Breast Cancer RESEARCH

REVIEW

Vitamin D and the mammary gland: a review on its role in normal development and breast cancer

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Abstract

Breast cancer is a heterogeneous disease associated with diverse biological behaviours and clinical outcome. Although some molecular subgroups of breast cancer have a targeted therapy, the most aggressive tumours still lack a molecular target. Despite vitamin D being classically associated with the physiological role of calcium regulation and phosphate transport in bone metabolism, several studies have demonstrated a wide range of functions for this hormone, which are particularly important in the field of cancer. The mechanisms underlying the protective actions of vitamin D in cancer development are only sparsely understood, but evidence shows that vitamin D participates in cell growth regulation, apoptosis and cell differentiation. In addition, it has been implicated in the suppression of cancer cell invasion, angiogenesis and metastasis. Most of vitamin D biological actions are mediated by the vitamin D receptor and the synthesis and catabolism of this hormone are regulated by the enzymes CYP27B1 and CYP24A1. In the present review we highlight research data concerning the function of this hormone in the mammary gland, with a special focus on breast carcinogenesis. Hence, and although the available data are controversial, we consider not only updated information on the epidemiology of vitamin D in breast cancer and its potential value as a therapeutic agent or prophylactic (with an emphasis on molecular mechanisms and effectors of vitamin D action), but include data on its role in other stages of breast cancer progression as well. Accordingly, we review data on the influence of vitamin D in the development of normal breast and the expression of vitamin D-related proteins (VDR, CYP27B1 and CYP24A21) in benign mammary lesions and ductal carcinomas *in situ*.

Introduction

Vitamin D was first identified in 1919 by Edward Mellanby as a lipid soluble substance with anti-rachitic properties and is classically associated with its physiological role of calcium and phosphate regulation in bone metabolism. Humans can obtain vitamin D from two main sources: from the diet and from sunlight exposure. Few natural foods contain vitamin D in significant amounts; among these, fatty fish, eggs and sun-dried mushrooms can be highlighted. Still, the majority (90 to 95%) of the required vitamin D is produced by the skin when exposed to sunlight (ultraviolet B radiation) [1], which has caused vitamin D to be nicknamed 'the sunshine vitamin'. The biologically active form of vitamin D (1α,25-dihydroxy-vitamin D_3 or 1α,25$(OH)_2D_3$) is synthesised in the kidney by the mitochondrial enzyme CYP27B1 [2]. In order to maintain the homeostasis of the organism, especially regarding the levels of calcium and phosphate, the amount of circulating vitamin D has to be tightly regulated. The enzyme CYP24A1 plays a key role in this process, and is activated by 1α,25$(OH)_2D_3$ whenever there is an increase in the levels of this hormone. This enzyme is responsible for converting 1α,25$(OH)_2D_3$ to biologically inactive metabolites [2]. In this review, for simplicity purposes, whenever we state vitamin D, we are referring to the biologically active form (1α,25$(OH)_2D_3$) unless stated otherwise.

Vitamin D is a steroid hormone that exerts most of its biological activities by binding to a specific high-affinity receptor, the vitamin D receptor (VDR). VDR belongs to the superfamily of nuclear receptors for steroid hormones and regulates gene expression by acting as a ligand-activated transcription factor [3]. However, vitamin D can also induce VDR-independent effects, indicated by the fact that the anti-proliferative effects of vitamin D in MCF-7 cells are not exclusively dependent on the presence of the VDR [4]. Vitamin D is also known to exert rapid effects that are not dependent on gene transcription [5]. Presumably, these effects are mediated by cell surface

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membrane receptors. Two proteins have been implicated in expression-independent vitamin D action: membrane VDR and the membrane-associated rapid response steroid binding ($1,25D_3$-MARRS) protein. The evidence for the existence of a vitamin D membrane receptor came from two observations: first, the existence of vitamin D analogues that can cause rapid actions of vitamin D, but show low levels of affinity to the VDR [6,7]; and second, the existence of a vitamin D binding protein that has been described in the basolateral membrane of rat and chick enterocytes [8]. Besides the role of $1,25D_3$-MARRS as a vitamin D binding protein, the VDR can mediate non-transcriptional effects [5]. The most striking evidence supporting this hypothesis was the demonstration that the vitamin D-induced rapid actions are lost in osteoblasts from *Vdr* knockout mice [9]. Moreover, VDR has been identified within caveolae-enriched plasma membrane fractions from various cell types [10], suggesting that it can also work as a membrane receptor.

The role of vitamin D in health and disease is far from understood and the literature is full of contradictory findings. The role of vitamin D in cancer is particularly controversial. Still, evidence that vitamin D is capable of modulating several features of cancer exist. Anti-carcinogenic properties of this hormone include the inhibition of cell proliferation, invasion, metastasis and angiogenesis, and the induction of apoptosis and differentiation [5]. These issues will be further elaborated on in the light of breast cancer development in the following sections and controversial issues discussed.

Vitamin D in breast carcinogenesis

Epidemiology of vitamin D in breast cancer

There has been a great amount of information in the literature regarding a protective role of vitamin D in breast cancer. Two major types of epidemiological studies have been conducted: first, those that focused on the association between solar radiation and breast cancer risk; and second, those that analysed the relationship between vitamin D intake and breast cancer risk.

The first set of epidemiological studies demonstrated that decreased sunlight exposure, and consequent diminished vitamin D production by the skin, was correlated with higher breast cancer incidence and mortality [11]. One study described that women with breast cancer had, on average, lower vitamin D blood levels than women without breast cancer [12], hence proposing a potential protective effect of vitamin D. In line with this observation, early stage breast cancer patients show higher serum levels of vitamin D than those who have advanced bone metastatic disease and these changes in hormonal levels preceded clinical signs of progression and predicted disease response [13]. Additional evidence is provided by an inverse association between the circulating levels of 25-hydroxyvitamin D_3 (the inactive circulating form of vitamin D, which is used to measure the levels of vitamin D in the blood stream) and the risk for developing breast cancer, suggesting that high levels of 25-hydroxyvitamin D_3 may be associated with a reduced risk of breast cancer [14]. Furthermore, it has been described that patients with the most aggressive type of breast cancer (triple-negative and basal-like) display the lowest levels of 25-hydroxyvitamin D_3 [15,16]. These results are supported by a recent study where it was observed that breast cancer patients with suboptimal vitamin D levels are more likely to have tumours with aggressive profiles and worse prognostic markers [17]. Hence, these reports indicate that vitamin D deficiency may be linked with increased breast cancer risk, as well as with the development of more aggressive carcinomas. In contrast, other studies found no associations of vitamin D plasma levels or intake with reductions in breast cancer risk. For example, it has been reported that supplementation with calcium and vitamin D did not reduce invasive breast cancer incidence [18,19]. In line with this, it has been observed that circulating levels of vitamin D were not significantly associated with breast cancer risk [19-21].

Although some of these studies demonstrate a potential protective role for vitamin D in breast cancer, suggesting that disruption of the vitamin D signalling pathway may be a predisposition to develop the disease, others have failed to support a causal relationship between vitamin D intake or supplementation and breast cancer risk. Thus, further studies are required in order to clarify this issue.

Role of vitamin D in normal breast development

The VDR is expressed in the normal mammary gland and vitamin D has been shown to play an important role in the development and function of the mammary gland. Most studies conducted to elucidate the role of vitamin D in breast development have been based on the use of *Vdr* knockout mice. Zinser and colleagues [22] performed an elegant study on the role of the vitamin D signalling pathway in the growth regulation of the mammary gland during pubertal development. They showed that *Vdr* knockout female mice display more extensive ductal elongation and branching when compared with their wild-type counterparts. Furthermore, they observed that this enhanced morphogenesis was not associated with the deregulation of cell proliferation and apoptosis pathways in terminal end buds [22]. Moreover, they saw that VDR ablation leads to an increase of breast responsiveness to exogenous hormones (oestrogen and progesterone), represented by an increase in cell growth [23]. Collectively, these data indicate that VDR could have an important impact on breast development and suggest that the vitamin D signalling pathway participates

in the negative growth regulation of the mammary gland. In another study published by the same group [24], it was shown that *Vdr* knockout mice had delayed mammary gland regression after weaning and that this effect was associated with reduced apoptosis in the epithelial cell compartment, hence highlighting the contribution of VDR to mammary gland biology during the reproductive cycle.

Immunohistochemical studies on human material have demonstrated that the VDR protein is expressed in samples from normal breast tissues [25,26]. However, there are some discrepancies concerning the expression of the main enzymes involved in vitamin D metabolism (CYP27B1 and CYP24A1) in normal breast, probably due to the use of different methodologies. Studies assessing the mRNA of the two genes confirmed their expression in the normal mammary gland [27-30] and demonstrated that both enzymes are functionally active in normal breast [30]. In contrast, our group showed, by immunohistochemistry, that both enzymes are detectable in normal breast, but not in all cases [26]. In fact, although the majority of the cases (over 60%) presented CYP27B1 expression, only a small percentage (about 30%) of these presented detectable CYP24A1 protein expression (Figure 1). This work provided evidence for an imbalance in the enzymes that favours the presence of vitamin D in the normal mammary gland. Together with the fact that VDR has an important impact on breast development, it supports the role of the vitamin D signalling pathway in the growth and developmental control of the mammary gland.

Vitamin D in benign lesions of the mammary gland

Benign lesions of the breast can be associated with distinct clinical behaviours and their accurate classification is applicable to patient management in terms of surgical treatment and prophylaxis [31]. In contrast to the extensive studies addressing the expression of Vitamin D signalling and metabolic pathways in breast cancer, the studies in benign lesions of the mammary gland have been ignored. Recently, we have evaluated the expression of VDR, CYP27B1 and CYP24A1 in a series of breast benign lesions [26]. This series consisted of usual and atypical ductal hyperplasias, columnar cell lesions, papillomatosis and adenosis. The results we obtained indicate that the most benign lesions express VDR and CYP27B1 and less than 20% of these cases detectably express CYP24A1. Decreased levels of the three proteins was observed in benign lesions when compared with normal breast, although the results were not statistically significant. This observation may indicate a disturbance in the levels of the proteins that regulate vitamin D metabolism and signalling in early stages of breast cancer development (Figure 1).

Vitamin D in breast cancer models

Suppression of cell growth by vitamin D paved the way for vitamin D to be considered as a potential therapy in cancer research. Since then, numerous studies have been conducted in various cancer models in order to identify the signalling pathways and molecular mediators associated with such effects and these have shown the ability of vitamin D to affect different features of cancer (Figure 2). It was convincingly demonstrated in many of these studies that vitamin D has a prominent role in tumour cell proliferation, and cell cycle genes have consequently become the centre of attention to decrypt the molecular mechanisms of vitamin D in cancer.

Treatment of breast cancer cell lines with vitamin D elicits a change in the expression of proteins involved in cell cycle regulation, such as cyclins, cyclin dependent kinases (CDKs) and CDK inhibitors [5]. Vitamin D and analogues induce increased expression of the CDK inhibitors p21 and p27, thus blocking cell cycle progression [32-34]. Concomitantly, treatment with this hormone leads to impaired expression and activity of CDK2, CDK4, cyclin D1, cyclin D3, cyclin A1 and cyclin E1, which provoked cell cycle arrest at the G_0-G_1 transition [32-34], and this effect was correlated with hypophosphorylation of the retinoblastoma protein pRB. Together with these effects on cell cycle proteins, a downregulation of c-Myc oncoprotein was reported, further contributing to the inhibition of cell proliferation [35]. Altogether, these results demonstrate that vitamin D hampers proliferation by targeting several key masters of cell cycle progression. Additionally, it has been proposed that the transcription factor CCAAT enhancer binding protein alpha (C/EBPα) may mediate vitamin D growth inhibitory effects, since C/EBPα is induced by the treatment with vitamin D. A decrease in cell proliferation was observed in C/EBPα-negative MDA-MB-231 cells transfected with C/EBPα, while the knockdown of C/EBPα suppressed the antiproliferative effects of vitamin D in MCF-7 cells, thus providing evidence for the involvement of this transcription factor in breast cancer proliferation [36]. The breast cancer tumour suppressor TCF-4 is another molecular target of vitamin D action, as it has been reported that this transcriptional regulator is present at lower levels in *Vdr* knockout mice, which suggests a role for TCF-4 in the antiproliferative effects induced by vitamin D [37]. Furthermore, vitamin D induction of the *BRCA1* gene (Breast cancer 1) has also been inversely correlated with cell proliferation [38], while it has been described that vitamin D decreases aromatase expression [39] and, thus, can modulate oestrogen receptor-positive breast cancer growth.

Together with growth arrest, vitamin D has a role in the induction of apoptosis in breast cancer cells (Figure 2), since cell shrinkage, chromatin condensation

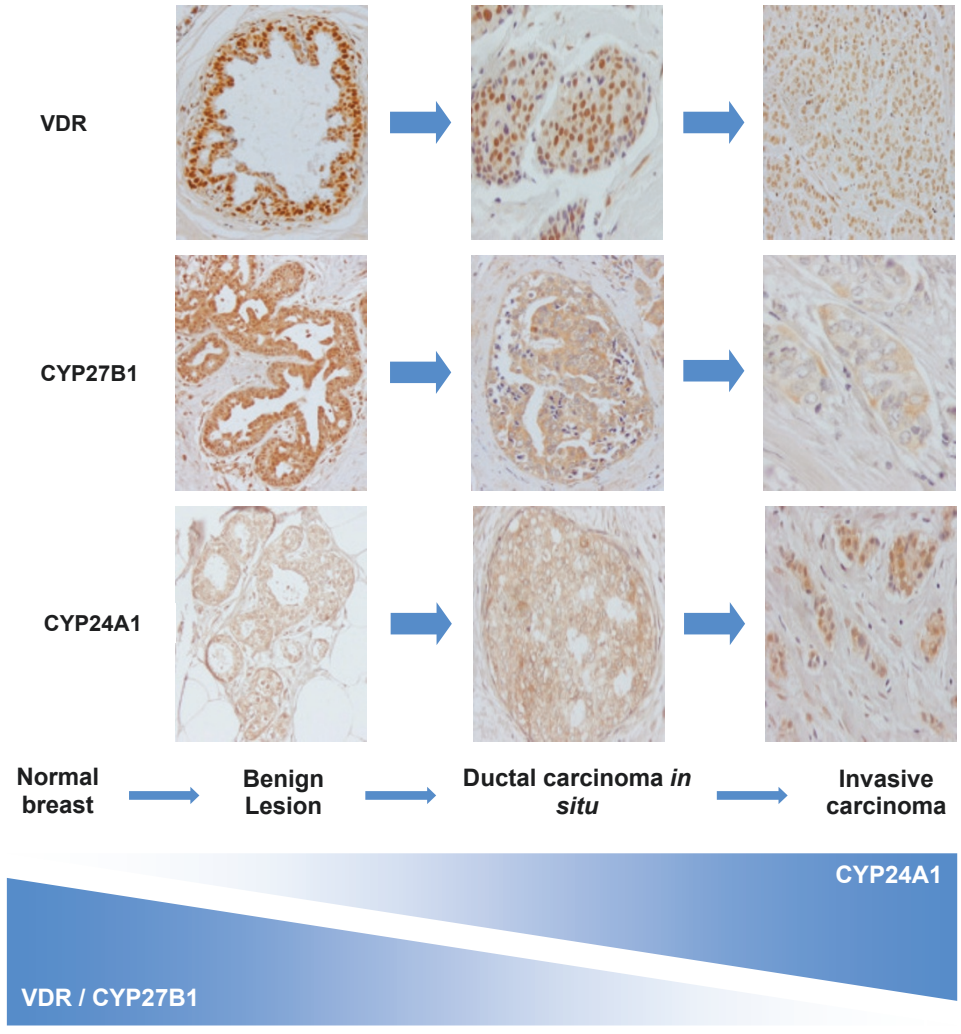

Figure 1. Representation of the differences in the expression of the vitamin D receptor, CYP27B1 and CYP24A1 during breast carcinogenesis. Vitamin D receptor (VDR) and CYP27B1 expression decreases with breast carcinogenesis, while CYP24A1 expression is augmented (brown represents positive staining). (Adapted from [26].)

and DNA fragmentation are observed in MCF-7 cells treated with this hormone [40]. The most probable mechanism of such vitamin D-induced apoptosis is through the downregulation of the anti-apoptotic protein Bcl-2 [5]. Vitamin D is able to enhance tumour necrosis factor alpha through caspase-dependent (increased caspase 3 activity) and caspase-independent mechanisms [41]. In support of the role of caspase-independent cell death mediated by vitamin D, it was shown that induction of apoptosis in MCF-7 cells was correlated with disruption of mitochondrial function, which was associated with Bax translocation from the cytosol to the mitochondria, cytochrome C release and production of reactive oxygen species. These mitochondrial effects did not require caspase activation, since these were not blocked by a specific caspase inhibitor [42]. Another caspase-independent cell death mechanism induced by vitamin D relies upon cytosolic calcium accumulation associated with an increase in lysosomal protease activity [43]. Finally, vitamin D was still described as a pro-oxidant in breast cancer cells, causing an increase in the overall cellular redox potential [44], which may also be an important mechanism underlying the pro-apoptotic effects of this hormone. Vitamin D is able to potentiate and enhance the morphological effects of apoptosis when administered to MCF-7 cells in combination with tamoxifen [45]. Effects of other anticancer agents are improved by the administration of vitamin D: it has been reported that pre-treatment of breast cancer cells with vitamin D enhances the cytotoxicity of doxorubicin [46], lowers the threshold for taxol-induced cell death [47] and enhances the growth inhibition effect induced by

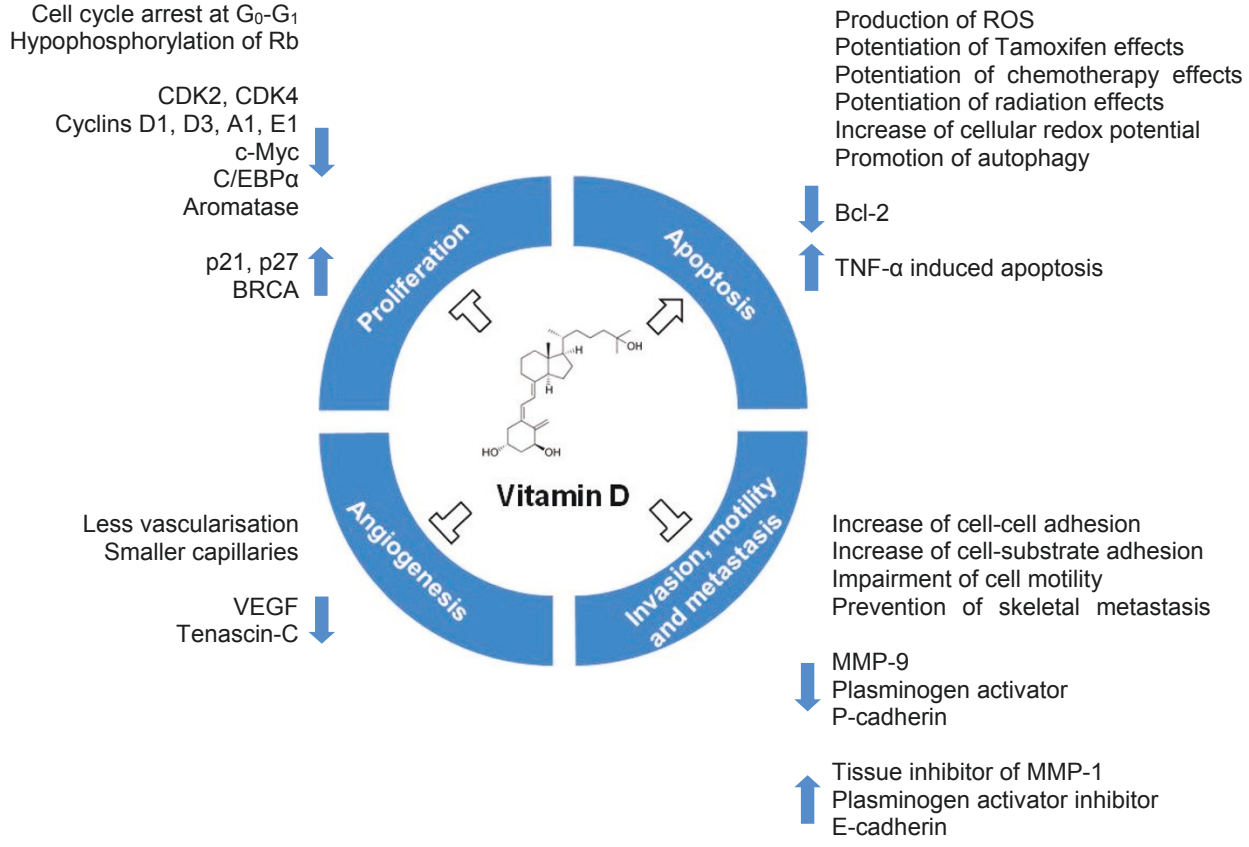

Figure 2. Schematic view of vitamin D effects in breast cancer. C/EBPα, CCAAT enhancer binding protein alpha; CDK, cyclin dependent kinase; MMP, matrix metalloproteinase; Rb, retinoblastoma; ROS, reactive oxygen species; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor.

cisplatin in MCF-7 cells [48]. Importantly, a recent study demonstrates that the interaction between p53 and VDR provides a mechanism for mutant p53 (the most common genetic alteration in human cancers) gain-of-function. This may have clinical implications and suggests that p53 status should be considered when studying vitamin D for cancer therapy [49].

Vitamin D plays an important role in the modulation of cancer invasion and metastasis as well (Figure 2). Hansen and collaborators [50] demonstrated that vitamin D has the ability to inhibit the invasive potential of human breast cancer cells *in vitro*. This reduced invasiveness was found to be associated with diminished activity of the metalloproteinase MMP-9 and downregulation of the plasminogen-activator, simultaneously with increased tissue inhibitor of MMP-1 activity and the induction of plasminogen-activator inhibitor [51]. This is indicative of the dual effect of vitamin D in the invasive process: on one hand, it decreases the activity and expression of metalloproteinases and serine proteases; at the same time, it induces their inhibitors. *In vitro* experiments using vitamin D analogues have demonstrated that they can inhibit the invasive potential of mammary cancer cells [52], as well as prevent skeletal metastasis and prolong survival time in nude mice transplanted with human breast cancer cells [53]. This is in line with the observation that vitamin D induces cell adhesion as well as impairs *in vitro* motility [54], hence contributing to a less aggressive phenotype in breast cancer cell lines. Furthermore, vitamin D is able to downregulate the expression of P-cadherin [54], an invasion promoter molecule in breast cancer cells [55]. We have addressed the effects of vitamin D in E-cadherin-negative breast cancer cell lines and have observed that treatment with vitamin D induces the *de novo* expression of E-cadherin in MDA-MB-231 cells by *CDH1* promoter demethylation [56], providing further evidence for a vitamin D role in invasion and metastasis.

Angiogenesis is yet another cancer feature that vitamin D can modulate (Figure 2). An analogue of vitamin D was able to inhibit angiogenesis at low concentrations *in vivo* [57]. Using xenografted mice with vascular endothelial growth factor (VEGF)-overexpressing MCF-7 breast cancer cells, it was demonstrated that the administration

of vitamin D results in reduced vascularisation of tumours [58]. The tumours formed in the treated animals displayed smaller capillaries when compared with their littermates, suggesting that vitamin D may also inhibit vessel growth and maturation. Additional evidence for angiogenesis inhibition by vitamin D and analogues has shown that it occurs through a decrease in VEGF and tenascin-C expression [59,60].

The anti-proliferative, pro-apoptotic, anti-invasion and anti-angiogenic properties of vitamin D indicate that it could serve as a potential therapeutic agent. However, breast cancer is not one disease and is divided into many different molecular subtypes [61]. This may partly explain the many different functions described above for vitamin D. In order to transform vitamin D into a (targeted) therapy, a better understanding of the role and function of this hormone in solid tumours is required, accompanied by an upfront stratification of the different patient cohorts in the vitamin D research field.

Conclusion

The data described provide evidence for an essential role of vitamin D in normal development of the mammary gland and breast cancer. Epidemiological studies are conflicting and their results not consistent, with the current view indicating no protective role for vitamin D in breast cancer development. Still, the different functions and effects of vitamin D on cancer cell biology, such as the cell cycle, apoptosis, invasion and metastasis, as well as angiogenesis, virtually bring together the entire spectrum of tumour development under its purview.

Abbreviations

C/EBPα, CCAAT enhancer binding protein alpha; CDK, cyclin dependent kinase; MARRS, membrane-associated rapid response steroid binding; VDR, vitamin D receptor; VEGF, vascular endothelial growth factor.

Competing interests

The authors declare that they have no competing interests.

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