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Vitamin K effects in human health: new insights beyond bone and cardiovascular health.

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Response to Reviewers:	Dear Professor Gambaro, Thank you for accepting our manuscript for submission. We appreciate the reviewer comments and have addressed them below. Sincerely, Pascale Khairallah and Maria Fusaro

	Reviewer #1: The new version is improved. Comment 1: Please note, line 300 edit ‘those’ into ‘whose’ or change the phrase. Thank you’. We have changed ‘those’ to ‘whose’ as highlighted in the text. Reviewer #2: The authors provide an overview on actions of vitamin k beyond bone and cardiovascular health. Now, the authors have improved the readability and the scope of the manuscript becomes way more clear. I have few comments left. Comment 1: Passage on MGP- first sentence- revised. I still do not agree with the phrasing. ‘MGP….after carboxylation shows 5 gamma carboxyglutamic acid residues’- it can show up to 5 carboxylated residues, as also explained later in this passage. Thank you for your comment. We have changed the sentence from ‘MGP is a 14 kDa vitamin K-dependent protein which after carboxylation shows 5 gamma-carboxyglutamic acid residues’ to ‘MGP is a 14 kDa vitamin K-dependent protein which after carboxylation can have up to 5 gamma-carboxyglutamic acid residues’ Comment 2: Vitamin K as ligand of Nuclear Receptors I think the connection between vitamin D and vitamin K is highly interesting, especially as a passage on CKD follows. A remark on the pathology of CKD-MBD would strengthen the importance of this pathway. Thank you for this comment, indeed it’s very intriguing the connection between Vitamin D and Vitamin K highlighted by an enhanced vitamin D3 effects on BGP gene expressiona and osteoblast precursor following supplementation MKn (see Fusaro et al, Vitamin K and bone: Clin Cases Miner Bone Metab 2017 - Review. PMID 29263734 Free PMC article). However, Vitamin D involves one aspect of CKD-MBD. Discussing the pathology of CKD-MBD only briefly will not be sufficient to highlight the different underlying processes and their importance. Comment 3: Periostin Contrary to the other VKD proteins, the passage lacks information on the carboxylation state of the described effects. As this is unknown, this should be stated. Thank you for this comment. We have added the following sentence ‘Similar to other VKDPs, the carboxylation of periostin is dependent on vitamin K. However, it is unknown how the carboxylation status of periostin influences its functions in different tissues.’ Comment 4: Conclusion The authors want to ‘develop techniques that can directly measure vitamin K’. Why? First, the technique is available- second, it just mainly reflects the short term intake of vitamin K and not the general vitamin K status. Thank you for the comment. The thechnique is available but it is not standardized. We have added in the sentence standardized.

Vitamin K effects in human health: new insights beyond bone and cardiovascular health

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Abstract:

Vitamin K is a cofactor for the function of the enzyme γ-glutamyl carboxylase, necessary for the activation of multiple vitamin K dependent-proteins. Vitamin K dependent-proteins (VKDPs) have important roles in bone health, vascular health, metabolism, reproduction as well as in cancer progression. Vitamin K deficiency is common in different conditions, including kidney disease, and it may influence the activity of VKDPs. This review discusses vitamin K status in human health and the physiologic and pathologic roles of VKDPs, beyond the established effects in skeletal and cardiovascular health.

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Introduction:

The vitamin K family is comprised of a group of fat-soluble molecules that share the 2-methyl-1,4-naphthoquinone (3-) groups. Vitamin K exists in 3 main forms, K1 and K2 which are the natural form, and K3 or menadione which is the synthetic form of the vitamin [1]. Vitamin K1, also known as phylloquinone, is found in vegetables, while vitamin K2, also known as menaquinone, is found in fermented food or produced by the intestinal microbiota. Vitamin K1 can be converted into vitamin K2. Two mechanisms of action of vitamin K have been described to date. It is an essential cofactor for the function of the enzyme γ-glutamyl carboxylase, and it acts as a ligand of the steroid and xenobiotic receptor (SXR) and pregnane X receptor (PXR, murine ortholog) [2].

Vitamin K-dependent proteins (VKDPs) play important roles in human physiology and can be an important link between the bone and the vasculature. This link becomes particularly important in patients with chronic kidney disease (CKD) who have a high prevalence of both mineral bone disorders (MBD) and vascular calcification (VC) [3] and whose primary cause of death is cardiovascular disease. Osteocalcin (OCN) is a VKDP known to be involved in bone mineralization, while Matrix GLA protein (MGP) is a known VC inhibitor whose deficiency is associated with increased risk for VC in CKD. New VKDPs have been discovered, and they have been found to play important roles in various cancers and their therapies.

While many questions have been answered, many more remain regarding the roles of the VKDPs in bone and vascular physiology. This review will discuss the roles of VKDPs and vitamin K in different pathologies.

Vitamin K Dependent Protein (VKDPs)

Vitamin K is an essential cofactor required for the activation of the gamma glutamyl carboxylase which converts glutamic acid to γ-glutamic acid residues. There are several vitamin K dependent proteins (VKDPs) [4]. These include the coagulation factors proteins C, S, M, Z, factors VII, IX, X and prothrombin. VKDPs also include Bone Gla Protein (BGP, or osteocalcin), Matrix Gla Protein (MGP), Gas6 (Growth Arrest-Specific 6 Protein), GRP (Gla Rich Protein) and Periostin. VKDPs play established roles in coagulation, in bone health and in cardiovascular health.

Bone Gla Protein (BGP): Beyond Skeletal Health

BGP or osteocalcin is the most abundant protein in bone. It is mainly secreted by osteoblasts, with a smaller amount secreted by chondrocytes[5]. BGP undergoes three carboxylation events to be transformed from the undercarboxylated form into the fully functional form. These carboxylation events require vitamin K as a cofactor[6]. Several mechanisms describing the BGPs role in bone

physiology have been proposed, including the inhibition of bone mineralization[7], the regulation of the rate of mineral maturation[8], and the formation of a complex between bone matrix and collagen in order to increase bone toughness[9]. However, none of these mechanisms are fully proven.

More recently, the relationship between BGP and glucose metabolism has been elucidated. In this role, BGP is thought to be released into the circulation and to exert an action similar to a hormonal effect[10]. This shed light into the peripheral functions of BGP and led to increased interest in this protein, therefore uncovering a wide range of functions.

The role of BGP in glucose metabolism and insulin signaling was first discovered by Lee et al[11] whose experiments showed that BGP knockout mice develop glucose intolerance, insulin resistance, and increased adipose tissue. The circulating form of BGP exerting the metabolic effects is mostly the undercarboxylated form (ucBGP). By binding to the receptor *Gprc6a*, in animals ucBGP acts on the pancreatic beta cells [10]. The influence of BGP on insulin sensitivity may be mediated via its effect on adiponectin, independent of insulin secretion[11]. Human studies have not shown this metabolic effect, however. When Basu et. al administered insulin to seven diabetic and seven non-diabetic patients and assessed the association with bone turnover markers, the change in the insulin levels did not influence BGP and ucBGP levels[12]. In humans, BGP also acts on Leydig cells thereby affecting the reproductive function of males[13].

Beyond the metabolic functions, BGP is involved in vascular calcification (VC) modulation through its effect on adiponectin[11]. Adiponectin inhibits osteoblastic differentiation of vascular smooth muscle cells, therefore protecting against VC[14]. In apolipoprotein E-deficient mice, daily injections of BGP for 12 weeks resulted in endothelium protection from atherosclerosis, but whether this was also mediated by the concomitant improvement in glucose metabolism is unknown[15]. Similarly, diabetic rats given daily injections of BGP had an improvement in arterial stiffness as assessed by pulse wave velocity[16].

The role of BGP in modulating and possibly preventing VC was confirmed in humans. BGP may exert this effect through its interaction with adiponectin, as seen by Bacchetta et al. when they found a significant association between BGP and adiponectin in CKD patients[17].

In human cardiovascular tissues, BGP was found in higher concentrations in calcified aorta and valves as compared to non-calcified tissue[18]. Fusaro et al. found lower BGP levels in patients with aortic and iliac calcifications as compared to patients without calcifications[19]. In men aged 51-85

years old in the MINOS study, higher total BGP levels were associated with slower progression of abdominal aortic calcification after a 10 year follow up[20].

In contrast to the above findings, in the Study of Osteoporotic Fractures (SOF) which enrolled 363 elderly women, total BGP levels were not associated with abdominal aortic calcification[21]. Moreover, in a meta-analysis of 46 clinical studies evaluating the relationship between BGP and VC, no definite associations could be found between the different forms of BGP (ucBGP, cBGP and total BGP) and VC. However, sound physiological conclusions cannot be drawn based on these findings. In fact, 44% of the included studies did not adjust for confounding variables and the BGP forms were measured using different assays in the different studies[22]. Moreover, BGP displays a circadian rhythm with levels falling in the morning and reaching the peak in the evening [23]. Therefore, the timing of blood draws may impact the results of the studies. It is also important to note that BGP is cleared by the kidneys[24]. Therefore any decline in renal function results in an elevation in BGP levels [24]. This is particularly notable when the glomerular filtration rate drops below 20 ml/min[24] . Additionally, based on the aforementioned studies, gender appears to be a confounding factor with the effects of BGP being differential between males and females. Vitamin K levels are obvious confounders. Moreover, menopausal status, adipose tissue, diabetic status are all expected to be confounders as well [25]. If we want studies that more accurately unravel the effect of BGP on the vasculature, we should standardize our BGP serum measurements and understand more carefully the confounders that should be accounted for.

Matrix Gla Protein (MGP): Beyond Cardiovascular Health

MGP is a 14 kDa vitamin K-dependent protein which after carboxylation can have up to 5 gamma-carboxyglutamic acid residues [26]. In addition to gamma-carboxylation, MGP requires post-translational serine phosphorylation. Phosphorylation occurs at 3 serine residues via the enzyme casein kinase[26, 27]. Phosphorylation regulates the protein secretion into the extracellular environment[26]. Based on the degree of carboxylation and phosphorylation, multiple forms of MGP can be found in the circulation and the extracellular matrix **(Figure 1).** MGP is released from vascular smooth muscle cells and chondrocytes [28]. It was the first calcification inhibitor to be characterized[28]. The exact mechanism through which MGP inhibits VC is not completely understood. However, the carboxylated active form of MGP is believed 1) to bind to calcification crystals in blood vessels forming vesicles and apoptotic bodies, 2) to directly prevent calcium phosphate precipitation, and 3) to prevent the trans-differentiation of vascular smooth muscles cells into an osteogenic phenotype [26, 29].

The different forms of MGP can be used as a biomarker of vitamin K deficiency[30]. Vitamin K deficiency in CKD leads to a decrease in the levels of the phosphorylated-carboxylated MGP (p-cMGP) and a rise in the levels of dephosphorylated undercarboxylated MGP (dp-ucMGP) [31]. Plasma dp-ucMGP levels increase as CKD advances with the highest levels found in CKD stage 5[31]. Plasma dp-ucMGP is positively associated with VC and might be utilized as an early marker for vascular calcification in CKD patients[30, 31].

Beyond the well-established effects of MGP in VC[32] studies also suggest that it has a role in skeletal health. Mice deficient in MGP develop diffuse VC as well as inappropriate calcification of the growth plate[28]. Mice overexpressing *Mgp* in osteoblasts have a decrease in bone mineralization particularly in the tooth dentin and cementum. Thus, MGP affects bone mineralization[33]. MGP interacts with both osteoblasts and osteoclasts. Phosphate regulates MGP expression in osteoblast cultures via the ERK1/2-Fra-1 pathway [34]. Via Src/Rac1 signaling, MGP modulates osteoclastogenesis; MGP depletion favors while MGP excess inhibits osteoclast differentiation [35].

In clinical studies, homozygosity of the MGP rs1800802 minor allele, but not total serum MGP levels was associated with 0.56 times lower prevalence of hand osteoarthritis compared with having ≥ 1 major allele at this locus (95% CI 0.32-0.99, $p<0.05$), suggesting a role for MGP in osteoarthritis[36]. Among 145 participants in the European Vertebral Osteoporosis Study, men with the homozygous MGP-7AA polymorphism had significantly more femoral bone loss as compared to those with genotypes -7GG and -7GA [37]. Those homozygous for MGP 83Ala-Ala had significantly more femoral neck loss as well as a greater tendency to vertebral fractures as compared to those with the genotypes 83Thr-Thr and 83Thr-Ala. A decrease in BMD was observed only in MGP-7AA and MGP 83Ala-Ala genotypes. These associations were not found in the 151 women who participated in the study possibly because 94% of the women were post-menopausal and had independent post-menopausal bone loss that could have confounded the effect of the MGP polymorphisms.
The effect of MGP on fractures and bone density was similarly seen following kidney transplantation. Evenepoel et al. evaluated vitamin K deficiency as measured by dp-ucMGPlevels in 468 de novo kidney transplant recipients. The patients with the highest tertile of dp-ucMGPlevels had lower bone mineral density and had higher incident fractures independently of common fracture determinants (HR 2.21; 95% CI, 1.00 to 4.91; $p < 0.05$) [38].

Studies evaluating the relationship between renal clearance and MGP levels are rare. In 842 outpatients with stable cardiovascular disease and a mean GFR of 76±23 mL/min, each 10 mL/ min

lower GFR was associated with a 79 nM lower ucMGP serum level ($p < 0.001$), and a 0.1 mg/L higher cystatin-C was associated with a 39 nM lower ucMGP serum levels ($p < 0.001$) in multivariate adjusted models [39]. However, when Rennenberg et al. looked at this association, they found no significant correlations between total MGP levels in renal arterial and venous blood and renal clearance of 90 patients with hypertension[40]. It is important to note however that none of the patients in this cohort had a GFR <26 mL/min[40]. A relationship between MGP levels and renal clearance at a GFR <26 mL/min is therefore still possible.

Vitamin K as Ligand of Nuclear Receptors

Vitamin K can act as ligand of the nuclear Steroid and Xenobiotic Receptor (SXR) and its murine ortholog, Pregnane X Receptor (PXR)[41]. SXR/PXR is present in different tissues, including osteoblastic cell lines [42, 43]. The presence of SXR/PXR in osteoblastic tissue is important as it could be the pathway through which vitamin K improves bone health [44].

Transcriptome analysis has revealed a number of bone-related genes which are involved in the vitamin K-SXR pathway. These include tsukushi and matrilin-2, which are involved in collagen and extracellular matrix assembly [45, 46]. In sarcoma cells, vitamin K up-regulates osteoblastic bone markers [43]. SXR/PXR knockout mice have increased bone resorption and decreased bone formation [47].

SXR is additionally involved in bone metabolism via its effect on vitamin D metabolism. In this role, SXR activation can have two effects. SXR activation by some drugs can lead to CYP3A4 expression (exerting 24- and 25-hydroxylase activity) and resultant vitamin D metabolism and deficiency. SXR activation can also lead to inhibition of CYP24A1 (24-hydroxylase activity) in the kidney therefore increasing 1,25(OH)D levels [48]. These data suggest that SXR/PXR is another pathway through which vitamin K is involved in bone homeostasis.

Vitamin K in Chronic Kidney Disease (CKD)

The western diet does not provide enough vitamin K to activate VKDPs in all tissues[49]. This deficiency is more pronounced in adults over the age of 40. Patients with CKD have even greater rates of vitamin K deficiency as compared to the general population. The number of CKD patients who have vitamin K deficiency reaches 70-90% of that population [50, 51, 52] **(Table 1).** Poor oral intake of vitamin K is the main cause of deficiency [50, 53]. When compared to healthy individuals, the vitamin K intake of HD patients is particularly low on days of dialysis and the weekend[54]. The

use of phosphorus binders in the dialysis population contributes to vitamin K deficiency as well [55]. Being lipophilic, vitamin K should not be removed via dialysis. However, studies to validate this hypothesis are needed, because serum levels of 25(OH)-vitamin D, another lipophilic molecule, decreased in patients who were switched from conventional hemodialysis to online hemodiafiltration [56].

There are known implications of vitamin K deficiency in population-based studies and in kidney disease patients [57, 58]. In the Rotterdam study of 7983 men and women over the age of 55, intake of menaquinone protected against incident coronary heart disease (RR of highest tertile of menaquinone intake as compared to lowest tertile = 0.59, p=0.007), and against coronary heart disease related mortality (RR of highest tertile of menaquinone intake as compared to lowest tertile = 0.43, p=0.005). Additionally, the odds ratio of severe aortic calcification was significantly lower in the patients with the highest intake of menaquinone intake as compared to those with lowest intake (OR 0.48, p <0.001) [59]. In the VIKI study, a cohort of 387 dialysis patients, 35.4% of patients had menaquinone-7 deficiency, 23.5% of patients had vitamin K1 deficiency and 14.5% of patients had menquinone-4 deficiency [57]. Patients with menaquinone-4 deficiency had significantly higher aortic calcification (10.6% versus 1.3%, $p = 0.01$). Menaquinone-7 deficiency was associated with significantly higher iliac calcifications (41% versus 28.2%, $p = 0.009$) [57].

There is no gold-standard for the measurement of vitamin K levels and there is a lack in standardization. Instead, functional deficiency of vitamin K is used as a surrogate of vitamin K status in individuals. Vitamin K deficiency in CKD leads to a decrease in the levels of active MGP, a rise in the levels of dp-ucMGP, as well as a rise in the levels of ucBGP[37]. Plasma dp-ucMGP levels increase as CKD advances with highest levels being in CKD stage 5[38]. A dp-ucMGP level of >500 pmol/L, ucBGP>4.5 ng/mL[59] or protein induced by vitamin K absence-II (PIVKA-II) >2 nM/L are indicative of vitamin K deficiency [30, 60].

In 53 dialysis patients, vitamin K2 supplementation resulted in a dose dependent decrease in functional vitamin K deficiency. After a 6-week supplementation regimen, dp-ucMGP levels were reduced 77% and 93% in the groups receiving daily oral administration of 135 μg and 360 μg of K2, respectively[61]. In 200 HD patients receiving vitamin K2 at dose of 360, 720 or 1080 μg thrice weekly for 8 weeks, dp-uc-MGP levels decreased by 17%, 33% and 46% respectively[62]. Several studies show the same pattern **(Table 2).**

Although kidney transplantation is associated with an improvement in vitamin K levels[55], a deficiency in vitamin K was still found in up to 91% of kidney transplant patients. This deficiency may persist as long as 188 months post transplantation[38,–63]. Moreover, in at least one study, vitamin K deficiency in kidney transplant patients was associated with an almost 3 times increase in all-cause mortality [63].

How Current therapy of MBD in CKD Influences Vitamin K levels and VKDPs

While MBD derangements contribute to renal osteodystrophy and to VC in CKD [64], treatments of MBD have not been sufficiently successful at reversing VC, improving cardiovascular events or decreasing mortality. We hypothesize that this might be partly explained by the negative impact of some of the MBD treatments on vitamin K levels. One such treatment is sevelamer. Sevelamer is thought to bind fat-soluble vitamins [65, 66]. Since vitamin K is a fat-soluble vitamin, Jansz et al. assessed the impact of sevelamer on vitamin K in patients who received a kidney transplantation. They found that sevelamer is associated with higher dpu-cMGP levels reflecting vitamin K deficiency [55]. This finding points to the possible need of giving vitamin K supplements to patients treated with sevelamer, but this approach should first be substantiated by a specific study.

However, some MBD treatments are associated with improvements in VKDPs. In an analysis of the VIKI study [57], the use of calcimimetics and vitamin D analogs was associated with higher levels of BGP. Calcimimetic use was also associated with higher levels of total MGP [19]. Therefore, this data suggests that calcimimetics and vitamin D analogs can help preserve or improve the activity of VKDPs.

VKDPs Beyond Bone and Vascular Health

Growth Arrest-Specific Protein 6 (Gas6)

Gas6 is a gamma-carboxyglutamic acid (Gla) domain-containing protein, member of the VKDPs family, which is present in several different tissues (e.g. vascular endothelium, kidney, heart, and the bone marrow). It is a ligand for the TAM (Tyro3-Axl-Mer) receptor family [67] and is thought to be involved in the stimulation of cell proliferation, migration and apoptosis [68, 69].

Gas6 and protein S are two homologous secreted proteins depending on vitamin K for a wide range of their biological functions. A discrete subset of these functions is mediated through their binding to and activation of the receptor tyrosine kinases Axl, Sky and Mer; in particular, the vitamin K-dependent protein Gas6 activates receptor tyrosine kinases of the Axl family [69].

A hallmark of the Gas6-Axl system is the unique ability of both Gas6 and protein S to tether their non receptor-binding regions to the negatively charged membranes of apoptotic cells. A relevant amount of evidence suggests that the Gas6-Axl system is able to regulate cell survival, proliferation, migration, adhesion and phagocytosis. Consequently, an altered expression, or a compromised activity of its components have been detected in a variety of diseases, including different cancer types. Moreover, Axl overactivation can equally occur without ligand binding, which has implications for tumorigenesis. [70]

Upregulation of Gas6 has been described in different malignancies [71], and an increased expression of either Gas6 or TAM receptor proved to be predictive of poor prognosis[72]. A number of animal studies highlighted the role of Gas6 in the processes of carcinogenesis [71, 72, 73], while clinical studies are rarer, but ultimately show consistent findings. Ovarian cancer samples from 90 patients had significantly higher expression of Gas6 and Axl as compared to normal ovarian tissue [73], RNA PCR from 42 glioblastoma frozen sections demonstrated that Gas6 and Axl are overexpressed both in the tumoral, as well as in the surrounding vascular, tissue [74]. Furthermore, glioblastoma patients whose tumors expressed higher Gas6 and Axl levels had significantly higher risk of tumor relapse as well as shorter time to relapse [74]. A similar observation has been reported in osteosarcoma; indeed, in 62 osteosarcoma patients, Axl was highly expressed in 43.5% of the cases, characterized by a significantly higher rate of recurrence, lung metastases, as well as a lower survival [75]. Gas6-Axl is also important as mechanisms of resistance to anticancer therapy; indeed, resistance to tyrosine kinase inhibitors in non-small cell cancer and renal cell carcinoma (RCC) was found to be driven by Axl [76].

As far as RCC, the Axl protein proved to be highly expressed in clear cell RCC cells deficient in functional von Hippel-Lindau (VHL) protein, a tumor suppressor gene often inactivated in ccRCC. VHL reconstituted cells expressed decreased levels of Axl protein, but not Axl mRNA, suggesting that VHL may regulate Axl expression. Furthermore, Gas6-mediated activation of Axl in ccRCC cells resulted in Axl phosphorylation, receptor down-regulation, decreased cell-viability, as well as migratory capacity, whilst no effects of the Gas6/Axl system could be detected on invasion. Moreover, in ccRCC tumor tissues, Axl was phosphorylated and Gas6 gamma-carboxylated, suggesting these molecules to be active in vivo. [77]

All the above has practical therapeutic implications, as targeting the Gas6-Axl pathway through the multikinase inhibitor cabozantinib proved to be an active treatment option for metastatic RCC patients progressing on standard antiangiogenic therapy [78].

Periostin

Periostin is another member of the VKDP family. Similar to other VKDPs, the carboxylation of periostin is dependent on vitamin K. However, it is unknown how the carboxylation status of periostin influences its functions in different tissues. Periostin is an extracellular matrix protein that binds integrins playing a role in cellular adhesion and migration[79]. It plays a role in collagen assembly in several tissues and is upregulated when tissues are subjected to stress[79-81]. Following cardiac injury, periostin is expressed in cardiac myofibroblasts and vascular smooth muscle cells contributing to a profibrotic phenotype[81-83]. Similar to other VKDPs, periostin has also been found in many cancers [84-86]. Periostin induces tumor angiogenesis[84, 85] and lymphangiogenesis [85], and its association with cancer confers a worse prognosis to patients[85]. The role of periostin in breast cancer has been described. Periostin is expressed in invasive ductal carcinoma cells [87]. Its expression increases with the cancer grade, suggesting that periostin may play a role in cancer progression[88]. Periostin can also serve as marker of breast cancer metastasis. Human breast cancer exosomes contain periostin. Further, periostin enriched exosomes were found in patients with lymph node metastasis as compared to those with localized disease[89]. Finally, periostin may have a role in breast cancer prognostication. In 259 breast cancer patients who underwent surgical and radiation therapy, local recurrence-free survival, distant metastasis-free survival and overall survival were significantly lower in the patients whose tumors expressed periostin as compared to those whose tumors were negative for periostin [90].

Gla-Rich Protein (GRP)

GRP is one of the newest members of the VKDP family. Its name derives from the large amount of Gla residues, which comprise 22% of its composition[91], and which make it the VKDP with the highest concentration of Gla residues. Since its discovery, GRP has been found to have a role as an anti-inflammatory protein[92]. In vivo, it prevents osteoarthritis progression[93]. It additionally plays a role in mineralization. In both animal models and in humans, GRP has been found to colocalize with mineral deposits at sites of calcification[94]. Further work demonstrates that similar to MGP, GRP in its carboxylated but not in its undercarboxylated form is a calcification inhibitor [95]. Although GRP role in cancer is less established as compared to other VKDPs, there is growing interest surrounding this protein. The undercarboxylated form as compared to the carboxylated form of GRP is found in more abundance in skin and breast cancer cells, particularly in microcalcifications associated with these tumors [96]. Therefore, GRP may be involved in cancer-related calcifications and as such may prove to be a therapeutic target for some types of cancer.

Vitamin K in Cancer

Several VKDPs are involved in tumorigenesis[71, 84, 85] **(Table 3).** Vitamin K2 administration in vivo inhibits the cellular proliferation of several cancers [96, 97]. This led to a number of studies investigating the role of vitamin K intake and supplementation in preventing cancer development, progression and recurrence. In the European Prospective Investigation into Cancer and Nutrition-Heidelberg cohort study which included 24,340 cancer-free participants followed up for 10 years, there was a significant inverse association between vitamin K2 intake and cancer mortality, but not cancer incidence[98]. Similarly, in the Prevención con Dieta Mediterránea study, which enrolled 7216 participants followed up for a median of 4.8 years, subjects who increased their dietary intake of both vitamin K1 and K2 had decreased cancer incidence[99].

The undercarboxylated form of prothrombin (PIVKAII), a VKDP, is upregulated in hepatocellular carcinoma (HCC) [100]. Vitamin K2 supplementation in patients who underwent curative hepatectomy or radiofrequency ablation for HCC suppressed HCC recurrence, though this effect did not reach statistical significance in any of these studies[101, 102]. In contrast, 45 mg per day of vitamin K2 supplementation resulted in significantly lower risk of HCC development in 21 women who had viral cirrhosis as compared to 19 women with viral cirrhosis who did not receive supplementation[103]. This suggests that vitamin K2 may play a role in preventing the development of HCC in high risk patients. Overall, the association and the relationship of vitamin K with cancer is still uncertain and under investigation. Further studies are needed to define this role of vitamin K.

Conclusion

Substantial research has made it clear that VKDPs or Vitamin-K related pathways can be used in the future to diagnose, treat and prognosticate a number of health conditions. There are still more vitamin K-related roles to be uncovered and which will further our understanding of the physiological and pathological importance of vitamin K status. It will also prove important to recognize the differential actions of vitamin K1 and vitamin K2, and to develop standardized techniques that can directly measure vitamin K levels instead of our current reliance on functional vitamin K status as measured by VKDPs levels [104]. This will allow to develop trials that can evaluate selective and optimal vitamin K supplementation strategies in order to further understand their effect on clinical outcomes.

Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no conflict of interest.

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TABLES AND FIGURES

Table 1. Vitamin K and VKDP levels in Kidney Disease

AUTHOR, YEAR	NUMBER OF PARTICIPANTS	KIDNEY DISEASE STAGE	VITAMIN K FORM MEASURED	% OF PATIENTS WITH VITAMIN K DEFICIENCY	VKDP MEASURED	% OF PATIENTS WITH MEASURED VKDP
Kolheimer M., 1997 [105]	68	ESKD -HD	Phylloquinone	33%		
Pilkey RM., 2007 [106]	142	ESKD	Phylloquinone	29%	ucBGP	93%
Holden RM, 2008 [52]	21	ESKD- PD	Phylloquinone	24%	ucBGP	60%
Holden RM, 2010 [107]	172	CKD 3-5	Phylloquinone	6%	ucBGP PIVKAII	60% 97%
Schurgers LJ, 2010 [108]	107	CK2-5 and ESKD-HD			dp-ucMGP	50%
Schlieper G, 2011 [109]	188	ESKD-HD			PIVKA-II dp-ucMGP	63% 100%
Cranenburg EC, 2012 [54]	40	ESKD-HD	Phylloquinone Menaquinone	45% 100%	PIVKAII dp-ucMGP	82.5% 100%
Westendfeld R, 2012 [61]	53	ESKD-HD	Menaquinone	100%	PIVKAII dp-ucMGP	92.5% 100%
Fusaro M, 2012 [57]	387	ESKD-HD	Phylloquinone Menaquinone-4 Menaquinone-7	23.5% 14.5% 35.4%	ucBGP	100%
Boxma PY, 2012 [110]	60	Post-Transplantation			dp-ucMGP	80%
Caluwe R, 2013 [62]	165	ESKD-HD			dp-ucMGP	100%
Delanaye P, 2014 [111]	160	ESKD-HD			dp-ucMGP	100%
CA, 2015 [63]	518	Post-Transplantation			dp-ucMGP	91%
Aoun M, 2017 [112]	50	ESKD-HD			dp-ucMGP	100%

Table 2. Effect of Vitamin K supplementation on dephosphorylated-undercarboxylated MGP levels in ESKD

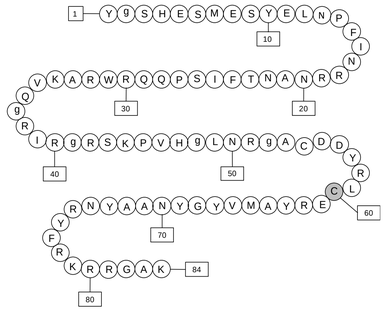
AUTHOR, YEAR	STUDY DESIGN	NUMBER OF PARTICIPANTS	KIDNEY DISEASE STAGE	INTERVENTION	OUTCOMES MEASURED	RESULTS
Schlieper G., 2011 [109]	Prospective	17	ESKD	Vitamin K2 at 135 µg/d for 6 weeks	dp-ucMGP level	Vitamin K2 supplementation resulted in a 27% reduction in dp-ucMGP levels p = 0.0027
Westenfeld R., 2012 [61]	Prospective	53	ESKD	Vitamin K2 at 45, 135, or 360 µg/d for 6 weeks	dp-ucMGP level	Vitamin K2 supplementation resulted in a dose-dependent decrease in the levels of dp-uc-MGP by 17.9%, 36.7%, and 61.1% in the 45-, 135-, and 360-µg groups, respectively, compared with baseline values. p<0.005
Caluwe R, 2014 [62]	Prospective	200	ESKD	Vitamin K2 at 60, 720 or 1080 µg thrice weekly for 8 weeks	dp-uc-MGP level	Vitamin K2 resulted in a dose-dependent decrease in the levels of dp-uc-MGP by 17%, 33% and 46% in the 360-, 720- and 1080-µg groups, respectively, compared to baseline values. p < 0.001
Aoun M, 2017 [112]	Prospective	50	ESKD	360 µg of vitamin K2 (menaquinone-7) for 4 weeks	dp-uc-MGP level	Vitamin K2 reduced dp-ucMGP by 86% P<0.05

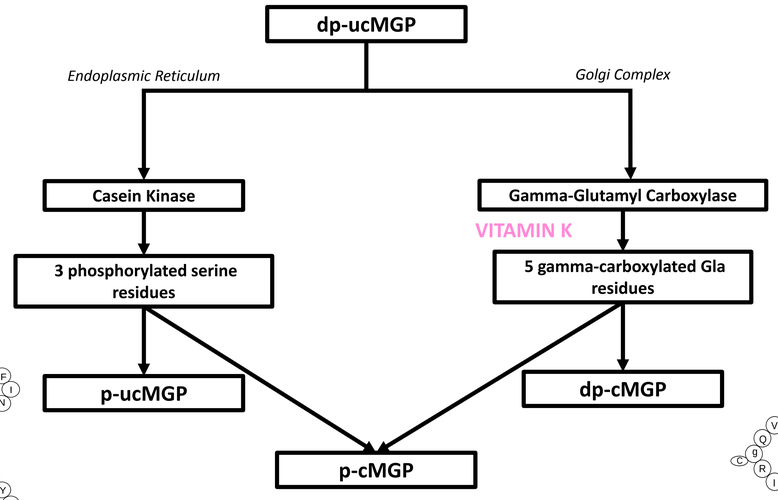
Table 3. Effects of VKDPs on cancer development and progression.

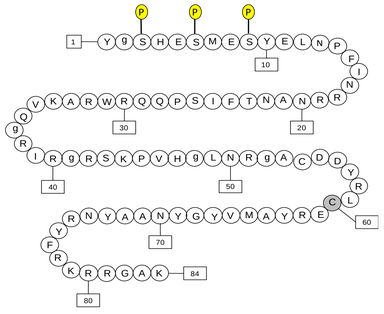
Vitamin K dependent proteins activity may modulate cancer behavior	
Gas 6	• Angiogenesis • Tumor Progression • Higher Tumor Recurrence • Metastasis and Poorer Prognosis • Cancer Therapy Resistance
Periostin	• Angiogenesis • Lymphangiogenesis • Tumor Progression • Poorer Prognosis
PIVKAII	• Tumor Progression

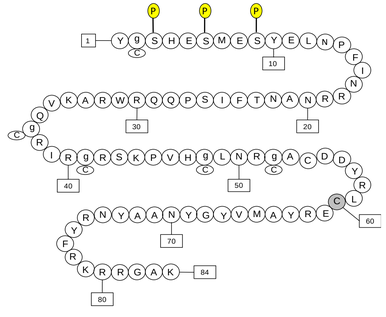
Figure legends

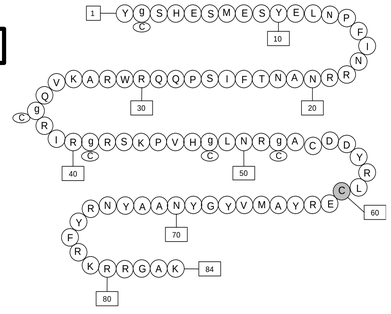
Figure 1. Different Forms of matrix Gla protein (MGP).











Dear Professor Gambaro,

Thank you for accepting our manuscript for submission. We appreciate the reviewer comments and have addressed them below.

Sincerely,
Pascale Khairallah and Maria Fusaro

Reviewer #1: The new version is improved.
Comment 1: Please note, line 300 edit 'those' into 'whose' or change the phrase.
Thank you'. We have changed 'those' to 'whose' as highlighted in the text.

Reviewer #2: The authors provide an overview on actions of vitamin k beyond bone and cardiovascular health. Now, the authors have improved the readability and the scope of the manuscript becomes way more clear.
I have few comments left.

Comment 1: Passage on MGP- first sentence- revised.
I still do not agree with the phrasing. 'MGP….after carboxylation shows 5 gamma carboxyglutamic acid residues'- it can show up to 5 carboxylated residues, as also explained later in this passage.
Thank you for your comment. We have changed the sentence from 'MGP is a 14 kDa vitamin K-dependent protein which after carboxylation shows 5 gamma-carboxyglutamic acid residues' to 'MGP is a 14 kDa vitamin K-dependent protein which after carboxylation can have up to 5 gamma-carboxyglutamic acid residues'

Comment 2: Vitamin K as ligand of Nuclear Receptors
I think the connection between vitamin D and vitamin K is highly interesting, especially as a passage on CKD follows. A remark on the pathology of CKD-MBD would strengthen the importance of this pathway.
Thank you for this comment, indeed it's very intriguing the connection between Vitamin D and Vitamin K highlighted by an enhanced vitamin D3 effects on BGP gene expressiona and osteoblast precursor following supplementation MKn (see Fusaro et al, Vitamin K and bone: Clin Cases Miner Bone Metab 2017 - Review. PMID 29263734 Free PMC article). However, Vitamin D involves one aspect of CKD-MBD. Discussing the pathology of CKD-MBD only briefly will not be sufficient to highlight the different underlying processes and their importance.

Comment 3: Periostin
Contrary to the other VKD proteins, the passage lacks information on the carboxylation state of the described effects. As this is unknown, this should be stated.
Thank you for this comment. We have added the following sentence 'Similar to other VKDPs, the carboxylation of periostin is dependent on vitamin K. However, it is unknown how the carboxylation status of periostin influences its functions in different tissues.'

Comment 4: Conclusion
The authors want to 'develop techniques that can directly measure vitamin K'. Why? First, the technique is available- second, it just mainly reflects the short term intake of vitamin K and not the general vitamin K status.
Thank you for the comment. The thechnique is available but it is not standardized. We have added in the sentence standardized.

Vitamin K effects in human health: new insights beyond bone and cardiovascular health

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Abstract:

Vitamin K is a cofactor for the function of the enzyme γ-glutamyl carboxylase, necessary for the activation of multiple vitamin K dependent-proteins. Vitamin K dependent-proteins (VKDPs) have important roles in bone health, vascular health, metabolism, reproduction as well as in cancer progression. Vitamin K deficiency is common in different conditions, including kidney disease, and it may influence the activity of VKDPs. This review discusses vitamin K status in human health and the physiologic and pathologic roles of VKDPs, beyond the established effects in skeletal and cardiovascular health.

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Introduction:

The vitamin K family is comprised of a group of fat-soluble molecules that share the 2-methyl-1,4-naphthoquinone (3-) groups. Vitamin K exists in 3 main forms, K1 and K2 which are the natural form, and K3 or menadione which is the synthetic form of the vitamin [1]. Vitamin K1, also known as phylloquinone, is found in vegetables, while vitamin K2, also known as menaquinone, is found in fermented food or produced by the intestinal microbiota. Vitamin K1 can be converted into vitamin K2. Two mechanisms of action of vitamin K have been described to date. It is an essential cofactor for the function of the enzyme γ-glutamyl carboxylase, and it acts as a ligand of the steroid and xenobiotic receptor (SXR) and pregnane X receptor (PXR, murine ortholog) [2].

Vitamin K-dependent proteins (VKDPs) play important roles in human physiology and can be an important link between the bone and the vasculature. This link becomes particularly important in patients with chronic kidney disease (CKD) who have a high prevalence of both mineral bone disorders (MBD) and vascular calcification (VC) [3] and whose primary cause of death is cardiovascular disease. Osteocalcin (OCN) is a VKDP known to be involved in bone mineralization, while Matrix GLA protein (MGP) is a known VC inhibitor whose deficiency is associated with increased risk for VC in CKD. New VKDPs have been discovered, and they have been found to play important roles in various cancers and their therapies.

While many questions have been answered, many more remain regarding the roles of the VKDPs in bone and vascular physiology. This review will discuss the roles of VKDPs and vitamin K in different pathologies.

Vitamin K Dependent Protein (VKDPs)

Vitamin K is an essential cofactor required for the activation of the gamma glutamyl carboxylase which converts glutamic acid to γ-glutamic acid residues. There are several vitamin K dependent proteins (VKDPs) [4]. These include the coagulation factors proteins C, S, M, Z, factors VII, IX, X and prothrombin. VKDPs also include Bone Gla Protein (BGP, or osteocalcin), Matrix Gla Protein (MGP), Gas6 (Growth Arrest-Specific 6 Protein), GRP (Gla Rich Protein) and Periostin. VKDPs play established roles in coagulation, in bone health and in cardiovascular health.

Bone Gla Protein (BGP): Beyond Skeletal Health

BGP or osteocalcin is the most abundant protein in bone. It is mainly secreted by osteoblasts, with a smaller amount secreted by chondrocytes[5]. BGP undergoes three carboxylation events to be transformed from the undercarboxylated form into the fully functional form. These carboxylation events require vitamin K as a cofactor[6]. Several mechanisms describing the BGPs role in bone

physiology have been proposed, including the inhibition of bone mineralization[7], the regulation of the rate of mineral maturation[8], and the formation of a complex between bone matrix and collagen in order to increase bone toughness[9]. However, none of these mechanisms are fully proven.

More recently, the relationship between BGP and glucose metabolism has been elucidated. In this role, BGP is thought to be released into the circulation and to exert an action similar to a hormonal effect[10]. This shed light into the peripheral functions of BGP and led to increased interest in this protein, therefore uncovering a wide range of functions.

The role of BGP in glucose metabolism and insulin signaling was first discovered by Lee et al[11] whose experiments showed that BGP knockout mice develop glucose intolerance, insulin resistance, and increased adipose tissue. The circulating form of BGP exerting the metabolic effects is mostly the undercarboxylated form (ucBGP). By binding to the receptor *Gprc6a*, in animals ucBGP acts on the pancreatic beta cells [10]. The influence of BGP on insulin sensitivity may be mediated via its effect on adiponectin, independent of insulin secretion[11]. Human studies have not shown this metabolic effect, however. When Basu et. al administered insulin to seven diabetic and seven non-diabetic patients and assessed the association with bone turnover markers, the change in the insulin levels did not influence BGP and ucBGP levels[12]. In humans, BGP also acts on Leydig cells thereby affecting the reproductive function of males[13].

Beyond the metabolic functions, BGP is involved in vascular calcification (VC) modulation through its effect on adiponectin[11]. Adiponectin inhibits osteoblastic differentiation of vascular smooth muscle cells, therefore protecting against VC[14]. In apolipoprotein E-deficient mice, daily injections of BGP for 12 weeks resulted in endothelium protection from atherosclerosis, but whether this was also mediated by the concomitant improvement in glucose metabolism is unknown[15]. Similarly, diabetic rats given daily injections of BGP had an improvement in arterial stiffness as assessed by pulse wave velocity[16].

The role of BGP in modulating and possibly preventing VC was confirmed in humans. BGP may exert this effect through its interaction with adiponectin, as seen by Bacchetta et al. when they found a significant association between BGP and adiponectin in CKD patients[17].

In human cardiovascular tissues, BGP was found in higher concentrations in calcified aorta and valves as compared to non-calcified tissue[18]. Fusaro et al. found lower BGP levels in patients with aortic and iliac calcifications as compared to patients without calcifications[19]. In men aged 51-85

years old in the MINOS study, higher total BGP levels were associated with slower progression of abdominal aortic calcification after a 10 year follow up[20].

In contrast to the above findings, in the Study of Osteoporotic Fractures (SOF) which enrolled 363 elderly women, total BGP levels were not associated with abdominal aortic calcification[21]. Moreover, in a meta-analysis of 46 clinical studies evaluating the relationship between BGP and VC, no definite associations could be found between the different forms of BGP (ucBGP, cBGP and total BGP) and VC. However, sound physiological conclusions cannot be drawn based on these findings. In fact, 44% of the included studies did not adjust for confounding variables and the BGP forms were measured using different assays in the different studies[22]. Moreover, BGP displays a circadian rhythm with levels falling in the morning and reaching the peak in the evening [23]. Therefore, the timing of blood draws may impact the results of the studies. It is also important to note that BGP is cleared by the kidneys[24]. Therefore any decline in renal function results in an elevation in BGP levels [24]. This is particularly notable when the glomerular filtration rate drops below 20 ml/min[24] . Additionally, based on the aforementioned studies, gender appears to be a confounding factor with the effects of BGP being differential between males and females. Vitamin K levels are obvious confounders. Moreover, menopausal status, adipose tissue, diabetic status are all expected to be confounders as well [25]. If we want studies that more accurately unravel the effect of BGP on the vasculature, we should standardize our BGP serum measurements and understand more carefully the confounders that should be accounted for.

Matrix Gla Protein (MGP): Beyond Cardiovascular Health

MGP is a 14 kDa vitamin K-dependent protein which after carboxylation can have up to 5 gamma-carboxyglutamic acid residues [26]. In addition to gamma-carboxylation, MGP requires post-translational serine phosphorylation. Phosphorylation occurs at 3 serine residues via the enzyme casein kinase[26, 27]. Phosphorylation regulates the protein secretion into the extracellular environment[26]. Based on the degree of carboxylation and phosphorylation, multiple forms of MGP can be found in the circulation and the extracellular matrix **(Figure 1).** MGP is released from vascular smooth muscle cells and chondrocytes [28]. It was the first calcification inhibitor to be characterized[28]. The exact mechanism through which MGP inhibits VC is not completely understood. However, the carboxylated active form of MGP is believed 1) to bind to calcification crystals in blood vessels forming vesicles and apoptotic bodies, 2) to directly prevent calcium phosphate precipitation, and 3) to prevent the trans-differentiation of vascular smooth muscles cells into an osteogenic phenotype [26, 29].

The different forms of MGP can be used as a biomarker of vitamin K deficiency[30]. Vitamin K deficiency in CKD leads to a decrease in the levels of the phosphorylated-carboxylated MGP (p-cMGP) and a rise in the levels of dephosphorylated undercarboxylated MGP (dp-ucMGP) [31]. Plasma dp-ucMGP levels increase as CKD advances with the highest levels found in CKD stage 5[31]. Plasma dp-ucMGP is positively associated with VC and might be utilized as an early marker for vascular calcification in CKD patients[30, 31].

Beyond the well-established effects of MGP in VC[32] studies also suggest that it has a role in skeletal health. Mice deficient in MGP develop diffuse VC as well as inappropriate calcification of the growth plate[28]. Mice overexpressing *Mgp* in osteoblasts have a decrease in bone mineralization particularly in the tooth dentin and cementum. Thus, MGP affects bone mineralization[33]. MGP interacts with both osteoblasts and osteoclasts. Phosphate regulates MGP expression in osteoblast cultures via the ERK1/2-Fra-1 pathway [34]. Via Src/Rac1 signaling, MGP modulates osteoclastogenesis; MGP depletion favors while MGP excess inhibits osteoclast differentiation [35].

In clinical studies, homozygosity of the MGP rs1800802 minor allele, but not total serum MGP levels was associated with 0.56 times lower prevalence of hand osteoarthritis compared with having ≥ 1 major allele at this locus (95% CI 0.32-0.99, $p<0.05$), suggesting a role for MGP in osteoarthritis[36]. Among 145 participants in the European Vertebral Osteoporosis Study, men with the homozygous MGP-7AA polymorphism had significantly more femoral bone loss as compared to those with genotypes -7GG and -7GA [37]. Those homozygous for MGP 83Ala-Ala had significantly more femoral neck loss as well as a greater tendency to vertebral fractures as compared to those with the genotypes 83Thr-Thr and 83Thr-Ala. A decrease in BMD was observed only in MGP-7AA and MGP 83Ala-Ala genotypes. These associations were not found in the 151 women who participated in the study possibly because 94% of the women were post-menopausal and had independent post-menopausal bone loss that could have confounded the effect of the MGP polymorphisms.
The effect of MGP on fractures and bone density was similarly seen following kidney transplantation. Evenepoel et al. evaluated vitamin K deficiency as measured by dp-ucMGPlevels in 468 de novo kidney transplant recipients. The patients with the highest tertile of dp-ucMGPlevels had lower bone mineral density and had higher incident fractures independently of common fracture determinants (HR 2.21; 95% CI, 1.00 to 4.91; $p < 0.05$) [38].

Studies evaluating the relationship between renal clearance and MGP levels are rare. In 842 outpatients with stable cardiovascular disease and a mean GFR of 76±23 mL/min, each 10 mL/ min

lower GFR was associated with a 79 nM lower ucMGP serum level ($p < 0.001$), and a 0.1 mg/L higher cystatin-C was associated with a 39 nM lower ucMGP serum levels ($p < 0.001$) in multivariate adjusted models [39]. However, when Rennenberg et al. looked at this association, they found no significant correlations between total MGP levels in renal arterial and venous blood and renal clearance of 90 patients with hypertension[40]. It is important to note however that none of the patients in this cohort had a GFR <26 mL/min[40]. A relationship between MGP levels and renal clearance at a GFR <26 mL/min is therefore still possible.

Vitamin K as Ligand of Nuclear Receptors

Vitamin K can act as ligand of the nuclear Steroid and Xenobiotic Receptor (SXR) and its murine ortholog, Pregnane X Receptor (PXR)[41]. SXR/PXR is present in different tissues, including osteoblastic cell lines [42, 43]. The presence of SXR/PXR in osteoblastic tissue is important as it could be the pathway through which vitamin K improves bone health [44].

Transcriptome analysis has revealed a number of bone-related genes which are involved in the vitamin K-SXR pathway. These include tsukushi and matrilin-2, which are involved in collagen and extracellular matrix assembly [45, 46]. In sarcoma cells, vitamin K up-regulates osteoblastic bone markers [43]. SXR/PXR knockout mice have increased bone resorption and decreased bone formation [47].

SXR is additionally involved in bone metabolism via its effect on vitamin D metabolism. In this role, SXR activation can have two effects. SXR activation by some drugs can lead to CYP3A4 expression (exerting 24- and 25-hydroxylase activity) and resultant vitamin D metabolism and deficiency. SXR activation can also lead to inhibition of CYP24A1 (24-hydroxylase activity) in the kidney therefore increasing 1,25(OH)D levels [48]. These data suggest that SXR/PXR is another pathway through which vitamin K is involved in bone homeostasis.

Vitamin K in Chronic Kidney Disease (CKD)

The western diet does not provide enough vitamin K to activate VKDPs in all tissues[49]. This deficiency is more pronounced in adults over the age of 40. Patients with CKD have even greater rates of vitamin K deficiency as compared to the general population. The number of CKD patients who have vitamin K deficiency reaches 70-90% of that population [50, 51, 52] **(Table 1).** Poor oral intake of vitamin K is the main cause of deficiency [50, 53]. When compared to healthy individuals, the vitamin K intake of HD patients is particularly low on days of dialysis and the weekend[54]. The

use of phosphorus binders in the dialysis population contributes to vitamin K deficiency as well [55]. Being lipophilic, vitamin K should not be removed via dialysis. However, studies to validate this hypothesis are needed, because serum levels of 25(OH)-vitamin D, another lipophilic molecule, decreased in patients who were switched from conventional hemodialysis to online hemodiafiltration [56].

There are known implications of vitamin K deficiency in population-based studies and in kidney disease patients [57, 58]. In the Rotterdam study of 7983 men and women over the age of 55, intake of menaquinone protected against incident coronary heart disease (RR of highest tertile of menaquinone intake as compared to lowest tertile = 0.59, p=0.007), and against coronary heart disease related mortality (RR of highest tertile of menaquinone intake as compared to lowest tertile = 0.43, p=0.005). Additionally, the odds ratio of severe aortic calcification was significantly lower in the patients with the highest intake of menaquinone intake as compared to those with lowest intake (OR 0.48, p <0.001) [59]. In the VIKI study, a cohort of 387 dialysis patients, 35.4% of patients had menaquinone-7 deficiency, 23.5% of patients had vitamin K1 deficiency and 14.5% of patients had menquinone-4 deficiency [57]. Patients with menaquinone-4 deficiency had significantly higher aortic calcification (10.6% versus 1.3%, $p = 0.01$). Menaquinone-7 deficiency was associated with significantly higher iliac calcifications (41% versus 28.2%, $p = 0.009$) [57].

There is no gold-standard for the measurement of vitamin K levels and there is a lack in standardization. Instead, functional deficiency of vitamin K is used as a surrogate of vitamin K status in individuals. Vitamin K deficiency in CKD leads to a decrease in the levels of active MGP, a rise in the levels of dp-ucMGP, as well as a rise in the levels of ucBGP[37]. Plasma dp-ucMGP levels increase as CKD advances with highest levels being in CKD stage 5[38]. A dp-ucMGP level of >500 pmol/L, ucBGP>4.5 ng/mL[59] or protein induced by vitamin K absence-II (PIVKA-II) >2 nM/L are indicative of vitamin K deficiency [30, 60].

In 53 dialysis patients, vitamin K2 supplementation resulted in a dose dependent decrease in functional vitamin K deficiency. After a 6-week supplementation regimen, dp-ucMGP levels were reduced 77% and 93% in the groups receiving daily oral administration of 135 μg and 360 μg of K2, respectively[61]. In 200 HD patients receiving vitamin K2 at dose of 360, 720 or 1080 μg thrice weekly for 8 weeks, dp-uc-MGP levels decreased by 17%, 33% and 46% respectively[62]. Several studies show the same pattern **(Table 2).**

Although kidney transplantation is associated with an improvement in vitamin K levels[55], a deficiency in vitamin K was still found in up to 91% of kidney transplant patients. This deficiency may persist as long as 188 months post transplantation[38,–63]. Moreover, in at least one study, vitamin K deficiency in kidney transplant patients was associated with an almost 3 times increase in all-cause mortality [63].

How Current therapy of MBD in CKD Influences Vitamin K levels and VKDPs

While MBD derangements contribute to renal osteodystrophy and to VC in CKD [64], treatments of MBD have not been sufficiently successful at reversing VC, improving cardiovascular events or decreasing mortality. We hypothesize that this might be partly explained by the negative impact of some of the MBD treatments on vitamin K levels. One such treatment is sevelamer. Sevelamer is thought to bind fat-soluble vitamins [65, 66]. Since vitamin K is a fat-soluble vitamin, Jansz et al. assessed the impact of sevelamer on vitamin K in patients who received a kidney transplantation. They found that sevelamer is associated with higher dpu-cMGP levels reflecting vitamin K deficiency [55]. This finding points to the possible need of giving vitamin K supplements to patients treated with sevelamer, but this approach should first be substantiated by a specific study.

However, some MBD treatments are associated with improvements in VKDPs. In an analysis of the VIKI study [57], the use of calcimimetics and vitamin D analogs was associated with higher levels of BGP. Calcimimetic use was also associated with higher levels of total MGP [19]. Therefore, this data suggests that calcimimetics and vitamin D analogs can help preserve or improve the activity of VKDPs.

VKDPs Beyond Bone and Vascular Health

Growth Arrest-Specific Protein 6 (Gas6)

Gas6 is a gamma-carboxyglutamic acid (Gla) domain-containing protein, member of the VKDPs family, which is present in several different tissues (e.g. vascular endothelium, kidney, heart, and the bone marrow). It is a ligand for the TAM (Tyro3-Axl-Mer) receptor family [67] and is thought to be involved in the stimulation of cell proliferation, migration and apoptosis [68, 69].

Gas6 and protein S are two homologous secreted proteins depending on vitamin K for a wide range of their biological functions. A discrete subset of these functions is mediated through their binding to and activation of the receptor tyrosine kinases Axl, Sky and Mer; in particular, the vitamin K-dependent protein Gas6 activates receptor tyrosine kinases of the Axl family [69].

A hallmark of the Gas6-Axl system is the unique ability of both Gas6 and protein S to tether their non receptor-binding regions to the negatively charged membranes of apoptotic cells. A relevant amount of evidence suggests that the Gas6-Axl system is able to regulate cell survival, proliferation, migration, adhesion and phagocytosis. Consequently, an altered expression, or a compromised activity of its components have been detected in a variety of diseases, including different cancer types. Moreover, Axl overactivation can equally occur without ligand binding, which has implications for tumorigenesis. [70]

Upregulation of Gas6 has been described in different malignancies [71], and an increased expression of either Gas6 or TAM receptor proved to be predictive of poor prognosis[72]. A number of animal studies highlighted the role of Gas6 in the processes of carcinogenesis [71, 72, 73], while clinical studies are rarer, but ultimately show consistent findings. Ovarian cancer samples from 90 patients had significantly higher expression of Gas6 and Axl as compared to normal ovarian tissue [73], RNA PCR from 42 glioblastoma frozen sections demonstrated that Gas6 and Axl are overexpressed both in the tumoral, as well as in the surrounding vascular, tissue [74]. Furthermore, glioblastoma patients whose tumors expressed higher Gas6 and Axl levels had significantly higher risk of tumor relapse as well as shorter time to relapse [74]. A similar observation has been reported in osteosarcoma; indeed, in 62 osteosarcoma patients, Axl was highly expressed in 43.5% of the cases, characterized by a significantly higher rate of recurrence, lung metastases, as well as a lower survival [75]. Gas6-Axl is also important as mechanisms of resistance to anticancer therapy; indeed, resistance to tyrosine kinase inhibitors in non-small cell cancer and renal cell carcinoma (RCC) was found to be driven by Axl [76].

As far as RCC, the Axl protein proved to be highly expressed in clear cell RCC cells deficient in functional von Hippel-Lindau (VHL) protein, a tumor suppressor gene often inactivated in ccRCC. VHL reconstituted cells expressed decreased levels of Axl protein, but not Axl mRNA, suggesting that VHL may regulate Axl expression. Furthermore, Gas6-mediated activation of Axl in ccRCC cells resulted in Axl phosphorylation, receptor down-regulation, decreased cell-viability, as well as migratory capacity, whilst no effects of the Gas6/Axl system could be detected on invasion. Moreover, in ccRCC tumor tissues, Axl was phosphorylated and Gas6 gamma-carboxylated, suggesting these molecules to be active in vivo. [77]

All the above has practical therapeutic implications, as targeting the Gas6-Axl pathway through the multikinase inhibitor cabozantinib proved to be an active treatment option for metastatic RCC patients progressing on standard antiangiogenic therapy [78].

Periostin

Periostin is another member of the VKDP family. Similar to other VKDPs, the carboxylation of periostin is dependent on vitamin K. However, it is unknown how the carboxylation status of periostin influences its functions in different tissues. Periostin is an extracellular matrix protein that binds integrins playing a role in cellular adhesion and migration[79]. It plays a role in collagen assembly in several tissues and is upregulated when tissues are subjected to stress[79-81]. Following cardiac injury, periostin is expressed in cardiac myofibroblasts and vascular smooth muscle cells contributing to a profibrotic phenotype[81-83]. Similar to other VKDPs, periostin has also been found in many cancers [84-86]. Periostin induces tumor angiogenesis[84, 85] and lymphangiogenesis [85], and its association with cancer confers a worse prognosis to patients[85]. The role of periostin in breast cancer has been described. Periostin is expressed in invasive ductal carcinoma cells [87]. Its expression increases with the cancer grade, suggesting that periostin may play a role in cancer progression[88]. Periostin can also serve as marker of breast cancer metastasis. Human breast cancer exosomes contain periostin. Further, periostin enriched exosomes were found in patients with lymph node metastasis as compared to those with localized disease[89]. Finally, periostin may have a role in breast cancer prognostication. In 259 breast cancer patients who underwent surgical and radiation therapy, local recurrence-free survival, distant metastasis-free survival and overall survival were significantly lower in the patients whose tumors expressed periostin as compared to those whose tumors were negative for periostin [90].

Gla-Rich Protein (GRP)

GRP is one of the newest members of the VKDP family. Its name derives from the large amount of Gla residues, which comprise 22% of its composition[91], and which make it the VKDP with the highest concentration of Gla residues. Since its discovery, GRP has been found to have a role as an anti-inflammatory protein[92]. In vivo, it prevents osteoarthritis progression[93]. It additionally plays a role in mineralization. In both animal models and in humans, GRP has been found to colocalize with mineral deposits at sites of calcification[94]. Further work demonstrates that similar to MGP, GRP in its carboxylated but not in its undercarboxylated form is a calcification inhibitor [95]. Although GRP role in cancer is less established as compared to other VKDPs, there is growing interest surrounding this protein. The undercarboxylated form as compared to the carboxylated form of GRP is found in more abundance in skin and breast cancer cells, particularly in microcalcifications associated with these tumors [96]. Therefore, GRP may be involved in cancer-related calcifications and as such may prove to be a therapeutic target for some types of cancer.

Vitamin K in Cancer

Several VKDPs are involved in tumorigenesis[71, 84, 85] **(Table 3).** Vitamin K2 administration in vivo inhibits the cellular proliferation of several cancers [96, 97]. This led to a number of studies investigating the role of vitamin K intake and supplementation in preventing cancer development, progression and recurrence. In the European Prospective Investigation into Cancer and Nutrition-Heidelberg cohort study which included 24,340 cancer-free participants followed up for 10 years, there was a significant inverse association between vitamin K2 intake and cancer mortality, but not cancer incidence[98]. Similarly, in the Prevención con Dieta Mediterránea study, which enrolled 7216 participants followed up for a median of 4.8 years, subjects who increased their dietary intake of both vitamin K1 and K2 had decreased cancer incidence[99].

The undercarboxylated form of prothrombin (PIVKAII), a VKDP, is upregulated in hepatocellular carcinoma (HCC) [100]. Vitamin K2 supplementation in patients who underwent curative hepatectomy or radiofrequency ablation for HCC suppressed HCC recurrence, though this effect did not reach statistical significance in any of these studies[101, 102]. In contrast, 45 mg per day of vitamin K2 supplementation resulted in significantly lower risk of HCC development in 21 women who had viral cirrhosis as compared to 19 women with viral cirrhosis who did not receive supplementation[103]. This suggests that vitamin K2 may play a role in preventing the development of HCC in high risk patients. Overall, the association and the relationship of vitamin K with cancer is still uncertain and under investigation. Further studies are needed to define this role of vitamin K.

Conclusion

Substantial research has made it clear that VKDPs or Vitamin-K related pathways can be used in the future to diagnose, treat and prognosticate a number of health conditions. There are still more vitamin K-related roles to be uncovered and which will further our understanding of the physiological and pathological importance of vitamin K status. It will also prove important to recognize the differential actions of vitamin K1 and vitamin K2, and to develop standardized techniques that can directly measure vitamin K levels instead of our current reliance on functional vitamin K status as measured by VKDPs levels [104]. This will allow to develop trials that can evaluate selective and optimal vitamin K supplementation strategies in order to further understand their effect on clinical outcomes.

Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no conflict of interest.

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TABLES AND FIGURES

Table 1. Vitamin K and VKDP levels in Kidney Disease

AUTHOR, YEAR	NUMBER OF PARTICIPANTS	KIDNEY DISEASE STAGE	VITAMIN K FORM MEASURED	% OF PATIENTS WITH VITAMIN K DEFICIENCY	VKDP MEASURED	% OF PATIENTS WITH MEASURED VKDP
Kolheimer M., 1997 [105]	68	ESKD -HD	Phylloquinone	33%		
Pilkey RM., 2007 [106]	142	ESKD	Phylloquinone	29%	ucBGP	93%
Holden RM, 2008 [52]	21	ESKD- PD	Phylloquinone	24%	ucBGP	60%
Holden RM, 2010 [107]	172	CKD 3-5	Phylloquinone	6%	ucBGP PIVKAII	60% 97%
Schurgers LJ, 2010 [108]	107	CK2-5 and ESKD-HD			dp-ucMGP	50%
Schlieper G, 2011 [109]	188	ESKD-HD			PIVKA-II dp-ucMGP	63% 100%
Cranenburg EC, 2012 [54]	40	ESKD-HD	Phylloquinone Menaquinone	45% 100%	PIVKAII dp-ucMGP	82.5% 100%
Westendfeld R, 2012 [61]	53	ESKD-HD	Menaquinone	100%	PIVKAII dp-ucMGP	92.5% 100%
Fusaro M, 2012 [57]	387	ESKD-HD	Phylloquinone Menaquinone-4 Menaquinone-7	23.5% 14.5% 35.4%	ucBGP	100%
Boxma PY, 2012 [110]	60	Post-Transplantation			dp-ucMGP	80%
Caluwe R, 2013 [62]	165	ESKD-HD			dp-ucMGP	100%
Delanaye P, 2014 [111]	160	ESKD-HD			dp-ucMGP	100%
CA, 2015 [63]	518	Post-Transplantation			dp-ucMGP	91%
Aoun M, 2017 [112]	50	ESKD-HD			dp-ucMGP	100%

Table 2. Effect of Vitamin K supplementation on dephosphorylated-undercarboxylated MGP levels in ESKD

AUTHOR, YEAR	STUDY DESIGN	NUMBER OF PARTICIPANTS	KIDNEY DISEASE STAGE	INTERVENTION	OUTCOMES MEASURED	RESULTS
Schlieper G., 2011 [109]	Prospective	17	ESKD	Vitamin K2 at 135 µg/d for 6 weeks	dp-ucMGP level	Vitamin K2 supplementation resulted in a 27% reduction in dp-ucMGP levels p = 0.0027
Westenfeld R., 2012 [61]	Prospective	53	ESKD	Vitamin K2 at 45, 135, or 360 µg/d for 6 weeks	dp-ucMGP level	Vitamin K2 supplementation resulted in a dose-dependent decrease in the levels of dp-uc-MGP by 17.9%, 36.7%, and 61.1% in the 45-, 135-, and 360-µg groups, respectively, compared with baseline values. p<0.005
Caluwe R, 2014 [62]	Prospective	200	ESKD	Vitamin K2 at 60, 720 or 1080 µg thrice weekly for 8 weeks	dp-uc-MGP level	Vitamin K2 resulted in a dose-dependent decrease in the levels of dp-uc-MGP by 17%, 33% and 46% in the 360-, 720- and 1080-µg groups, respectively, compared to baseline values. p < 0.001
Aoun M, 2017 [112]	Prospective	50	ESKD	360 µg of vitamin K2 (menaquinone-7) for 4 weeks	dp-uc-MGP level	Vitamin K2 reduced dp-ucMGP by 86% P<0.05

Table 3. Effects of VKDPs on cancer development and progression.

Vitamin K dependent proteins activity may modulate cancer behavior	
Gas 6	• Angiogenesis • Tumor Progression • Higher Tumor Recurrence • Metastasis and Poorer Prognosis • Cancer Therapy Resistance
Periostin	• Angiogenesis • Lymphangiogenesis • Tumor Progression • Poorer Prognosis
PIVKAII	• Tumor Progression

Figure legends

Figure 1. Different Forms of matrix Gla protein (MGP).