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EFFECT OF VITAMIN D SUPPLEMENTATION ON SERUM OSTEOCALCIN AS A BONE TURNOVER MARKER

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Abstract

The most abundant non-collagenous protein in bone matrix is osteocalcin, which is a key biochemical marker of osteoblastic activity and bone formation. The 1,25-dihydroxyvitamin D_3-induced regulation of osteocalcin gene expression via the vitamin D responsive element (VDRE) provides a molecular connection between vitamin D status and the production of osteocalcin. This review is a comprehensive overview of the impact of vitamin D supplementation on serum osteocalcin levels in various populations, based on molecular biology, clinical trials and meta-analyses. The vitamin D receptor (VDR) complexed with its ligand is able to associate with the vitamin D receptor element (VDRE) at nucleotide positions -466 to -437 of the rat osteocalcin gene promoter and consequently stimulate transcription in a cooperative manner with other basal elements of the promoter such as the TATA box and the CCAAT box. But, when translated into the clinic, this molecular relationship is quite complex. Meta-analytical evidence shows that, in women, vitamin D supplementation is associated with a significant decrease in serum osteocalcin (mean difference -0.610; high heterogeneity between studies, likely due to different age, baseline vitamin D status, supplementation dosage and duration, calcium supplementation given concurrently, and menopausal status). In particular, increased levels of vitamin D and/or higher baseline levels of 25-hydroxyvitamin D are associated with improvements in circulating osteocalcin, possibly due to increased carboxylation and improved bone matrix incorporation along with reduced bone turnover. A further twist on the vitamin D status-vitamin K aspect is that vitamin K is required for γ-carboxylating osteocalcin, which otherwise cannot bind to hydroxyapatite. Administration of antiresorptive therapy, calcium, and exercise also modulate the osteocalcin response to vitamin D, with differential osteocalcin response by age group(postmenopausal women versus premenopausal women and men), and with unique pathophysiological considerations in some populations, such as chronic kidney disease, obesity, and diabetes. Interpretation of serum measurements is complicated by methodological issues such as assay standardization, diurnal variation and the presence of osteocalcin fragments. This review aims to present the current knowledge on vitamin D supplementation and serum osteocalcin in an integrated fashion and to identify gaps and unanswered questions. This

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discrepancy in the effects of vitamin D on osteocalcin serves as yet another reminder that tailored approaches to vitamin D supplementation are necessary and that osteocalcin should be interpreted with other bone turnover markers, not as a stand-alone marker. In the future, the potential research directions are: thorough dose-response studies taking into account vitamin K status; the study of the undercarboxylated versus carboxylated fraction of the osteocalcin; the incorporation of the measurement of osteocalcin in the comprehensive fracture risk assessment algorithms.

1. INTRODUCTION

The remarkable role of the skeletal system as a structural and protective organ is now complemented by its well-recognized role as a dynamic metabolic organ which remodels continuously throughout life. The remodeling process is one in which old or damaged bone is resorbed by osteoclasts and new bone is formed by osteoblasts, which keeps the bones intact, in calcium equilibrium, and supports adaptation to mechanical [1]. Metabolic bone disease such as osteoporosis is a disease affecting hundreds of millions people around the world, which places a huge burden on healthcare systems, and is caused by disruption of the delicate balance between bone resorption and formation. Biochemical markers of bone turnover have become an important tool in assessing bone dynamics, fracture risk and response to treatment. [2]Of these markers, osteocalcin is a unique one because it is the most abundant non-collagenous protein in bone and is a specific osteoblast product which reflects the activity of bone formation. Osteocalcin, also known as bone Gla protein, because of the presence of gamma-carboxylation acid residues, is a small (49 amino acids, about 5.8 kDa) protein.[3] It is made by mature osteoblasts and odontoblasts, and is made up of three vitamin K dependent gamma-carboxyglutamic acid residues at positions 17, 21, and 24 of the polypeptide chain. These residues provide the calcium binding properties and allow binding of the protein to hydroxyapatite crystals in the bone matrix; about 60–90 percent of newly synthesized osteocalcin is bound to hydroxyapatite crystals.

Osteocalcin that is not sequestered into bone is excreted into the circulation where it can be measured by immunoassay as a marker of osteoblastic activity and, thus, the rate of bone formation. However, it should be noted that bone resorption produces osteocalcin, which can also be a contributor to circulating levels of osteocalcin, and that the intact protein is rapidly metabolized in the liver and kidneys, resulting in analytical difficulties.[4] Osteocalcin gene expression is one of the best-studied vitamin D-regulated transcriptionally controlled genes. In the late 1980s and early 1990s, the osteocalcin gene promoter was cloned and characterized and a vitamin D responsive element (VDRE) was identified, consisting of nucleotide sequence -466 to -437 of the rat osteocalcin gene, that binds the vitamin D receptor (VDR) with sequence specificity . Because of the demonstration that 1,25-dihydroxyvitamin D_3, the hormonally-active form of vitamin D, stimulates osteocalcin transcription, a direct molecular pathway was identified that could link vitamin D status to osteocalcin production. The molecular relationship led to extensive clinical research into the effect of vitamin D supplementation on serum levels of osteocalcin and the impact this may have on bone health. This is a question with not only academic, but clinical significance.[5]

Vitamin D deficiency is a global problem and is prevalent in many populations across various latitudes, ages and socio-economic statuses. Optimum vitamin D status for skeletal health is important, as rickets has returned in certain areas and osteoporosis prevalence remains high. There is an increasing number of vitamin D supplement options and various professional organizations

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have recommended vitamin D supplementation for certain groups at risk. To understand such a supplementation effect on bone turnover, such as that of serum osteocalcin and other markers, is important for evidence-based clinical practice.

The vitamin D–osteocalcin connection, however, is far from being as simple as it sounds: just a gene stimulator. However, vitamin D administration has been found to have conflicting effects on serum osteocalcin levels, with some studies reporting increases, some decreases, and others no change.[6] This variation is due to the multiple factors that affect vitamin D metabolism, as well as osteocalcin biology, such as baseline vitamin D status, age, menopausal status, renal function, vitamin K status, compounding medications, and dosage and duration of supplementation. Several factors further muddy the waters surrounding serum osteocalcin measurements: fragments from osteocalcin catabolism circulate in the blood, and there are undercarboxylated forms of the protein as well as carboxylated ones; the different commercial assays available are not standardized. Meta-analysis more recently has shed some light: there is evidence for a significant association between vitamin D supplementation and reductions in serum osteocalcin in women, with significant heterogeneity owing to the heterogeneity of the studies included.[7] The bone turnover rate was reduced as shown by the reduction in osteocalcin, as well as in bone resorption markers such as serum C-terminal telopeptide of type I collagen (sCTX) and urinary type I collagen cross-linked N-telopeptide (uNTX), which indicated that the bone turnover rate was reduced overall, and not just bone formation. This discovery supports the premise that vitamin D, through its role in improving calcium uptake and regulating parathyroid hormone, lowers the drive towards more bone turnover during vitamin D deficiency. The purpose of this review is to present a comprehensive synthesis of the existing knowledge about the effect of vitamin D supplementation on serum osteocalcin as a bone turnover marker.[8]

It will explore the molecular mechanisms of the interaction between vitamin D and osteocalcin, the physiological roles of osteocalcin, the clinical trial evidence and meta-analysis, factors influencing the osteocalcin response, methodological issues of measuring osteocalcin, and the role of osteocalcin in the context of other bone turnover markers. In the hope of laying a scientifically sound basis for understanding this clinically relevant but frequently misinterpreted facet of bone biology, a review is provided here that combines molecular, physiological and clinical perspectives. The talk will be organized so that it covers the two aspects known facts and uncertainties and give a balanced and evidence-based examination of the state of knowledge.

2. Osteocalcin: Biochemistry, Physiology, and Role as a Bone Turnover Marker

2.1 Molecular Structure and Post-Translational Modifications

The molecular structure and post-translational modifications of proteins. Osteocalcin is a small protein containing 49 amino acid residues and a molecular weight of ~ 5.8 kDa, highly conserved in humans. The protein is being synthesized as a precursor that is cleaved by a proteolytic enzyme to form the secreted protein. It is characterized by the presence of three gamma-carboxyglutamic acid (Gla) residues at positions 17, 21 and 24 in its amino acid sequence. Such Gla residues are created during post-translational modification of the proteins by an enzyme called gamma-glutamyl carboxylase which requires reduced vitamin K (vitamin K hydroquinone) as a cofactor. In the carboxylation reaction, certain glutamic acid residues are modified to gamma-carboxyglutamic acid (Gla), which is responsible for the calcium binding properties of the protein and for its interaction with hydroxyapatite, the mineral part of bone. [9]Nuclear magnetic resonance spectroscopy has been used to determine the structure of osteocalcin, which shows a dramatic conformational change on calcium binding. Without calcium, the molecule is for the most part unordered and flexible.

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The binding of calcium ions, however, causes a reorientation to an alpha-helical conformation which brings the Gla residues on one side of the helix into close contact with the crystalline surface of hydroxyapatite. This calcium-dependent structural change underlies the role of osteocalcin as a protein of the mineralized matrix, and has led to preferential binding to bone mineral. Their ability to bind to hydroxyapatite means that the newly synthesized protein is trapped with about 60-90 percent in the bone matrix and the rest in the circulation. The state of carboxylation of osteocalcin is now becoming recognized as an important factor in the ability of osteocalcin to bind to bone, as well as its possible endocrine activity. Typically, osteocalcin molecules that are undercarboxylated (ucOC) are seen to have a lower affinity to hydroxyapatite and are more easily released into the bloodstream, usually because of insufficient vitamin K.[10] Undercarboxylated osteocalcin has been found to be a hormone secreted by bone, which acts on the receptor G protein-coupled receptor family C group 6 member A (GPRC6A), and thus regulates energy metabolism, glucose homeostasis, and male fertility. In the following paragraphs the endocrine activities of osteocalcin will be reviewed in more detail, with the most recent report that undercarboxylated osteocalcin might have a reciprocal effect on vitamin D metabolism by stimulating the production of 25-hydroxyvitamin D by the Leydig cells.

2.2 Role of 1,25-Dihydroxyvitamin regulation of Osteocalcin gene expression

Since the cloning and characterization of the rat and human osteocalcin genes in the late 1980s, the regulation of osteocalcin gene expression has been thoroughly investigated. It provided a pivotal advance in understanding the molecular action of vitamin D on bone when a study was reported that showed that 1,25-dihydroxyvitamin D_3 could strongly stimulate the transcription of the osteocalcin gene.A key study that came up with the discovery of 1,25-dihydroxyvitamin D_3's ability to potently induce osteocalcin gene transcription was an important advance in understanding vitamin D action in bone. A well-documented vitamin D responsive element (VDRE) exists in the osteocalcin gene promoter to which 1,25-dihydroxyvitamin D_3 binds and regulates the transcription of the gene.[11] The VDRE within the rat gene of osteocalcin is situated between the nucleotides -466 and -437 from the transcription start site. A motif of an imperfect direct repeat of the hexanucleotide sequence AGGTCA separated by 3 nucleotides, a form that is typical of classical VDREs. This sequence is bound by the vitamin D receptor (VDR) which belongs to a family of nuclear receptors that heterodimerize with retinoid X receptor (RXR). When no ligand is present, the VDR-RXR heterodimer can interact with the VDRE with corepressor proteins that keep the gene in a "repressed" state by deacetylation of histones.[12]Once 1,25-dihydroxyvitamin D3 has bound to the VDR, it undergoes a conformational change that facilitates the binding of coactivator proteins, such as members of the steroid receptor coactivator family, which in turn results in histone acetylation, chromatin remodeling and increased transcription.

The complexity of regulation of the osteocalcin gene is not limited to the VDRE. The effect of vitamin D on osteocalcin gene expression was found to be dependent on and related to basal gene expression by Bortell and colleagues, implying that interaction between the VDRE and other promoter elements results in both basal and vitamin D-enhanced gene transcription. The interaction of protein with the VDRE is evident by the sequence-specific and antibody-sensitive binding of endogenous vitamin D receptor in nuclear protein extracts of osteoblastic cells. In addition, a vitamin D-induced increase in gene transcription of osteocalcin is followed by an increase in protein-DNA interactions within the TATA box region (nucleotides -44 to +23) that also contains a potential glucocorticoid responsive element. [13]The cooperative action of the VDRE and the proximal promoter element including the CCAAT box of osteocalcin at position -99 to -76, suggests that vitamin D receptor and associated factors work together

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with the basal transcriptional machinery to regulate the amount of osteocalcin gene transcription. It seems that the architecture of the nucleus is important in order to facilitate such control interactions. Nuclear matrix attachment elements in the promoter of the rat osteocalcin gene which are sequence specific for nuclear matrix proteins can be involved in the conformation of the promoter to make the VDRE accessible to basal regulatory elements. Based on the results of micrococcal nuclease digestion and Southern blot analysis, it is estimated that about three nucleosomes fit in the sequence between the osteocalcin gene VDRE, the CCAAT box and the TATA/glucocorticoid responsive element, limiting the distance between the VDRE and the basal regulatory elements . The authors propose that the higher-order chromatin structure plays a role in the integration of vitamin D signals into other signals affecting the osteocalcin gene promoter. [14]

2.3 Physiological functions of osteocalcin

Physiological roles of osteocalcin have been extensively studied and, to some extent, still discussed. The classical role of osteocalcin as a bone matrix protein that regulates hydroxyapatite crystal size and maturation has been greatly expanded by the identification of its endocrine role. Both the skeletal and extraskeletal functions of osteocalcin will be discussed. The carboxylated osteocalcin in the skeleton binds strongly to the hydroxyapatite crystals via its Gla residues and is thought to control the size and orientation of the hydroxyapatite crystals in the collagen fibril structure.[15]Osteocalcin knockout mice originally showed that osteocalcin inhibited bone formation and that there was more bone mass in these animals than in wild-type controls, as well as better biomechanical properties.

Later studies, however, have shown a more complex pattern, in which osteocalcin seems to control the orientation of biological apatite crystals parallel to the collagen fibrils, and thus plays a role in the strength of bone without being linked with bone mass. The carboxylated version of osteocalcin could also act as a template for mineralization or regulator of the crystal growth kinetics when it is combined with calcium phosphate. In addition, osteocalcin secreted from bone matrix during osteoclastic resorption could be a chemoattractant for osteoclast progenitors implying a function in coupling bone resorption with bone formation. Recognition of osteocalcin as an endocrine hormone has changed the view of the skeletal system as an organ that is part of systemic physiology. Undercarboxylated OCN has been demonstrated to increase insulin secretion from pancreatic beta cells, increases the expression of adiponectin by adipocytes, and increases insulin sensitivity of peripheral tissues. [16] These effects are mediated via GPRC6A, which is expressed in various tissues such as the pancreas, adipose tissue, skeletal muscle and testes. It is clear that the metabolic functions of osteocalcin create a bone-pancreas endocrine loop that links the metabolic output of bone with the production and bioactivation of insulin in the pancreas, which in turn affects energy metabolism. In relevance to the topic of this review, there is evidence that osteocalcin could also affect vitamin D metabolism and that it could be affected by vitamin D metabolism. De Toni and colleagues showed that, in a mouse Leydig cell line, undercarboxylated osteocalcin is able to activate GPRC6A and induce 25-hydroxyvitamin D production . Undercarboxylated osteocalcin stimulated the expression of the important enzyme, CYP2R1, which is involved in vitamin D 25-hydroxylation, in a dose-dependent fashion, which led to a dose-dependent increase in release of 25-hydroxyvitamin D into the culture medium from MA-10 Leydig cells.

Blocking the receptors with anti-GPRC6A antibodies prevented this effect. These results are supported by the relevance found in vivo, as the serum levels of undercarboxylated osteocalcin were positively associated with the serum levels of 25-hydroxyvitamin D in overweight male patients and controls.[17]The observations indicate the presence of a regulatory feedback loop such that vitamin D leads to production of osteocalcin, and undercarboxylated osteocalcin stimulates

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hydroxylation of vitamin D to its circulating precursor form. The potential for this feedback mechanism has significant impact on the results of studies investigating the impact of vitamin D supplementation on serum osteocalcin.

2.4 Renal Phosphaturic hormone regulation of bone turnover

Circulating osteocalcin is widely used as a biochemical marker of bone formation since it is produced selectively by osteoblasts and has been correlated with histomorphometry findings of the bone formation process. But there are a number of factors to consider when using serum osteocalcin as a bone turnover marker. The advantages and disadvantages, and clinical uses, of osteocalcin measurement are addressed in the following discussion. Since osteocalcin comes from osteoblasts, it is regarded as a marker of bone formation and not bone resorption.[18] Osteocalcin in the circulation is related to indices of osteoblastic activity and bone formation rate in patients with different metabolic bone diseases in bone biopsy specimens.

Serum osteocalcin levels are generally elevated in conditions marked by high bone turnover where both the resorption and formation of bone is increased, including Paget disease of bone, hyperthyroidism and primary hyperparathyroidism. However, low bone turnover, such as that during corticosteroid excess, is associated with low levels of osteocalcin, and osteocalcin is especially sensitive to the effect of suppressed osteoblasts and is profoundly suppressed after a single dose of high doses of a corticosteroid. Some limitations must be taken into account when interpreting serum osteocalcin concentrations, however. First, only a small proportion of the osteocalcin that is synthesized is secreted in the blood, the rest remaining in the bone matrix. The percentage released may be altered with various physiological and pathological states, which could separate serum concentrations from actual bone formation rate.[19] Second, the circulating level of osteocalcin is rapidly degraded and its half-life is mainly determined by renal clearance. Immunoreactive fragments from the catabolism of osteocalcin may give inconsistent results based on the antibody specificity used. Third, the carboxylation status of circulating osteocalcin is influenced by vitamin K status, and assays may vary in their ability to detect carboxylated vs undercarboxylated osteocalcin.

Finally, there are large diurnal variations in serum osteocalcin levels, with peak levels in the night and early morning and a nadir in the afternoon, which means that the collection of samples needs to be standardized for valid comparisons. The challenges and methodological issues specific to measurement of osteocalcin will be covered later in this review in more detail (Section 5). Response to therapeutic interventions is commonly monitored by measuring osteocalcin, especially in the treatment of osteoporosis where large decreases are seen coupled with the inhibition of bone resorption. However, the use of osteocalcin as a marker of the effectiveness of vitamin D supplementation is complicated by the molecular link between vitamin D and osteocalcin gene expression, the pleiotropic actions of vitamin D on bone metabolism, and the clinical results of these studies are highly variable as will be described in this review. [20]

3. Vitamin D and Its Molecular Interaction with the Osteocalcin Gene

3.1 Vitamin D Metabolism

Vitamin D exists in two forms, a seco-steroid hormone, and can be obtained from two major sources: endogenous production in the skin upon exposure to ultraviolet-B radiation (290-315 nm) that converts 7-dehydrocholesterol to previtamin D_3 which then gets converted to vitamin D_3 (cholecalciferol); dietary intake of vitamin D_2 (ergocalciferol) from plant sources and vitamin D_3 from animal sources such as fortified foods and supplements. The transport of both vitamin D_2 and D_3 occurs in the circulation in complex with vitamin D-binding protein, and a dual hydstructural transition is crucial to the action of osteocalcin as a mineralized matrix protein, accounting for its preferential association with

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bone mineral. [21] The affinity of fully carboxylated osteocalcin for hydroxyapatite leads to a sequestration of about 60% to 90% of newly secreted protein in the bone matrix and the remaining 10% to 40% entering the circulation. The carboxylation of osteocalcin has been found to be an important factor in the binding of the molecule to bone, as well as in its possible endocrine activity. Osteocalcin molecules which are undercarboxylated (ucOC) demonstrate a decreased affinity for hydroxyapatite and are more likely to be found in the circulation, commonly because of a lack of vitamin K. Undercarboxylated osteocalcin has been found to be a hormone secreted from bone that acts on the G protein-coupled receptor family C group 6 member A (GPRC6A) to act on energy metabolism, glucose homeostasis, and male fertility. [22]The endocrine actions of osteocalcin will be referred to in more detail later: recently, it was found that undercarboxylated osteocalcin could regulate vitamin D metabolism in a reciprocal manner by stimulating the production of 25-hydroxyvitamin D in Leydig cells . 2.3 Actions of 1,25-Dihydroxyvitamin D_3 on the Osteocalcin.

3.2 Actions of 1,25-Dihydroxyvitamin D_3 on the Osteocalcin Gene.

Since the late 1980s when the rat and human osteocalcin genes were first isolated and sequenced, the regulation of osteocalcin gene expression has been extensively studied. Along the way, this research team made a key discovery: 1,25-dihydroxyvitamin D_3 is a potent inducer of osteocalcin gene transcription. The gene promoter of osteocalcin includes a well characterized vitamin D responsive element (VDRE) that transcribes in response to 1,25-dihydroxyvitamin D_3. The VDRE in the rat osteocalcin gene is from -466 to -437 from the start site. The imperfect direct repeat of the hexanucleotide sequence AGGTCA separated by 3bp is the DR3 motif found in classical VDREs. [23]

This sequence is bound by the vitamin D receptor (VDR) heterodimerized with retinoid X receptor (RXR) of the nuclear receptor superfamily. When the VDR-RXR heterodimer is not bound to the VDRE, it can bind to the VDRE with corepressor proteins that keep the gene repressed by deacetylation of histones. The VDR binding of 1,25-dihydroxyvitamin D3 causes a conformational change that facilitates the recruitment of coactivator proteins such as members of the steroid receptor coactivator family which then lead to histone acetylation, chromatin remodeling, and increased transcription. The VDRE is not the only complex regulatory element of the osteocalcin gene. Bortell and her colleagues showed that the ability of vitamin D to stimulate osteocalcin gene expression depends on and is linked to basal gene expression; that is, the Vitamin D Receptor (VDR) interacts with other promoter elements to enhance basal and vitamin D-mediated transcription. [24] The endogenous vitamin D receptor is directly bound and antibody-sensitive to the VDRE in nuclear protein extracts from osteoblastic cells, reflecting protein-DNA interactions at the VDRE. In addition, there is a vitamin D-dependent increase in the transcription of the osteocalcin gene, along with greater interactions of the protein with the TATA box region of the gene (-44 to +23) that also has a potential glucocorticoid responsive element

The cooperative interaction among the VDRE and the proximal promoter elements, such as the osteocalcin CCAAT box between -99 and -76, indicate that the vitamin D receptor and its associated factors act together with basal transcriptional machinery to regulate the level of osteocalcin gene transcription. Nuclear architecture seems to be important in supporting these regulatory interactions. Nuclear matrix attachment elements (NM-AE) of the rat osteocalcin gene promoter can bind sequence specifically to nuclear matrix proteins, and may impose structural constraints on the conformation of the promoter that bring the VDRE into proximity with basal regulatory elements. Micrococcal nuclease digestion and southern blot analysis suggest that only about three nucleosomes can fit in the region between

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the osteocalcin gene VDRE and the CCAAT box and TATA/glucocorticoid responsive element, thus limiting the distance between the VDRE and the basal regulatory elements. In this model, higher order chromatin structure plays a role in the integration of vitamin D input with other regulatory inputs on the osteocalcin gene promoter. [25]

3.3 Physiological Functions of Osteocalcin

The physiological role of osteocalcin has been extensively studied and in certain areas of interest, is still under debate. The recognition of osteocalcin's endocrine role has greatly expanded the classical role of the protein as a bone matrix protein that regulates the size and maturation of hydroxyapatite crystals. The skeletal and extraskeletal function of osteocalcin will be explored. In the skeleton, the carboxylated osteocalcin is tightly bound to hydroxyapatite crystals by its Gla residues and it is thought to control the size and orientation of the mineral crystals in the collagen fibril network. In the early days the role of osteocalcin in the inhibition of bone formation was suggested from studies in osteocalcin knockout mice, demonstrating that the absence of osteocalcin resulted in augmented bone mass and enhanced biomechanical properties. [26] Further research has shown that osteocalcin seems to control the orientation of the biological apatite crystals in parallel with the collagen fibrils, and therefore also plays a role in bone strength apart from the bone mass. The carboxylated form of osteocalcin could also act as a nucleating site for mineralization or act as a regulator of crystal growth kinetics, by binding calcium and hydroxyapatite. Furthermore, osteocalcin, which is released from bone matrix during osteoclastic resorption, may be a chemoattractant for osteoclast precursors, which could imply involvement in the coupling of osteoclastic resorption with new bone formation. [27] The discovery of osteocalcin as an endocrine hormone has led to a paradigm shift in the notion of the skeleton as part of the whole. It has been demonstrated that undercarboxylated osteocalcin stimulates insulin secretion by pancreatic beta cells, increases adiponectin expression by adipocytes and improves insulin sensitivity in peripheral tissues.

These effects occur via the binding to GPRC6A, which is found in several tissues such as the pancreas, adipose tissue, skeletal muscle and testes. Metabolic actions of osteocalcin set up a bone-pancreas endocrine loop that links the production of bone-derived osteocalcin with its effects on energy metabolism, and the role of insulin signaling in the modulation of osteocalcin production and its bioactivation. A particular aspect of this review is the recent evidence suggesting that osteocalcin can have a reciprocal effect on vitamin D metabolism. Undercarboxylated osteocalcin is shown to stimulate 25-hydroxyvitamin D production in a mouse Leydig cell line via a GPRC6A-dependant pathway by De Toni and colleagues. Undercarcosylated osteocalcin (uOC) stimulated the expression of CYP2R1, the major enzyme responsible for vitamin D 25-hydroxylation, in a dose-dependent manner and led to an increase in the release of 25-hydroxyvitamin D into the culture medium in a dose-dependent manner in MA-10 Leydig cells. This effect was abolished by receptor blockade with anti-GPRC6A antibodies. Supporting this in vivo finding, undercarboxylated osteocalcin levels were positively associated with 25-hydroxyvitamin D levels in overweight male patients and controls. [28] Based on these observations, a regulatory feedback loop is suggested that involves vitamin D stimulating osteocalcin production, and undercarboxylated osteocalcin stimulating the hydroxylation of vitamin D to its circulating precursor form. The impact this proposed feedback mechanism has on interpretation of the studies exploring the effect of vitamin D supplementation on serum osteocalcin is significant. 2.4 Osteocalcin as a Bone Turnover Marker Serum osteocalcin has been broadly utilized as a biochemical marker for bone formation because it is specifically synthesized by osteoblasts, and is correlated with histomorphometric indices of new bone formation. Several factors need to be considered

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when the serum osteocalcin level is used as a marker of bone turnover. [29]

Osteocalcin is thought to be a marker of bone formation, and is subject to hydroxylation to obtain its biological activity. 25-hydroxyvitamin D is formed primarily in the liver, with the enzyme responsible for the first hydroxylation being the cytochrome P450 enzyme CYP2R1 and 25 hydroxylases. This is the most common form of vitamin D in the blood, and has a half-life of about two to three weeks, making it the main clinical indicator of vitamin D. The second hydroxylation takes place predominantly in the proximal renal tubules, where the enzyme CYP27B1 (1-alpha-hydroxylase) converts 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D_3, the hormonally active form. The activity of CYP27B1 is firmly controlled by parathyroid hormone, calcium and phosphate levels, and by the fibroblast growth factor 23 (FGF23) to maintain vitamin D homeostasis. Another area of emerging interest is the fact that CYP27B1 is expressed in extrarenal tissues such as osteoblasts where local production of 1,25-dihydroxyvitamin D_3 may play paracrine or autocrine roles in bone metabolism. This idea proposes that the levels of 25-hydroxyvitamin D in the blood could affect bone cell function not just through their ability to produce 1,25-dihydroxyvitamin D in the kidneys and release it into the blood, but also through their local production in the bone microenvironment.

The effect of vitamin D on the regulation of osteocalcin, as demonstrated by De Toni et al., who showed that undercarboxylated osteocalcin increases in Leydig cells the expression of CYP2R1 and production of 25-hydroxyvitamin D through a GPRC6A-dependent mechanism, further complicates the vitamin D-osteocalcin relationship. [30] The biological activity of 1,25-dihydroxyvitamin D3 is exerted mainly via binding to its receptor, the vitamin D receptor (VDR), a member of the nuclear receptor superfamily of ligand-activated transcription factors. The VDR is found in nearly all tissues and cell types, where it is highly expressed in classical target tissues that regulate calcium and phosphate homeostasis such as the intestine, kidney and bone. In the nucleus, ligand bound VDR binds to vitamin D responsive elements (VDREs) and heterodimerizes with retinoid X receptor (RXR) to form a receptor complex that binds to and activates vitamin D responsive genes. The hexameric sequence AGGTCA is repeated in a direct repeat with a spacing of 3 bases, although there is a lot of diversity in the sequence, spacing and orientation of responsive elements in different genes. Binding of the VDR-RXR heterodimer to the VDRE kicks off a complicated series of events that include the recruitment of coregulatory proteins. When not bound to ligand, the free VDR or free VDR-RXR heterodimer can bind to corepressor proteins like nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid hormone receptors (SMRT) to bring histone deacetylases and keep the chromatin in a transcriptionally repressed state.[31]

Upon ligand binding, VDR undergoes conformational changes that allows corepressors to be removed and coactivator proteins, such as the members of the p160 steroid receptor coactivator (SRC) family and the vitamin D receptor-interacting protein (DRIP)/mediator complex are recruited. Coactivators have histone acetyltransferase activity or they bring acetyltransferase-containing complexes to the target gene promoters that result in histone acetylation, chromatin decompaction and increased accessibility of the transcription machinery. Eventually, the coordinated action of several co-activator complexes leads to the recruitment of RNA polymerase II and the start of transcription. Vitamin D does not only act as a transcriptional activator of gene expression. [32] However, a significant number of vitamin D target genes are negative targets and are regulated by less well understood mechanisms that could include direct transcriptional repression of the gene, interference in the ability of other transcription factors to bind/repress the gene, or epigenetic modification of the gene. Vitamin D's net effect on bone metabolism is the result of the

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combined transcriptional programs in various cell types and on various target genes.

3.4 The Vitamin D Responsive Element in the Osteocalcin

Gene Classical VDRE is found on the rat osteocalcin gene approximately 450 base pairs upstream of the transcription start site (nucleotides -466 to -437). This element is the same as the DR3 motif found in vitamin D-responsive promoters and has the sequence: CTGGGTGAATGAGGACATTACT. Using electrophoretic mobility shift assays with nuclear protein extracts from ROS 17/2.8 rat osteosarcoma (OS) cells, detailed characterization of protein-DNA interactions at this VDRE showed direct sequence specific binding of the vitamin D receptor (VDR), which was confirmed by antibody-mediated supershift experiments . These studies gave clear proof that the osteocalcin gene is a direct transcriptional target of the vitamin D receptor, and set the paradigm for understanding vitamin D mediated control of gene expression in bone. The osteocalcin VDRE has additional functions other than binding to the VDR-RXR heterodimer. Bortell and colleagues have shown that vitamin D induced increases in osteocalcin gene expression is dependent on basal gene expression and is inversely correlated to basal gene expression, suggesting that the VDRE does not act alone, but rather works with other promoter elements that affect basal transcriptional activity. [33]The osteocalcin gene promoter harbours a CCAAT box element at position -99 to -76 that can bind the CCAAT/enhancer-binding proteins and is involved in tissue-specific expression. Moreover, the TATA box region (nucleotides -44 to +23) has a potential glucocorticoid responsive element, thus allowing for multiple hormonal pathway coupling. This observation, together with the direct interaction of VDR with the VDRE, indicates that the vitamin D signal is propagated to the basal transcriptional machinery via cooperative interactions between regulatory complexes that bind at the VDRE and at nearby promoter elements.

Three-dimensional organization of the osteocalcin gene locus is important in the integration of these regulatory elements. The promoter of the osteocalcin gene appears to be bound to the nuclear scaffold and thereby conformationally constrained, thereby possibly promoting interactions between far separated regulatory elements. Evidences for positioned nucleosomes within the region comprising the VDRE, the CCAAT box and the TATA box/glucocorticoid responsive element have been obtained by chromatin studies based on limited micrococcal nuclease digestion and Southern blot analysis. This regulatory region is capable of accommodating three nucleosomes, thereby bringing the VDRE closer to the proximal promoter, which could facilitate cooperative interaction between the complex containing the VDR and the basal transcription factors that form at the TATA box.[34]This nuclear architectural model emphasizes the role played by the three-dimensional organization of chromatin to allow crosstalk among regulatory elements required for proper physiological regulation of the osteocalcin gene.Figure 1: The regulatory architecture of the rat osteocalcin gene promoter is shown with relative positions of key regulatory elements depicted. The vitamin D responsive element (VDRE) is located from nucleotides -466 to -437 and is an imperfect direct repeat of sequence AGGTCA separated by 3 nucleotides (DR3 motif). The 1,25-dihydroxyvitamin D_3-VDR heterodimerizes with retinoid X receptor (RXR) and specifically binds the element. The osteocalcin CCAAT box exists at -99 to -76 and is bound by CCAAT/enhancer-binding proteins which are involved in basal and tissue-specific transcription. The TATA box region is nucleotides -44 to +23, includes the TATA sequence necessary for the assembly of the RNA polymerase II pre-initiation complex and contains a potential glucocorticoid responsive element (GRE). The chromatin conformation is regulated by the attachment of the regulatory region of the promoter to the nuclear scaffold by nuclear matrix attachment elements flanking the regulatory region. Nucleosomes (cylinders) are

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positioned to allow three nucleosomes to exist between the VDRE and the TATA box, to bring the VDR-RXR-coactivator complex and the basal transcriptional machinery closer together and promote cooperative interactions. It is important for the vitamin D signaling to be integrated with other inputs that are all of a 3-D nature and determine the rate of osteocalcin gene transcription.

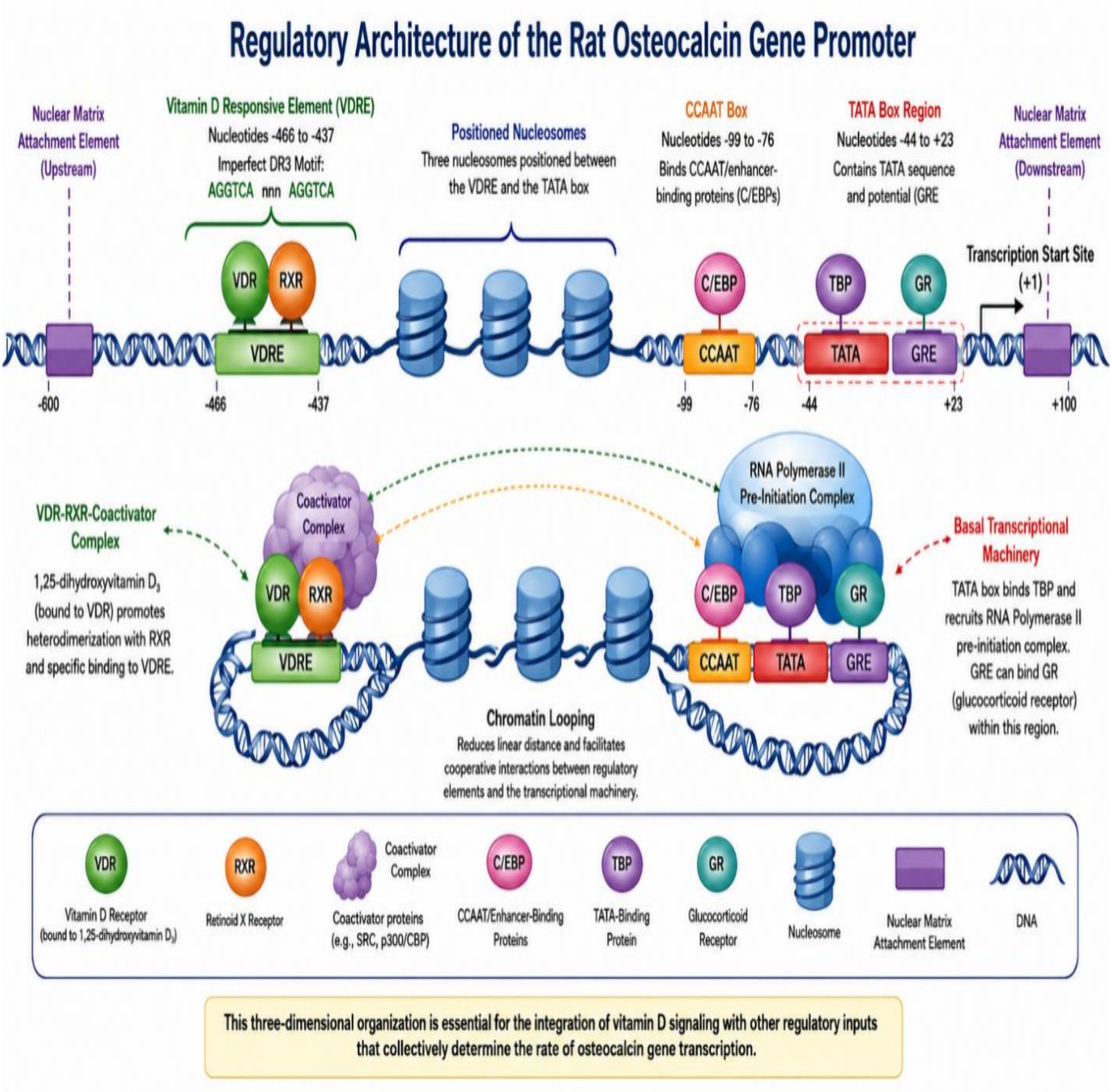


Figure 1: Schematic Representation of the Rat Osteocalcin Gene Promoter

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4. Clinical Evidence: Effects of Vitamin D Supplementation on Serum Osteocalcin

4.1 Systematic Reviews and Meta-Analyses Basics

The effects of vitamin D on bone turnover, and longer studies are needed to see the changes The most comprehensive assessment of the impact of vitamin D supplementation on the serum osteocalcin was provided by Nasimi et al. in a British Journal of Nutrition systematic review and meta-analysis published in 2024 . The authors performed a meta-analysis of the effects of vitamin D supplementation or fortification on a panel of bone turnover markers (BTMs) in women of age 18 years and older, including markers of bone resorption (serum C-terminal telopeptide of type I collagen [sCTX] and urinary type I collagen cross-linked N-telopeptide [uNTX]) and bone formation (osteocalcin, bone alkaline phosphatase [BALP] and procollagen type-1 intact N-terminal propeptide [P1NP]). The number of studies that reported osteocalcin outcomes in response to vitamin D administration is considerable and 24 studies were included in the meta-analysis. The vitamin D supplementation resulted in a significant difference in serum osteocalcin, with a mean difference of -0.610 and a 95% confidence interval excluding zero, in the pooled analysis. The result suggests that, overall, vitamin D supplementation was correlated with a statistically significant decrease in circulating osteocalcin concentration. It is interesting that this direction is unlike the stimulatory effect of 1,25-dihydroxyvitamin D_3 on the transcription of the osteocalcin gene shown in cellular and molecular studies [35].

The authors looked at sources of this heterogeneity in subgroup analyses and meta-regression models and found that age, sample size, vitamin D dose, duration of supplementation, baseline serum 25-hydroxyvitamin D levels, geographic region, and the quality of the study all had a significant impact on the variability observed. The potential age effect modifier was identified, with the effect of vitamin D potentially varying between younger and older women. Vitamin D dosage (usually in international units/day or micrograms/day) and duration of supplementation were important variables for the meta-regression. Heterogeneity also occurred with respect to baseline vitamin D status (defined as the serum 25-hydroxyvitamin D concentration before the beginning of the vitamin D supplementation), which may affect interpretation of vitamin D supplementation effects among populations with varying vitamin D nutriture. Subgroup analyses were used to add detail to the overall results. Decreases in a second measure of bone formation, P1NP, were significant in samples less than 600 micrograms per day and in studies with larger numbers of participants, more than 100. The effect on osteocalcin was more pronounced across the various subgroupings, but most often, none of the stratification variables was able to eliminate the subgroup heterogeneities. The meta-analysis also included the bone formation markers BALP, which had no significant change with vitamin D supplementation. [36] The decrease in bone resorption markers (sCTX (mean difference -0.038) and uNTX (mean difference -8.188) with no specific heterogeneity for uNTX) also suggests a widespread decrease in bone turnover rate and not simply inhibition of bone formation, thus explaining the decrease in osteocalcin. This interpretation is consistent with the physiological effect of vitamin D on enhancing calcium absorption and hence decreasing the need to mobilize calcium from the bone, thus lowering the stimuli to bone remodeling. A coupling theory, a basic concept in bone biology, which means that bone resorption is usually if not always matched by bone formation within a single remodeling cycle. Individual Randomized Controlled Trials In addition to the overall estimations from the metaanalyses, individual RCTs give valuable information on the conditions under which vitamin D supplementation affects serum osteocalcin.

In 2025, Alghadir et al. published a concurrent study in BMC Musculoskeletal Disorders that looked at the impact of high-intensity interval training and vitamin D supplementation on bone

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metabolism among women with osteoporosis. The study was a random controlled design with a pre test post test four arm experimental design and measured serum osteocalcin, BAP, calcium and 25-OH vitamin D levels before and after the intervention period. The combination of exercise and vitamin D supplementation allowed assessment for possible interaction between mechanical loading and vitamin D status in the modulation of bone turnover. The results showed that levels of osteocalcin in the blood change, but it remains unclear how vitamin D and exercise affected the changes, as well as how they worked together. These studies therefore underscore the need to take into account co-interventions and lifestyle factors if interpreting the vitamin D supplementation response in osteocalcin. In 2023, Reis et al. conducted a systematic review of randomized clinical trials (RCT) and analyzed their findings on the impact of vitamin D supplementation (alone or with calcium) on bone remodeling and fracture risk in postmenopausal women without osteoporosis in Nutrition.[37]

4.2 systematic review of randomized clinical trials (RCT)

The effect of vitamin D supplementation (with or without calcium) on bone remodeling and fracture risk in postmenopausal women without osteoporosis in Nutrition. Both bone turnover markers and fracture outcomes were included in this review, with focus on the difference between vitamin D and vitamin D plus calcium. The results added to the evidence that the impact of vitamin D on bone turnover markers, such as osteocalcin, could be influenced by concurrent calcium intake, vitamin D baseline levels and the use or not of antiresorptive therapy. In 2025, Migliorini and colleagues conducted a systematic review in the European Journal of Medical Research, which focused on vitamin D and calcium supplementation in women receiving pharmacological treatment for postmenopausal osteoporosis. This systematic review, at level I, of 37 RCTs involving 43,397 patients evaluated the association between various levels of vitamin D and calcium supplementation and bone mineral density, serum markers of osteoporosis, fracture rates, adverse events and mortality. A mean of 833.6 milligrams of calcium and 92.8 international units of vitamin D was supplemented to the patients with a mean follow-up of 25.8 months. This review emphasized the need to evaluate vitamin D supplementation along with current osteoporosis pharmacotherapy, since its effect on bone turnover markers can vary between patients on antiresorptive and anabolic treatment and those not taking any medications. [38]

4.3 Vitamin D metabolites and their effects on osteocalcin

Other factors that can influence the osteocalcin response to vitamin D. Meta-analyses of vitamin D supplementation on serum osteocalcin have shown significant heterogeneity in the results, emphasizing the need for the identification and characterization of factors that modulate the response to vitamin D supplementation. A discussion of important variables that have been recognized as having contributed to variability in study results follows. One of the most consistently recognized factors modulating the effects of vitamin D supplementation is baseline vitamin D status (serum 25-hydroxyvitamin D). Many vitamin D outcomes display the principle of diminishing returns, which means that those with the lowest levels of vitamin D tend to benefit more from a particular amount of vitamin D supplementation. [39] Considering BTM, it may be suggested that vitamin D deficiency populations will see more reduction in levels of BTM like osteocalcin after supplementation while populations with normal baseline BTM levels will have minimal or no change. This was shown in the meta-regression carried out by Nasimi et al., where baseline vitamin D level was found as a significant source of heterogeneity. Vitamin D dose and duration of supplementation are related; lower doses for longer period or higher doses for shorter period will affect the same factor of the osteocalcin effect. The relationship between vitamin D dose and changes in serum osteocalcin is not

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necessarily linear, although it may be stronger and more rapid; increased doses of vitamin D can lead to more and larger changes in serum osteocalcin, but not necessarily. The length of time supplied is important as bone remodeling is a relatively slow process, and takes about 3-6 months for a complete remodeling cycle. Short-term studies may not be long enough to and may, therefore, lead to higher levels of undercarboxylated osteocalcin. In this mechanistic context, it is important to take into account the vitamin K status when interpreting the effect of vitamin D supplementation on serum osteocalcin concentrations. [40]

4.4 Effects in Special Populations

Several special populations where there are specific pathophysiological factors that may alter vitamin D response to supplementation have been studied, specifically regarding serum osteocalcin. Postmenopausal women with osteoporosis are a clinical population of interest. M Migliorini et al. conducted a systematic review of vitamin D and calcium supplementation in women receiving pharmacological treatment for postmenopausal osteoporosis and analysed data from 37 randomised controlled trials (RCTs) involving more than 43,000 women. The findings suggested an association between vitamin in remodeling rate. Age and menopausal status are known factors for bone turnover and are higher in older people and in postmenopausal women as a consequence of the lack of estrogen. [41] The effect of vitamin D supplementation on osteocalcin levels may be variable depending on individual baseline turnover levels, and could be even larger in persons with higher baseline levels. In the meta-analysis by Nasimi et al., there was some heterogeneity with regard to age, but it remains unclear what this age effect is. Postmenopausal women is a population different from premenopausal women and men in terms of bone metabolism; and how they will respond to vitamin D is different from premenopausal women and men.

Concurrently administered calcium supplementation has important considerations for interpretation of studies on vitamin D and bone turnover markers. Vitamin D and calcium act synergistically in the control of PTH secretion and bone remodelling with vitamin D augmenting calcium absorption from the gut and calcium serving as a substrate for mineralisation. There might be a greater effect of vitamin D on bone turnover markers, such as osteocalcin, when used in combination with calcium than when used alone. Therefore, studies reporting vitamin D supplementation alone may underestimate the effect of optimizing vitamin D status in calcium-replete individuals and studies reporting both nutrients may see effects that are not attributable to vitamin D alone. Vitamin D has complex interaction with vitamin K status (causing confusion when interpreting measurements of serum osteocalcin). Thus, vitamin K is needed as a cofactor for the gamma-carboxylation of osteocalcin and persons with reduced vitamin K status might be more likely to have uncarboxylated osteocalcin in the circulation. [42] The transcription of the osteocalcin gene increases with vitamin D supplementation, whereas the fate of the transcription product is determined by the presence of vitamin K. For people with normal levels of vitamin K, newly synthesized osteocalcin is readily carboxylated into it and can be included in the bone matrix, meaning there may be no change, or even a decrease, in serum levels. In vitamin K-insufficient individuals there is more newly synthesized osteocalcin that could be undercarboxylated and, therefore, be released into the circulation D supplementation and reduced gastrointestinal adverse events and mortality, with some studies having less consistent findings related to bone turnover markers. Fluorescence bone markers in this population are significantly altered by the use of antiresorptive drugs, including bisphosphonates, denosumab, and selective estrogen receptor modulators, which make it difficult to assess a vitamin D-only effect on the bone marker osteocalcin.

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Calcium homeostasis and parathyroid hormone regulation likely play a larger role in the action of vitamin D on bone turnover markers in treated osteoporotic patients than direct effects on osteocalcin production. People with chronic kidney disease (CKD) have special concerns about vitamin D metabolism and how osteocalcin is measured. Secondary hyperparathyroidism and renal osteodystrophy are caused by impaired renal 1-alpha-hydroxylation of 25-hydroxyvitamin D, which leads to decreased production of 1,25-dihydroxyvitamin D_3. [43] In addition, the removal of osteocalcin fragments by the kidneys is impaired, leading to the detection of immunoreactive fragments in the circulation by assays which can result in spuriously elevated levels of osteocalcin in the circulation of individuals with CKD that do not necessarily indicate a higher rate of bone formation. This CKD-specific vitamin D metabolism and clearance kinetic alteration affects the vitamin D supplementation effects on serum osteocalcin, which are usually given as 1-alpha-hydroxylated metabolite instead of vitamin D compounds.Concentrations of 25-hydroxyvitamin D are lower in the obese when compared with lean individuals, likely due to the binding to the lipophilic vitamin in the fat, and with complex changes in bone metabolism. Changes in the pharmacokinetics of vitamin D and the endocrine effects of undercarboxylated osteocalcin on energy metabolism may affect the relationship between vitamin D supplementation and osteocalcin in obese people.[44] The positive correlations between serum undercarboxylated osteocalcin and 25-hydroxyvitamin D in overweight male patients reported by the De Toni team suggests that the interplay between osteocalcin and vitamin D metabolism might be different in obesity. Attenuation of Bone Turnover Versus Direct Regulation One of the main problems for interpretation of the clinical literature on vitamin D and osteocalcin is the apparent discrepancy between the stimulatory effect on osteocalcin gene transcription of 1,25-dihydroxyvitamin D_3, which has been established at the molecular level, and the overall reduction or lack of change in serum osteocalcin in clinical supplementation trials. This seeming paradox can be explained by the difference between the direct transcriptional effects of vitamin D replacement and the integrated physiological response to vitamin D replacement.[45] The decrease in serum osteocalcin after vitamin D supplementation in individuals with vitamin D deficiency is most likely due to a dampening effect on secondary hyperparathyroidism and/or increased bone turnover that occurs in vitamin D deficient individuals. With low vitamin D status, there is a tendency to hypocalcemia, which triggers the secretion of parathyroid hormone, resulting in low calcium absorption in the intestine. These increases in bone remodeling result from high PTH levels, and are accompanied by an increase in both bone resorption and formation, as well as an elevation in markers of bone turnover (such as osteocalcin). [46]

Vitamin D supplementation corrects the normal calcium absorption and this decreases the stimulus to secreting parathyroid hormone and consequently the high turnover state. The decrease in bone formation marker levels is more likely to be the result of normalization of bone turnover activity, rather than the direct inhibition of osteoblastic activity. In this model, reduced bone turnover is the dominant effect on the osteocalcin gene and overrides the molecular stimulation caused by 1,25-dihydroxyvitamin D_3.[47] Supplementation would not be anticipated to lower osteocalcin in people with a normal baseline vitamin D level and a normal level of bone turnover, and, if the effect was at the level of the transcription, it may actually raise the level. The variability seen in clinical trials, therefore, may be the result of the interplay between these opposing effects that depend on baseline vitamin D status, concurrent calcium intake, and other factors that affect bone turnover. Another factor that is important is the carboxylation status of osteocalcin. Vitamin D supplementation might affect the gamma-carboxylation of newly-synthesised osteocalcin directly or indirectly on the gamma-carboxylation

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machinery or through vitamin K status.[48] Higher carboxylation would lead to a greater proportion of osteocalcin being incorporated into bone matrix, therefore a smaller proportion of free circulating osteocalcin in the absence of any alteration in total osteocalcin production. Measurement of total osteocalcin without differentiation between carboxylated and undercarboxylated osteocalcin might thus fail to capture changes in the bone/blood osteocalcin partition.[49] Table 1 summarizes the key characteristics and findings of studies examining the effect of vitamin D supplementation on serum osteocalcin. The table includes data from the systematic review and meta-analysis by Nasimi et al. (2024) and representative individual randomized controlled trials.[50] Abbreviations: MD, mean difference; CI, confidence interval; OC, osteocalcin; sCTX, serum C-terminal telopeptide of type I collagen; uNTX, urinary type I collagen cross-linked N-telopeptide; P1NP, procollagen type-1 intact N-terminal propeptide; BALP, bone alkaline phosphatase; RCT, randomized controlled trial; IU, international units; 25(OH)D, 25-hydroxyvitamin D.[51]

Table 1: Summary of Key Studies Examining the Effect of Vitamin D Supplementation on Serum Osteocalcin

Study	Design	Population	Intervention	OC Outcome	Key Findings
Nasimi et al. (2024)	Meta-analysis of 24 studies	Women ≥18 years	Vitamin D supplementation/ fortification	MD -0.610	Significant reduction in OC; high heterogeneity
Nasimi et al. (2024)	Meta-analysis of 22 studies	Women ≥18 years	Vitamin D supplementation/ fortification	sCTX MD -0.038	Significant reduction in resorption marker
Nasimi et al. (2024)	Meta-analysis of 6 studies	Women ≥18 years	Vitamin D supplementation/ fortification	uNTX MD -8.188	Significant reduction in resorption; no heterogeneity
Migliorini et al. (2025)	Systematic review of 37 RCTs	Postmenopausal women with osteoporosis on antiresorptive therapy	Vitamin D + calcium (mean 92.8 IU + 833.6 mg)	Not primary outcome	Vitamin D associated with lower GI events and mortality

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Study	Design	Population	Intervention	OC Outcome	Key Findings
Alghadir et al. (2025)	Four-arm RCT	Women with osteoporosis	High-intensity interval training + vitamin D	Changes observed	Interactions between vitamin D and exercise

5. Methodological Considerations and Challenges

5. 1 Analytical issues in the measurement of osteocalcin

A precise measurement of osteocalcin in serum is a difficult task which has implications for the interpretation of clinical studies of the effects of vitamin D supplementation. The main methodological issues when measuring osteocalcin will be discussed below. Osteocalcin is found in several forms: intact 49 amino-acid protein, amino-terminal mid-molecular fragments resulting from proteolytic degradation, carboxylated and undercarboxylated forms.[52] Intact protein is rapidly cleared from the circulation (minutes) by renal clearance and proteolytic degradation. As a result, there are large amounts of immunoreactive osteocalcin present as fragment. All commercial immunoassays use antibodies of varying specificity that can target a range of intact protein and fragments. Assays that employ monoclonal antibodies against the intact amino-terminus and the intact carboxyl-terminus will only detect intact osteocalcin, while assays that use polyclonal antibodies or antibodies against only one epitope can detect both osteocalcin fragments and the intact form.[53] There is considerable inter-assay variability because there is no standardisation of assays used, making it difficult to compare results across studies. However, undercarboxylated osteocalcin is also found in the bloodstream, which complicates the picture for assays. The carboxylation status of osteocalcin is dependent upon the availability of vitamin K, and assays can vary in their relative reactivity to carboxylated and undercarboxylated osteocalcin.[54]

Several specific assays have been developed to measure underscorboxylated osteocalcin, which has been suggested as a measure of vitamin K status and a predictor of fracture risk and metabolic outcomes. However, when vitamin D supplementation studies are undertaken, it is not possible to differentiate between carboxylated and undercarboxylated osteocalcin, and this can mask physiologically relevant changes in the distribution of these forms.[55] Intra-individual variability due to diurnal fluctuation of serum osteocalcin is well established and needs to be taken into consideration in the design of studies. Osteocalcin concentrations have been found to have a circadian rhythm with peaks during night and early morning and valleys during the afternoon, the difference in some studies being as high as 20 percent. Thus, it is vital to standardize the time of blood sampling between subjects and within subjects over time for valid comparisons. The lack of diurnal control would add the noise to osteocalcin measurements, which would decrease the statistical power to detect any real treatment effect. Measurements of osteocalcin are influenced by pre-analytical factors such as freeze-thawing and specimen handling, and processing delays. Blood serum should be drawn from the clot as soon as possible and should not be subjected to repeated freeze-thaw.[56]Collection tubes and anticoagulants should remain the same for a study; the use of the protease inhibitors may be considered to reduce the ex vivo degradation.

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5.2 Study Design Considerations

Several methodological pitfalls could have an impact on the validity and generalizability of vitamin markers, it may be beneficial to stratify by baseline vitamin D status or to only include participants who are vitamin D deficient or insufficient in the studies. The dose and formulation of vitamin D to D study outcomes exploring the effect of vitamin D supplementation on osteocalcin. As vitamin D status, age, menopausal status, renal function, and concurrent medications all play a role in the effect of vitamin D supplementation, the choice of study population is highly relevant. Studies with heterogeneous baseline characteristics can yield divergent findings and mask true findings. [57]To obtain better clarity on the effect of vitamin D supplementation on bone turnover use, and the length of treatment, should be chosen to provide physiologically relevant changes in vitamin D status. Low doses of vitamin D might fail to induce a change in the levels of 25(OH)D that could be detected to see a change in bone turnover markers, and very high doses may have effects that are not reflective of typical doses used in vitamin D supplementation. Treatment should be given for the time necessary for changes in bone remodeling, which takes months. Research that focuses on a short time frame (< 3 months) may not reflect the complete impact of vitamin D on bone turnover. The statistical power of a study to detect changes in osteocalcin is dependent on the magnitude of the effect size, the variability of osteocalcin measurement and sample size.[58] Heterogeneity observed in meta-analyses indicates that effect sizes will likely be relatively small and will be somewhat variable, suggesting the need for sufficiently large studies (n ≥ 200) to draw firm conclusions. The results from many individual trials in this area have been inconsistent, and small sample sizes have made them underpowered in many cases, thus requiring synthesis through meta-analysis. Another key aspect of the design is the type of comparator. Randomized controlled trials with placebo controls are the most convincing evidence of efficacy while the comparison of various doses or forms of vitamin D are dose-response studies and do not have a negative control. Open label study without placebo could be subject to bias due to participant expectations and interpretation with appropriate caution.[59] **:** Table 2 summarizes key methodological recommendations for the design, conduct, and reporting of future studies examining the effect of vitamin D supplementation on serum osteocalcin and other bone turnover markers.[60] Recommendations are based on the methodological challenges identified in the literature and aim to enhance the comparability, validity, and clinical relevance of study findings.

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Table 2: Recommendations for the Design and Conduct of Studies Examining Vitamin D Supplementation and Osteocalcin

Methodological Aspect	Recommendation
Study population	Characterize baseline 25(OH)D status; consider enrichment for deficient/insufficient participants
Vitamin D intervention	Specify dose (IU/day), formulation (D_3 vs D_2), and duration; consider dose-response design
Co-interventions	Document and control for calcium intake, vitamin K status, and concurrent medications
Comparator	Use placebo-controlled design when ethically feasible
Outcome measurement	Standardize blood sampling time; specify assay methodology and recognition of OC fragments
OC measurement	Consider measurement of both total and undercarboxylated OC; document assay inter- and intra-assay CV
Statistical considerations	Power study for anticipated effect size; account for multiplicity if multiple BTMs measured
Reporting	Adhere to CONSORT guidelines; report individual-level changes and responder analyses

6. Integration with Other Bone Turnover Markers

6.1 The Bone Turnover Marker Panel

The broader profile of bone turnover markers, together offering a more comprehensive view of skeletal response to vitamin D supplementation, enhances interpretation of osteocalcin and other markers following vitamin D supplementation.[61] Bone turnover markers are generally classified as markers of bone formation associated with the activity of the osteoblasts, and markers of bone resorption associated with the activity of the osteoclastic cells. In the following sections, the most frequently used markers in each category are discussed. Osteocalcin is one of the markers of bone formation, besides the procollagen type-1 intact N terminal propeptide (P1NP) and bone-specific alkaline phosphatase (BALP). BALP is an ectoenzyme that is expressed on the surface of osteoblasts and is involved in mineralization as it breaks down pyrophosphate, an inhibitor of hydroxyapatite crystal growth.[62] Immunoassay of BALP in serum has the advantage that it is relatively stable, that is, it has a longer half-life than osteocalcin, and it is less influenced by renal function. P1NP is a peptide

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produced when procollagen I is converted to collagen I and is located on the amino terminal end of the procollagen. P1NP is a specific and sensitive marker of bone formation, as it is a reflection of the rate of collagen synthesis and the most abundant protein of the bone matrix. A significant effect on vitamin D supplementation on P1NP was seen in the meta-analysis by Nasimi and colleagues when the dosage was low (less than 600 micrograms/day) or when the sample size was large (> 100 participants); a significant effect was not observed for BALP . Collagen degradation products sCTX and uNTX are markers of bone resorption.[63] In sCTX degradation of type I collagen by cathepsin K (catK), the main bone collagen-degrading protease in osteoclastic resorption, produces the peptide fragment sCTX. It is present in serum, and has a diurnal rhythm, with higher levels at night and early morning. Another type I collagen degradation product excreted in urine and normalized to urinary creatinine concentration is uNTX. In the meta-analysis by Nasimi and colleagues, there were significant reductions in both sCTX (MD -0.038) and uNTX (MD -8.188) with vitamin D supplementation. The uNTX showed no significant heterogeneity, whereas there was high heterogeneity in osteocalcin and sCTX.[64]

6.2 The Bioinformatic Approach and Coupling of Remodeling

A principle of bone biology, the coupling of bone resorption and formation simplifies the understanding of bone turnover marker changes after an intervention. Osteoblastic formation is normally coupled with osteoclastic resorption in the basic multicellular unit of bone remodeling. A bone remodeling transient is the coupled decrease in bone formation seen in interventions that lead to a decrease in bone resorption, but see a higher rate of bone formation during a period preceding the decrease in bone resorption. On the other hand, interventions which mainly increase bone formation can be followed by a coupled process of bone resorption.[65] The effect of vitamin D on bone turnover markers, decreasing both resorption markers (sCTX and uNTX) and formation markers (osteocalcin and P1NP under some conditions), suggests a global effect on the rate of bone remodeling rather than on either bone formation or bone resorption. This interpretation is consistent with the vitamin D supplementation hypothesis, which suggests that calcium supplementation may be beneficial because it leads to enhanced calcium absorption and a decrease in parathyroid hormone secretion, thereby reducing the drive for bone remodeling. In postmenopausal osteoporosis and other hyperremodeling states, such as elevated remodeling states, a reduced bone turnover could be beneficial, if not excessive, as long as it does not become adynamic, and is associated with bone loss and an increase in fracture risk. The change in the relative magnitude of the resorption marker and formation marker could give some clues as to the bone balance at the single remodeling unit level.[66] The larger the decrease of resorption markers when compared with formation markers, the more positive the bone balance and the larger the increase of bone tissue in each remodeling cycle; the opposite would indicate a negative bone balance. The decrease in uNTX was more consistent between studies in the meta-analysis by Nasimi et al. than the decrease in osteocalcin, but because those analyses were not done at the individual level, definitive conclusions about bone balance cannot be drawn.[67]

6.3 Clinical Utility in Monitoring Vitamin D Supplementation

Several factors restrict the use of bone turnover markers such as osteocalcin for the monitoring of the effect of vitamin D supplementation in single patients. The amount of change in markers after vitamin D treatment is relatively small and can be near the biological and analytical variation range. The day to day variability of bone turnover markers (measured by the least significant change) can approach the magnitude of the effect of the treatment making it difficult to assess treatment response in individual patients.[68] Moreover, the clinical relevance of vitamin D-

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induced bone turnover markers changes in terms of fracture risk reduction or bone mineral density improvements is not well established, as it is for anti-resorptive or anabolic therapies. While such limitations cannot be overcome, bone turnover markers could be useful in certain clinical settings. A decrease in the levels of bone turnover markers after vitamin D repletion could be considered objective evidence of a biological response in patients with proven vitamin D deficiency and some evidence of secondary hyperparathyroidism with elevated bone turnover markers. This is all in favor of a normalized bone remodeling rate, because the rates of bone destruction (resorption markers) and bone formation(formation markers) are both reduced.[69] Failure to respond can be due to non-compliance, insufficient dosage or other possible causes of high bone turnover, including lack of estrogen, excess of glucocorticoids, and primary hyperparathyroidism. The measurement of osteocalcin in particular may be of particular interest in areas where vitamin K status is a concern, as the ratio of undercarboxylated/ carboxylated osteocalcin has been suggested as a functional measure of vitamin K sufficiency. If this is the case, however, then the response to vitamin D and vitamin K may give clues which are different from those derived from other bone turnover markers. But this is a technique that needs to be further validated for use in clinical practice.[70]

7. Clinical Implications and Future Directions

7.1 Implications for the Management of Metabolic Bone Diseases

The results on the impact of vitamin D supplementation on osteocalcin serum levels have relevance to the treatment of metabolic bone diseases, especially osteoporosis. Vitamin D supplements help to decrease bone turnover rates as evidenced by the steady decrease in bone resorption markers, and the less consistent, but significant, decrease in bone formation markers such as osteocalcin, suggesting the beneficial effects of vitamin D on bone health in those with increased bone remodeling.[71] There has been a lot of study and some debate about the importance of vitamin D for fracture prevention. Meta-analyses of randomized controlled trials have provided conflicting results, some showing that vitamin D and calcium combination therapy reduces fracture rate, especially in institutionalized elderly patients who are at high risk for vitamin D deficiency and who do not consume sufficient calcium, and others reporting no benefit of vitamin D alone. The reduced bone turnover seen in biomarker studies is potentially a biological mechanism for fracture benefit as increased bone turnover has been shown to have a negative effect on bone strength, beyond the effect of bone mineral density, because of its effect on bone microarchitecture, mineralisation homogeneity and collagen cross-linking.[72]

There are areas still under investigation relating to the use of BTM in osteoporosis care, such as the role of BTM in the evaluation of the response to vitamin D supplementation. P1NP has been used in algorithms for monitoring anabolic therapy, and increases in P1NP in these algorithms have been shown to be reliable predictors of future bone mineral density improvement. The use of osteocalcin monitoring for vitamin D supplementation is less clear, but a decrease in elevated osteocalcin along with a decrease in resorption markers may support a biological response for the individual.[73] Proper calcium (food or supplement) is essential to the actions of vitamin D on bone metabolism. The skeletal effects of vitamin D are mainly due to its stimulation of intestinal calcium absorption, and the presence of calcium in the intestinal lumen is a requirement for this. The studies that have shown the efficacy of vitamin D supplementation in fracture prevention have routinely co-supplemented calcium, and it is possible that some of the negative studies conducted in vitamin D alone have been because calcium was not co-supplemented.

7.2 Unresolved Questions and Future Research Directions

Although there is significant literature on the vitamin D/osteocalcin connection, there are

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some important questions left unanswered and should be explored in the future. The dose-response relationship of vitamin D supplementation to changes in osteocalcin has not yet been well described. Dosage is a source of variation in effect size as the majority of studies have used a fixed dose of vitamin D, and there was considerable variation in the meta-analysis conducted by Nasimi et al..[74] Dose response studies, preferably using several dose levels in one randomized controlled trial, would be useful to establish the recommended dose of vitamin D for bone health outcomes. Studies like this should consider initial 25-hydroxyvitamin D levels; the dose needed to induce a specific change in osteocalcin would be expected to be dependent on initial vitamin D levels. It is necessary to explore systematically the interaction of vitamin D with vitamin K in terms of osteocalcin carboxylation and serum levels. Measuring both total and undercarboxylated osteocalcin and other bone turnover markers in combination with bone mineral density following combined vitamin D and vitamin K supplementation would test the hypothesis that vitamin K sufficiency is necessary for optimal incorporation of the vitamin D stimulated osteocalcin in bone matrix.[75]

Some observational studies support the clinical potential of combined supplementation for fracture prevention; however, this has not been proven in RCTs. Reciprocal relationship between osteocalcin and vitamin D metabolism was proposed by the results of De Toni and colleagues and needs further investigation. The osteocalcin response to Vitamin D supplementation may be a regulatory feedback mechanism that affects Vitamin D metabolism as the undercarboxylated osteocalcin physiologically stimulates the expression of CYP2R1 and 25-hydroxyVitamin D production. Study of this feedback mechanism in humans, such as CYP2R1 activity or expression in readily accessible tissues, would yield information about the physiological coupling of bone and vitamin D metabolism in humans.[76] Vitamin D supplementation has an emerging interest in respect to the role of osteocalin and its undercarboxylated form in the endocrine system. The resulting change in the ratio of undercarboxylated to carboxylated osteocalcin might impact metabolic parameters such as insulin secretion, insulin sensitivity and adiponectin expression. Changes in undercarboxylated osteocalcin should be included as a possible mediator in clinical trials aimed at vitamin D's effect on metabolic parameters. The establishment and validation of a standard method for the measurement of intact osteocalcin and the identification of carboxylated and undercarboxylated variants and quantitation of the fragments would greatly benefit the field. Study results are currently not easily comparable and results from research are not easily translated to clinical use, due to a lack of assay standardization. Harmonization and development of reference standards and ranges should be considered a priority.Figure 2 indicates that $_3$ can activate the osteocalcin gene transcription via the VDR-VDRE pathway. In vitamin K sufficient patients, newly synthesized osteocalcin may be efficiently carboxylated, and preferentially deposited in the bone matrix, which may help to explain the decrease in serum osteocalcin. [77]The net effect on serum osteocalcin is the result of reducing bone turnover (decreasing OC) and vitamin D stimulated transcription (increasing OC) with further modulation by vitamin K-dependent carboxy: An integrated model was created which summarizes the proposed effects of vitamin D supplementation on the osteocalcin axis from gene transcription to serum measurement to clinical outcome. In vitamin D deficient state (Panel A), low 25-hydroxyvitamin D results in low levels of 1,25-dihydroxyvitamin D_3 production, decreased intestinal calcium absorption and secondary hyperparathyroidism. A higher parathyroid hormone (PTH) is associated with heightened bone turnover, by adding to both markers of bone resorption and bone formation such as osteocalcin. With vitamin K deficiency, more osteocalcin is undecarboxylated (ucOC), with a lower affinity for bone matrix, and it is

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preferentially released into the circulation. This is because the high levels of serum osteocalcin (not due to vitamin D stimulated transcription) signify high bone turnover. In the vitamin D-replete state after vitamin D supplementation, 25(OH)D is sufficient to produce 1,25(OH)D_3 (Panel B).[78] Increased calcium absorption in the intestine restores the calcium homeostatis, which decreases PTH production and the high turnover bone state. Bone resorbtion and formation markers return to normal levels. At the same time, 1,25-dihydroxyvitamin D acts as a growth factor for OC that is directed towards bone. Undercarboxylated osteocalcin might regulate the metabolism of vitamin D through the stimulation of 25 hydroxylation by CYP2R1 in a feedback manner. The green arrows show stimulation, red bars inhibition or attenuation.[79]

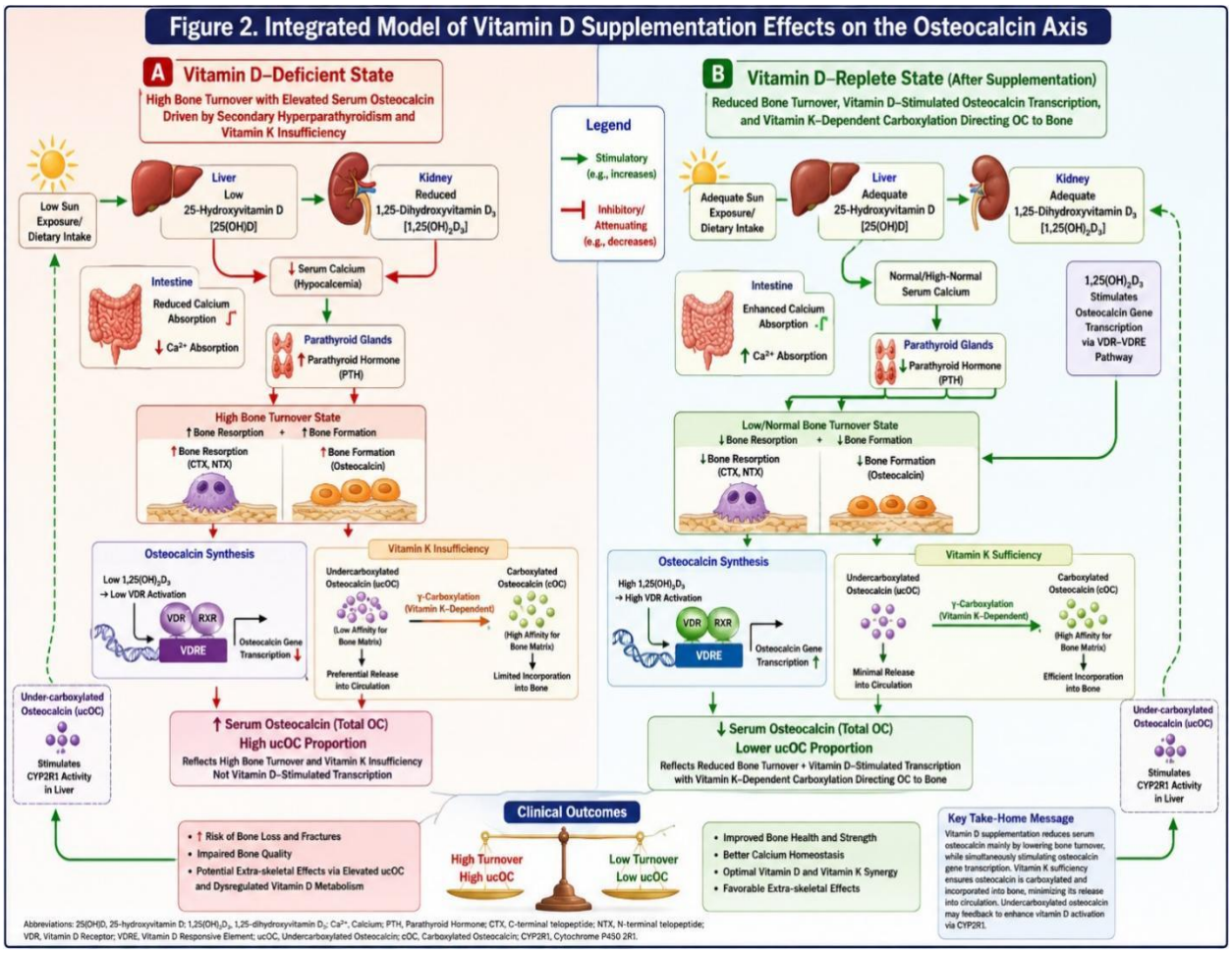


Figure 2: Integrated Model of Vitamin D Supplementation Effects on the Osteocalcin Axis

8. Conclusion

Vitamin D supplementation and serum osteocalcin is an interesting case study of the complexity of molecular regulatory mechanisms to clinical responses in biomarkers. The regulation of osteocalcin gene expression by 1,25-dihydroxyvitamin D_3 through the vitamin D responsive element is one of the best understood vitamin D receptor mediated mechanisms of transcriptional control at the molecular level. The binding of the VDR-RXR heterodimer to the VDRE at nucleotides -466 to -437 of the rat osteocalin gene promoter, together with the cooperative interaction with basal regulatory elements such as the CCAAT box and the TATA box region, elucidates the vitamin-D-mediated

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transcription of osteocalin in a clear mechanistic manner. [80].However, when the clinical translation of these molecular relationships is considered, they show that vitamin D supplementation has more of an effect on decreasing circulating osteocalcin levels. The results of the meta-analysis (24 studies on vitamin D supplementation or fortification in women) show a significant reduction in serum osteocalcin, -0.610, but with high heterogeneity as a result of several modifying factors. The parallel decrease in markers of bone resorption (sCTX and uNTX) suggests that the loss of osteocalcin is not due to a specific suppression of osteoblastic activity, but rather to a general inhibition of bone turnover rate. The apparent contradiction of molecular stimulation and clinical reduction is explained by the recognition that the physiological response to the repletion of vitamin D is the correction of the secondary hyperparathyroidism and the increase in bone turnover associated with the vitamin D deficient state. Although there is continued stimulation of transcription of the osteocalcin gene, there is a net decrease in bone formation markers due to enhanced intestinal calcium absorption, decreased secretion of parathyroid hormone, and normalization of the high turnover state of bones[81]. Vitamin K availability determines the carboxylation status of osteocalcin, providing an additional layer of regulation, carboxylated osteocalcin is more readily incorporated into bone, while the undercarboxylated osteocalcin is more readily released into the circulation. Other analytical factors, such as the presence of immunoreactive fragments, diurnal variation, and nonstandardization of assays, further complicate the interpretation of serum osteocalcin measurements. The methodological challenges illustrate the need to measure osteocalcin in a panel of bone turn-over markers and in standard conditions that account for known sources of variability. These findings may have clinical implications in the management of metabolic bone diseases, as vitamin D could help to reduce fracture risk by slowing bone turnover with supplementation and maintaining b one strength when calcium intake is sufficient.[82] Changes in bone turnover markers were however small, and varied between populations, suggesting that, to date, it is not feasible to use measurement of osteocalcin as a sole marker of response to vitamin D supplementation in individual patients. Characterization of dose-response relationships, elucidation of vitamin D-vitamin K interactions to modulate osteocalcin carboxylation and fate, investigation of potential feedback loops between osteocalcin and vitamin D metabolism and development of standardized assays for comparison of dose-response across studies are recommended for future research.[83] As new insights into the endocrine role of undercarboxylated osteocalcin and its relationship with vitamin D emerge, new aspects of the vitamin D-osteocalcin connection that extend far beyond the realms of skeletal and bone health will become apparent. To summarise, vitamin D supplementation has a measurable effect on serum osteocalcin, and the effects on its levels are dependent on the baseline vitamin D status, the dose and duration of the vitamin D supplementation, the availability of vitamin K, the level of calcium supplementation during the treatment, and the population-specific factors. This clear understanding will give a scientific basis for interpreting vitamin D assays in research and clinical practice and will emphasize the ongoing relevance of vitamin D in bone biology.[84]

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