which may also account for the beneficial effects of MSK on health K and bone tissue (Kaneki, Hosoi, Ouchi, & Orimo, 2006).

3. Effects of Vitamin K on Bone Metabolism and Osteoporosis Prevention

Several studies indicate that Vitamin K may have a pivotal role in bone tissue homeostatis. This hypothesis is supported by the observations that vitamin K appears to be actively implicated in the carboxylation of glutamic acid residues of proteins which are present in bone tissue including osteocalcin (OC) and Protein S (Price, 1985; Hauschka et al., 1989; Maillard et al., 1992). The carboxylated residues of glutamic acid bind calcium and allow the binding of these proteins to hydroxyapatite present in the extracellular matrix. In the absence of this modification, OC lacks of structural integrity and cannot bind the mineral hydroxyapatite. As consequence, Vitamin K deficiency impairs OC γ-carboxylation as indicated by increased levels of circulating non carboxylated form of OC (uoOC) and a decrease in the ratio between carboxylated OC form (COC) and total OC (TOC) (Iwamoto et al., 2009). In healthy adult subjects, only a small fraction of coagulation factors is decarboxylated while most of circulating OC appears to be decarboxylated (Shearer, 2000; Bugel, 2003). The circulating form of uoOC closely reflects vitamin K status (Price, 1988). In fact serum levels < 4.0 ng/ml are indicative of a Vitamin K deficiency. On the other hand, it has been demonstrated that in post-menopausal women with serum levels of ucOC ≥ 4.0 ng/ml are associated with lower concentrations of vitamin K and higher levels of bone resorption markers, such as deoxypyridinoline crosslink (DPD), and with an increased incidence of vertebral fractures (Shiraki, Aoki, & Shiraki, 2002). Differences in the activity of γ-glutamyl carboxylase among the common genotypes may correlate with bone mineral density (BMD). In this context, clinical studies carried out on Japanese elderly women have shown a genetic polymorphism at a single nucleotide level which is the consequence of the substitution of glycine (GLY) in position 325 with arginine (ARG). Since the carboxylase activity of GLY325 is higher that of ARG, the carboxylation of Gla proteins is more efficient in GLY325 (Kinoshita et al., 2007). Therefore, the polymorphism of γ-glutamyl carboxylase may affect the carboxylase activity and the pharmacological activity of vitamin K. (Kimura et al., 2006) These findings may also, in part, explain the different degree of susceptibility of some subjects to the therapeutic response of vitamin K treatment (Iwamoto et al., 2009). Another important function of OC has been highlighted during some studies on GPRC6A receptor, a member of calcium-sensitive extracellular receptors family. These receptors are G-coupled proteins

which play a role in the regulation of calcium homeostasis (Pi et al., 2005; Pi & Quarles, 2012). In mice, the GPRC6A receptor is expressed in several tissues including bone and osteoblasts (Pi & Quarles, 2012). Interestingly, OC stimulates the activity of this receptor, although it may inhibits Ca Ca^{+2} - dependent activation of this receptor. It is likely that GPRC6A may function as a cation-sensitive receptor for OC (Pi & Quarles 2012). Therefore, it may be considered a potential "target" for calcium sensitive molecules that mediate extracellular responses in osteoblasts (Pi et al., 2005; Pi & Quarles, 2012). As mentioned above, some studies suggest that MKS, but not PK, may be endowed with biological functions which are not related to its γ-carboxylation activity. In fact, there is evidence that these molecules may also act as ligands for "orphans nuclear receptors", steroid receptors, and xenobiotic and pregnane-X receptors. Although the significance of these biological interactions needs to be better defined, experimental studies indicate that they probably contribute to increase OC production (Tabb et al., 2003). These observations are consistent with the findings that, despite its effects on γ-carboxylation, PK has a low affinity for these receptors. Similar results were obtained in vitro with MKS (Spronk et al., 2003). In fact, this molecule has been also shown to accelerate the hydroxylation of proline, an essential precursor for the formation of cross links of mature collagen which may induce marked changes in many of the structural parameters of the femoral trabecular bone (Matsumoto et al., 2009). Other studies have shown that vitamin K2 inhibits bone resorption by blocking the synthesis of PGE2. In addition, MKS side chain has been reported to play an important role in the inhibition of bone resorption (Hara, Akiyama, Tajima, & Shiraki, 1993; Hara, Akiyama, Nakamura, Murota, & Morita, 1995). These findings are in line with the observations that vitamin K2 may also inhibit the expression of NF-KB in osteoclasts (Yamaguchi et al., 2011; Takeuchi, Suzawa, Fukumoto, & Fujita, 2000) and may induce apoptosis in these cells (Yamaguchi et al., 2011; Kameda et al., 1996). It has also been hypothesized that the mechanism underlying the inhibitory effects of vitamin K2 on bone resorption may be independent from its γ-carboxylation activity, and are correlated to side chain (Notoya, Yoshida, Shirakawa, Taketomi, & Tsuda, 1995). Hara, Akiyama, Nakamura, Murota, & Morita, 1995). This hypothesis is supported by the observations that MKS inhibits in vitro bone resorption (Iwamoto, Takeda, & Sato, 2006).

3.1 Relationship between Vitamin K and Osteoporosis

The results mentioned above are suggestive of a possible relationship between the reduced expression or even the lacking of vitamin K and osteoporosis in post menopausal women. It is well known that in subjects older than 30 years, the bone undergoes a physiological reduction (Feng & McDonald, 2011). However, an excessive reduction of bone mass lead to osteoporosis and may facilitate the onset of bone fracture. (Feng & Mc Donald, 2011) Although this phenomenon occurs mainly in women after the menopause it may be observed also in male (Khosla, 2010; Adler, 2011). There is strong evidence that in women estrogens deficiency may contribute to the onset of this bone disorder (Feng & McDonald, 2011). However, low levels of serum vitamin D and a reduced intake and/or intestinal absorption of calcium may also contribute to the onset of osteoporosis in post-menopausal women In fact, hypovitaminosis D increases the risk of fractures following the onset of hyperparathyroidism to counteract this phenomenon (Nuti, Merletti, & Gennai, 2011; Nakano et al., 2011). As consequence these effects further contributes to the decrease of bone mineral density (BMD). In 1984, a case-control study, performed in subjects with femoral neck fractures and low levels of circulating vitamin K1 (Hart et al., 1984), demonstrated that subjects with low serum concentrations of vitamin K1 had a bone mineral density (BMD-DXA) lower than that measured in subjects not affected by vitamin K deficiency (Hart et al., 1985). These data, suggested that that low concentrations of vitamin K and the reduced BMD may be considered useful as markers of bone tissue damage. On the other hand both these parameters may account for the increased risk of bone fractures (Knapen, Schurgers, & Vermeer, 2007). Clinical studies reported that subjects administered vitamin K antagonists had low levels of DXA-BMD and showed an increased risk of fractures (Caraballo et al., 1999; Barnes et al., 2005; Gage, Birman-Deych, Radford, Nilasena, & Binder, 2006). However, a drawback in these studies consists in that investigations specifically directed also at assessing also the correlation with the intake of MKS are lacking. In fact, although in the western countries MKS represent only 10-20% of the total intake of vitamin K (Schugers et al., 1999 & 2000), they are absorbed by the food matrix more extensively than vitamin K1 (Shearer & Newman, 2008). Furthermore, vitamin K2 is also preferentially taken up by extrahepatic tissues such as bones and blood vessels (Shearer & Newman, 2008). Thus, the role of circulating PKs as a marker of altered bone metabolism remains still questionable (Knapen, Schurgers, & Vermeer, 2007). On the other hand, several investigations have been carried out in order to clarify the relationship between vitamin K deficiency and osteoporosis and to assess the relationship between biochemical markers of vitamin K deficiency and risk of fractures and/or BMD (Fang, Hu, Tao, Wan, & Tao, 2012). Some of these studies have shown that increased ucOC serum levels correlate with the increase of the incidence of fractures (Kim et al., 2012; Szulc, Chapuy, Meunier, & Delmas, 1993; Szulc, Chapuy, Meunier, & Delmas, 1996; Vergnaud et al., 1997) and with a low BMD (Szulc et al., 1994). In particular, the EPIDOS study, performed on

7598 healthy elderly women over an observation period of 22 months, showed that the circulating levels of ucOC and not those of TOC are crucial to predict the risk of fractures (Vergnaud et al., 1997). Furthermore, in postmenopausal women with osteoporosis, urinary concentrations of PYR and DPD were observed to be higher during the day and in the night without any increase a during the night/early morning period (Eastell et al., 1992). In fact, their excretion was significantly more elevated in those pathological conditions, such as in the case of primary osteoporosis, that are associated with an increased osteoclast activity (Delmas, Schlemmer, Gineyts, Riis, & Christiansen, 1991; Eastell et al., 1992; Eastell, Colwell, Hampton, & Reeve, 1997). In postmenopausal women with osteoporosis, the increased excretion of the DPD and PYR cross links has been shown to be inversely correlated with DXA-BMD of the hip, spine, vertebrae and forearm, (Civitelli, Armamento-Villareal, & Napoli, 2009; Melton, Khosla, Atkinson, O'Fallon, & Riggs, 1997). In a large cohort of older women, free DPD excretion above the upper normal limit, as determined in premenopausal women, was associated with an increased risk of hip fracture (Garnero et al., 1996). Although many studies have shown a significant correlation between risk of bone fractures and vitamin K levels (Hodges et al., 1991; Kaneki et al., 1995; Feskanich et al., 1999; Booth et al., 2003), the correlation between circulating vitamin K and BMD appears to be somewhat controversial (Kaneki, Hosoi, Ouchi, & Orimo, 2006). In fact, the correlation between the rate of vitamin K1 intake of and risk of fracture is much more elevated than that observed between this vitamin and BMD (Booth et al., 2003). This discrepancy can be, in part, explained with the differences in the methods of measurement used and type of subjects enrolled for this studies (Booth et al., 2003). Moreover, although most of the studies show an inverse relationship between circulating ucOC and BMD, it is noteworthy to point out that the increase in ucOC serum makes these subjects more susceptible to a risk of fracture that is independent from BMD values (Vergnaud et al., 1997; Yasui et al., 2006). This suggests that vitamin K deficiency may increase the probability of bone fractures by both BMD-dependent and BMD-independent mechanisms.

4. Vitamin K and Osteoporosis: Animal Studies

The effects of vitamin K on bone remodeling were studied in rats in which osteoporosis was experimentally induced by different means such as ovariectomy, orchiectomy, glucocorticoids, removal of the sciatic nerve, tail suspension and calcium or magnesium deficient diet.

4.1 Effects of a Single Treatment with Vitamin K2 on Ovariectomized Rats

The estrogen deficiency caused by ovariectomy in rats results in a loss of bone mass due to an increase in bone turnover (Yoon et al., 2012).In these animals, the administration of MKS is essential. In this regard, many studies have demonstrated that vitamin K2 is able to prevent the decrease of femoral BMD by 1) inhibiting osteoclast induced bone resorption (Booth et al., 2003; Akiyama, Hara, Kobayashi, Tomiuga, & Nakamura, 1999), 2) by increasing osteoblastic activity at the femoral metaphysis level (Booth et al., 2003; Asawa et al., 2004, Yamaguchi, 2011), 3) by preventing the loss of trabecular bone mass (Iwamoto, Matsumoto, Takeda, Sato, & Yeh, 2010) and 4) by reducing the loss of bone mineral content at the level of the lumbar vertebrae (Xin, Takemitsu, & Atsuta, 2001). MKS have been shown to be also effective in preventing the decrease of bone strength of the femoral diaphysis in ovariectomized rats (Shiraishi et al., 2002). However, some studies have shown that vitamin K2 is not able to affect the increase of bone turnover in ovariectomized rats (Fu et al., 2012). At the same time it does not reduce femoral BMD and bone strength of lumbar vertebral and femoral bones (Iwamoto, Takeda, & Sato, 2006). Taken together, the results indicate that MKS are involved in the regulation of bone metabolism and in maintaining the trabecular architecture of bone in ovariectomized rats.

4.2 Combined Treatment with Vitamin K2 and Vitamin D3 in Ovariectomized Rats

To date, few studies aimed at assessing the efficacy of this type of treatment on the prevention of osteoporosis have been performed (Matsunaga, Ito, & Sakou, 1999; Hara, Kobayashi, & Akiyama, 2001). These investigations have reported that this combination appears useful in preventing loss of bone mass at the level of proximal tibia metaphysis and in increasing bone strength of the femoral diaphysis in ovariectomized rats. These observations support the concept of a beneficial effect of these drug combination on the prevention of osteopenia in ovariectomised animals (Iwamoto, Takeda, & Sato, 2006). Interestingly recent experimental studies have shown that the association of Vitamin K and D stimulates in vitro osteoblast differentiation in fracture site derived human mesenchymal stem cells (Gigante et al., 2008). These findings are suggestive of an additional target for reparative treatments.

4.3 Combined Treatment with Vitamin K2 and Bisphosphonates on Ovariectomized Rats

Bisphosphonates are potent inhibitors of bone resorption (Russell, Watts, Ebetino, & Rogers, 2008). In humans, these molecules have been shown to increase bone density and reduce the risk of hip fractures and that of spine and other bone sites (Russell, Watts, Ebetino, & Rogers, 2008). Alendronate, risedronate, ibandronate and

zoledronic acid, are some of the molecules approved for the treatment of osteoporosis (Bikle, 2007; Räkel, Boucher, & Ste-Marie, 2011). Risedronate has been proven useful in preventing the deterioration of the trabeculae of bone in the proximal tibial metaphysis which result in an increase of trabecular thickening (Ito, 2002). However, few studies have been designed to investigate the effectiveness of the association vitamin K2-bisphosphonate in ovariectomized rats in this case too. These studies have demonstrated a preventive effect of this combination on the deterioration of the three-dimensional architecture of trabecular bone (Iwamoto, Takeda, & Sato, 2006). However recent observations by Matsumoto et al. (2009) showed that a prior treatment with MK-4 followed by risedronate significantly increased femur strength in comparison to the OVX controls. More recently Sasaki et al. (2010) results have shown that the administration of alendronate alone or in combination with vitamin K(2), induced a significant improvement in BMD. In addition the combination treatment was more effective than alendronate given as single agent in improving bone strength in OVX mice.

4.4 Combined Treatment with Vitamin K2 and Raloxifene on Ovariectomized Rats

In humans, several estrogen-like drugs have been used for the treatment of postmenopausal osteoporosis, including raloxifene which, interestingly, has been shown to exert a protective effect on vertebral fractures, but not on hip fracture. In vivo studies have reported that, in ovariectomized rats the administration of MK as single agent, increases osteoblastic activity, while raloxifene, administered alone or in combination ,decrease the rate of bone turnover (Iwamoto, Takeda, & Sato, 2006). Unlike vitamin K2, which appears to be more effective when associated to other drugs, raloxifene may prevent bone loss at both proximal and distal metaphysis. The combinations of the two drugs resulted in an increased bone strength of the femoral neck have complementary effects on bone resorption and induce a significant improvement of the femoral neck strenght (Iwamoto et al., 2005). In this context recent observations of Tasci et al. (Tasci, Bilgili, Altunay, Gecit, & Keskin, 2011) highlighted that Vitamin K2, given as single drug single or co-administered with Raloxifene was effective in preserving bone mechanical properties upon osteoporosis. Interestingly, Vitamin K2 was observed to also minimizedthe adverse effect of Raloxifene which lead to liver dysfunction.

4.5 Effect of Vitamin K2 in Rats Undergoing Orchiectomy

In males testosterone play a key role in promoting bone growth during the growth period and it is responsible for the maintenance of skeletal mass after development (Bertelloni, Baroncelli, Battini, Perri, & Saggese, 1995; Guo, Jones, & Eastell, 1997; Behre, Kliesch, Leifke, Link, & Nieschlag, 1997). The lack of steroid hormone production following orchiectomy, is responsible for the increased rate of bone turnover and a decrease of osteogenesis (Erben, Eberle, Stahr, & Goldberg, 2000; Prakasam et al., 1999). Testosterone deficiency also induces a cortical and spongy osteopenia due to the high turnover in trabecular and endocortical bone and in the proximal tibial metaphysis (Iwamoto, Yeh, Takeda, Ichimura, & Sato, 2003). The administration of MKS in these animals suppresses the turnover of trabecular bone and endocortical bone resorption, thereby alleviating the development of osteopenia (Iwamoto, Takeda, & Sato, 2006). The effect of vitamin K2 on the spongy osteopenia appeared to be i mediated primarily by its ability to decrease the reduction of the trabecular thickness in orchiectomized animals (Iwamoto, Takeda, & Sato, 2006).

4.6 Effects of Vitamin K2 in Rats Treated with Glucocorticoids

It is well established that glucocorticoids (GCs) treatments may cause to numerous clinical complications, including bone loss and increased risk of fracture (Feng & McDonald, 2011). The administration of glucocorticoids in rats causes a decrease of the osteoblastic activity (Feng & McDonald, 2011). This phenomenon leads to a decreased osteogenesis and, consequently, may cause the onset of cortical and spongy osteopenia of the tibia. Prednisone has been shown to cause a reduction of the tibial and femoral length and a decrease of bone density, bone strength and calcium content. These effects can be prevented by vitamin K2 treatment (Hara, Akiyama, Ohkawa, & Tajima, 1993; Hara, Kobayashi, Hara, & Akiyama, 2002). In line with these studies, Iwamoto et al. (2009) have recently shown that vitamin K (2) reduces the suppression in glucocorticoids treated rats-t The mechanism by which vitamin K2 induced GC bone loss appears to be due to its ability in preventing the reduction of osteoprotegerin (OPG) a cytokine which inhibits both the differentiation and functions of osteoclasts (Sasaki et al., 2005; Boyce & Xing, 2007). These findings further support the clinical value of Vitamin K2 in the treatment and prevention of GC induced osteoporosis.

4.7 Effect of Vitamin K2 in Rats Subjected to the Removal of the Sciatic Nerve

The immobilization of the hind limbs following sciatic neurectomy enhances osteoclast activity which results in a decreased osteogenesis (Iwamoto, Matsumoto, Takeda, Sato, & Yeh, 2010). This phenomenon leads to the onset of spongy and cortical osteopenia in the hind limbs in rats (Iwasaki et al., 2002a; Iwamoto, Yeh, & Takeda, 2003; Iwasaki-Ishizuka et al., 2003). In fact, the removal of the sciatic nerve is associated with a transient

increase in bone resorption and a resulting decrease of spongy bone mass in the proximal tibial metaphysis (Iwasaki et al., 2002), and a decrease of bone mineral density in the distal metaphysic of the femur and femoral diaphysis (Iwasaki-Ishizuka et al., 2003).The administration of vitamin K2 has been shown to prevent these effects (Iwasaki et al. 2002; Iwamoto, Yeh, & Takeda, 2003; Iwasaki-Ishizuka et al., 2003), Iwamoto, Matsumoto, Takeda, Sato, & Yeh, 2010). In fact, vitamin K2 suppresses osteoclast activity and lowers the reduction of trabecular thickness, which results in a reduced osteopenia (Iwamoto, Matsumoto, Takeda, Sato, & Yeh, 2010; Iwamoto, Takeda, & Sato, 2006). Other studies shown that in neurectomized rats, vitamin K2 causes an increase of BMD and that of serum γ-carboxylated OC (Iwasaki-Ishizuka et al., 2005). These results suggest that the administration of MKS may improve osteopenia by acting at osteoclastic level whose activity is reduced, and by accelerating γ-carboxylation reaction of OC in neurectomized osteopenic rats (Iwamoto, Takeda, & Sato, 2006; Iwamoto, Matsumoto, Takeda, Sato, & Yeh, 2010).

4.8 Effects of Vitamin K2 in the Rat Tail-suspension Model

The "tail-suspending" increases bone resorption and induces a decrease of of osteogenesis in cancellous bone and cortical bone in rat (Zhang et al., 1995). These effects results in a spongy and cortical osteopenia of the hind limbs (Kodama et al., 1997; Moriyama, Iwamoto, Takeda, & Toyama, 2002; Iwasaki et al., 2002b). The suppression of osteogenesis seems to play an important role in the induction of bone resorption (Morey & Baylink, 1978). In this case, the administration of vitamin K2 by antagonizing osteoclast activity may decrease bone resorption (Iwamoto, Takeda, & Sato, 2006). In particular, MKS counteract the loss of BMD and that of bone mass at the level of tibial spongy bone (Iwamoto, Takeda, & Sato, 2006; Iwasaki et al., 2002b).

4.9 Combined Treatment with Vitamin K2 and Bisphosphonate in "Tail-suspended" Rats

Studies by Iwasaki et al. (2003) demonstrated that, in rats tail-suspended bisphosphonates reduce the loss of cancellous bone tissue through a marked suppression of bone turnover at the level of the metaphysis of the proximal tibia.The combination of these drugs with MKS results in a further reduction of osteolysis and in an increased osteogenesis, as compared with bisphosphonates given as single drug (Iwamoto, Takeda, & Sato, 2006; Iwamoto, Matsumoto, Takeda, Sato, & Yeh, 2010).

4.10 Effects of Vitamin K2 in Rats Fed Diets Deficient in Calcium

It is well known that a marked reduction of calcium causes a decrease of bone mass in rats (Rude, Singer, & Gruber, 2009). Numerous studies that have reported the beneficial effects of MKS on osteopenia and calcium homeostasis in rats with calcium deficiency A diet low in calcium (80-100 mg /day versus 800-1200 mg /day) reduced femoral BMD in rats by 12%. This effect is thwarted by the administration of MKS (Kato et al., 2002). Other studies show that calcium deficiency (100 mg/day versus 500 mg/day) in rats causes hypocalcemia associated with an increase in serum parathyroid hormone (PTH) levels, 1.25-dihydroxyvitamin D (calcitriol) and in a decrease in 25-hydroxyvitamin D serum levels. The increased of calcitrol levels lead to an enhanced absorption of calcium from the intestine. On the other hand, PTH facilitates the renal resorption of calcium while, at the bone tissue level, this phenomenon results in a reduced cortical bone mass of the tibial diaphysis, with enlargement of the medullary cavity. The administration of MKS in these animals lead to an increase reabsorption of renal calcium and in a delay in the increase of PTH serum levels. Since MKS does not induce any substantial change in the concentrations of calcitriol, the intestinal absorption of calcium is not stimulated. Vitamin K2 also reduces the cortical bone loss at tibial level by a mechanism closely related to the inhibiton of bone resorption (Iwamoto, Takeda, & Ichimura, 2003). The onset of hypercalciuria in calcium-deficient rats which are also vitamin K deficient, may be counteracted by MKS administration (Robert et al., 1985). Finally, a diet deficient in calcium and magnesium (calcium: 100mg/day versus 500 mg/day in controls; magnesium: 30 mg/day versus 100 mg/day in controls) induced a decrease in serum levels of these ions and that of femoral bone mass, while PTH and renal excretion of calcium are increased. These alterations may be counteracted by vitamin K2 administration (Kobayashi, Hara, & Akiyama, 2002). These findings suggest that MKS may play an important role in calcium homeostasis, in particular, by facilitating the stimulation of renal calcium resorption and by reducing osteolysis induced by calcium-deficiency (Sogabe, Maruyama, Baba, Hosoi, & Goseki-Sone, 2011).

4.11 Effects of Vitamin K2 in Rats with Magnesium Deficiency

Magnesium deficiency reduces bone strength in the femur with no significant changes in the cortical BMD and cortical thickness (Rude, Singer, & Gruber, 2009). The administration of MKS in these rats does not alter the value of femoral BMD or the thickeness of bone tissue. However it inhibits the reduction of bone strenght suggesting that vitamin K2 may be used for the maintenance of this parameter in Mg-deficient rats (Kobayashi, Hara, & Akiyama, 2004). Study undertaken on 9 weeks old ovariectomized mice, pretreated with risedronate

(0,25 mg/kg /day up to 8 weeks) show that vitamin K2 administered as MK-4 (100mg/kg/dayup to 8 weeks) improves its effectiveness. On the other hand, risedronate, co-administered with estrogens or raloxifene, is more effective in comparison with a single drug treatment. As expected, ovariectomy results in a decrease of BMD and BMC and cortical thinning of the cortical thickness. In this study bone strength of the femoral shaft and the resistance to compression of the distal metaphysis of the femur have been also tested. The treatment with MK-4 alone does not significantly increase the mineral content and bone density unless this drug was associated to risedronate. The animals undergone pretreatment with vitamin K show the highest response rate (Matsumoto et al., 2009). In mice genetically deficient for OC, vitamin K also leads to an increase in bone mass (Ducy et al., 1996), suggesting that the osteoprotective activity of vitamin K is also due to mechanisms which are indepented from its γ-carboxylation activity (Matsumoto et al., 2009). In fact vitamin K, is known to stimulate the active transcription of osteoblast genes, steroid receptor, xenobiotic receptor and pregnane, receptors expressed in the extracellular matrix which mediate gene transcription in cooperation with ERα, one of the two estrogen receptors (Igarashi et al., 2007).

5. Vitamin K and Osteoporosis

In Japan, Korea, Thailand, Taiwan, vitamin K is widely used to treat osteoporosis (Kaneki, Hosoi, Ouchi, & Orimo, 2006). The major concerns in its clinical use regards the type of molecule (PK, MK-4 or MK-7) and the dosage to be administered. In a double blind study, post-menopausal women were administered a placebo or food supplement containing 500 mg of calcium, 10 mg of zinc, magnesium and 150 mg of 8 g of vitamin D, or a dietary supplement that, in addition to vitamin D, contained also vitamin K1 at a dose of 1mg/day. BMD values were monitored for up to 3 years. The results of these studies showed that, unlike in patients receiving placebo, the supplement containing mineral salts, vitamin D and PK significantly decreased the loss of bone mass in the neck of the femur and that of minerals associated with vitamin D. Furthermore, the administration of PK, at the dose level of 1mg/day, significantly reduced ucOC serum levels (Braam et al., 2003). Other clinical studies showed that patients suffering of osteoporosis treated with increasing doses of MK-4, namely15, 45, 90 and 135 mg/day respectively, and vitamin D3 at 0.75 g/day, showed a significant increase in the urinary excretion of Gla residues, a markers of vitamin K-dependent γ-carboxylation, compared to vitamin D3. However, the urinary excretion of Gla residues was more pronounced in those patients receiving higher doses of MK-4. These observations highlighted that the oral administration of 15 mg/day of MK-4 is not sufficient to achieve maximal production of Gla residues in vitamin K-dependent patients suffering from osteoporosis. The minimal effective concentrations for the treatment of osteoporosis has been established to be 45 mg. (Iwamoto et al., 2009). Vitamin K2 administered as menatetrenone, was not only effective as a dietary supplement, but also as drug, being its optimal dose (45 mg/day) for the treatment of osteoporosis, about 400 times higher than the suggested amount of the daily dose intake (Orimo, Fujita, Onomura, Inoue, Kushida, & Shiraki, 1992; Kaneki, Hosoi, Ouchi, & Orimo, 2006; Iwamoto et al., 2009). In patients with osteoporosis, the combination of MK-4 (45 mg /day) and calcium (150 mg /day), reduced the age-related decrease of bone BMD and the incidence of fractures, as compared to calcium supplementation alone (Iwamoto et al., 2009). In addition, a decrease of ucOC serum levels and an increase of TOC levels was noted (Shiraki, Shiraki, Aoki, & Miura, 2000). Other studies further confirmed these observations (Iwamoto, Yeh, & Takeda, 2003; Kaway & Ishida, 2004). Furthermore, clinical investigations have shown that vitamin K2 reduced the incidence of fractures in patients with glucocorticoids induced osteoporosis (Yonemura, Kimura, Miyaji, & Hishida, 2000; Inoue, Sugiyama, Matsubara, Kawai, & Furukawa, 2001; Tanaka & Oshima, 2001; Iwamoto, Matsumoto, Takeda, Sato, & Yeh, 2010) and increases BMD in the metacarpal bone and in the upper extremities in patients with postictal paralysis or elderly women with Parkinson's disease (Sato, Honda, Kuno, & Oizumi, 1998; Sato et al., 2002). Moreover, Vitamin K2 administration has been shown to decrease the incidence of fractures in elderly women with Alzheimer's disease who underwent treatments with supplements of calcium and vitamin D2 (Sato, Kanoko, Satoh, & Iwamoto, 2005). Finally, Vitamin K2 treatment was able to maintain lumbar BMD in patients with osteoporosis associated to liver diseases (Shiomi et al., 2002). The effects of administration of 45 mg/day of MK-4 for three years have also been studied in post-menopausal subjects with no clinical evidence of osteoporosis. In these subjects, the indices of resistance of the femoral neck and the bone metabolism markers (t-OC, ucOC, c-OC, bone-specific alkaline phosphatase (BAP), N-telopeptides of type I collagen (sNTX), 25-hydroxy-vitamin D, urinary DPD, calciuria) were evaluated. These studies highlighted significant changes in the serum concentration of ucOC and COC. t-OC and BAP were also significantly more elevated in these subjects than in the control group. On the other hand, no effect wasobserved on bone resorption markers and BMD. On the other hand HAL appeared to increase slightly with age, either in presence or in absence of MK-4, while FNW did not show any changes. Vitamin K2 administration induced an increase in BMC in the hip and in femoral neck width in post-menopause patients, allowing the maintenance of bone strength. Positive effects of MKS administration were also observed

in a group of younger postmenopausal group of women (55-65 years) than in older ones (65-75 years). On the contrary, in control group, bone strength showed a significant decrease (Knapen, Schurgers, & Vermeer, 2007). A WHO report in 2003 attributed to MK-4 a second level degree of effectiveness regarding BMD and bone fractures, while estrogen and bisphosphonates showed a first level degree of effectiveness (WHO, 2003). However, MK-4 decreased the risk of vertebral fractures at the same extent as bisphosphonates (reduced osteoclast- induced bone resorption and promoted osteoblast-mediated bone formation (Iwamoto, Takeda, & Ichimura, 2001; Ishida & Kawai, 2004; Shiraki, Shiraki, Aoki, & Miura, 2000; Iwamoto, Takeda, & Sato, 2004); Adams & Pepping, 2005). When compared with other drugs such as estrogens and bisphosphonates, the effects of MK-4 on osteogenesis was predominant over osteolysis (Iwamoto, Takeda, & Ichimura, 2001; Shiomi et al., 2002). Several studies indicate vitamin K2 may be considered an attractive molecule for combination therapy in association with other food supplements, such as calcium and vitamin D, which may result in positive effects on DXA-BMD and in potentiating the effects of other drugs (bisphosphonates) (Iwamoto et al., 2009). In fact, the effects of vitamin K on BMD are lower when administered as single drug (Knapen, Schurgers, & Vermeer, 2007). This suggest that a better therapeutic response can be obtained when given in combination with bisphosphonates, calcium and/or vitamin D, because of synergistic effects elicited by this combination (Bolton-Smith, Mole, McMurdo, Paterson, & Shearer, 2001; Braam et al., 2003; Iwamoto, Takeda, & Sato, 2004); Knapen, Schurgers, & Vermeer, 2007; Hirao, Hashimoto, Ando, Ono, & Yoshikawa, 2008; Iwamoto et al., 2009). The most effective therapeutic approach was obtained by administering first vitamin K2 followed by risedronate (Matsumoto et al., 2009). Regarding the most effective form of vitamin to be administered (K1 and K2), studies undertaken on 50 post menopausal women w/o osteopenia for periods of 2-3 years indicated that vitamin K2 is more effective on the geometry of the bone tissue, compared to vitamin K1 (Iwamoto et al., 2009). Several reasons may account for these differences, first the dose administered (500 μg to 10 mg for PK, versus 45mg for MKS (Knapen, Schurgers, & Vermeer, 2007). As, in normal conditions, the daily intake needed is 70 μg/day. Both doses administered are very high. Therefore, it is possible to hypothesize, mainly for MKS, that, in addition to the OC γ-carboxylation activity, they may elicit other pharmacological effects (Kaneki, Hosoi, Ouchi, & Orimo, 2006). Another explanation may rely on the difference in the metabolism of PK and MKS: The liver transfers MKS into the circulation mainly via LDL (Schurgers & Vermeer, 2002). Overall, these studies highlight that PKs and MKS decrease the circulating levels of ucOC. This phenomenon was not dose dependent. On the other hand discrepant results have been obtained regarding the serum levels of TOC. Vitamin K deficiency impairs OC γ-carboxylation. This results in an increase of circulating ucOC and probably in a decrease of TOC and that of COC ratio. These effects may lead to an increased risk of fractures in post-menopausal women and, possibly, to a loss of BMD in elderly subjects (Iwamoto et al., 2009). As bone strength is closely related to bone mass and bone quality (NIH, 2001), serum ucOC could be a potential indicator of bone quality which is independent from BMD. Low levels of PKs and MKS reduce ucOC levels. Therefore, the intake of food supplements containing both forms of vitamin K increases the concentration of ucOC and result in an increased bone strength in the femoral neck, and in a reduced risk of fracture (Iwamoto et al., 2009). However, even in those subjects where a significant change of BMD was not observed, vitamin K1 and K2, at doses of 5 and 45 mg/day, respectively, showed to improve bone strength of femoral neck, its width and maintain the compression and bending index by increasing the resistance to impact and reducing the risk of fracture (Iwamoto et al., 2009). In particular, some studies carried out in Japan showed a decrease in the incidence of vertebral fractures in women with postmenopausal osteoporosis who had previously at least 5 fractures at the same bone site (Inoue et al., 2009). A drawback of MK-4 is its short half-life (Shearer & Newman, 2008). For this reason this molecule needs 3 daily doses of 15 mg each. A possible alternative options to reduce the number of doses may be that to use MK-7 which is the form of vitamin K2 present in the “nattou” (Knapen, Schurgers, & Vermeer, 2007). MK-7 has probably effects similar to those of MK-4 (Kaneki et al., 2001), but it has also a longer half-life. This allow MK-7 to keep the plasma levels in the therapeutic range and to accumulate in various tissues. This may have as consequence the use low therapeutic doses may (Knapen, Schurgers, & Vermeer, 2007). 3 types of vitamin K may be present in food supplements: MK-4, prepared by organic synthesis and used almost exclusively in Japan, vitamin K1, a synthetic product used worldwide and MK-7, obtained by extraction from nattou) (Schurgers et al., 2007). Although MK-7 is currently attracting a great interest, Vitamin K1 is, by far, the most common form of vitamin K present in food supplements available on the market. (Kaneki et al., 2001; Suzuki et al., 2003; Katsuyama et al., 2004). The effectiveness of both forms of vitamin K with respect to the OC carboxylation was monitored using the ratio COC/ ucOC. Within 3 days both these vitamins caused an increase in the concentration of COC, but only MK-7 induced a constant increase of COC concentration over the entire period of observation, thus suggesting that MKS are the most effective drugs. The effect on the coagulation parameters was more marked with MK-7. This means that MK-7 is 3-4

times more potent than vitamin K1 as an antidote against oral anticoagulants. Thus, after oral ingestion, MK-7 is more effective in catalyzing the carboxylation of the OC in bone and in thwarting the anticoagulant effects of coumarin in the liver. This effect might be the consequence of the long half-life of MK-7 compared to vitamin K1 and therefore may be taken up by extrahepatic tissues (Schurgers et al., 2007). The use of vitamin K has been proven to be quite safe, although sufficient data to define a maximum tolerated dose are not currently available. (Iwamoto et al., 2009). Since 1995, MK-4 has been widely administered to a wide number of patients with osteoporosis in Japan, Corea, Thailand, and Taiwan. The reported side effects were negligible, even with the highest administered dose (Knapen, Schurgers, & Vermeer, 2007). Only a small percentage of patients experienced gastrointestinal effects including constipation and weight gain (Iwamoto et al., 2009). In summary, the administration of vitamin K may be considered safe, except in pregnant women. Further studies to better define the pharmacological and toxicological profile of these drugs also on pregnant women are currently in progress.

6. Conclusions

Early studies on vitamin K have shown the effectiveness of this vitamin in modulating the activity of several coagulation factors (factors II, VII, IX and X). Subsequent investigations identified the mechanisms underlying these effects which consisted in promoting the γ-carboxylation of these proteins. Furthermore, vitamin K has been shown to regulate other important biological functions such as bone mineralization, calcium homeostasis, apoptosis, cell growth and signal transduction. In particular, the protective effects of vitamin K on bone tissue are of clinical relevance. On the basis of these findings vitamin K has been challenged in the prevention and treatment of osteoporosis in post-menopausal women. Interestingly, growing evidence shows that vitamin K is involved in the processes of carcinogenesis. In fact experimental observations indicate that, this molecule may have synergistic inhibitory effects on tumor neovascularisation, when associated with anti-angionetic drugs. These data support the concept that Vitamin K may have pleiotropic effects and warrant further study to better assess the clinical role of Vitamin K in human diseases.

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