Vitamin D: Biological Significance and Diagnosis of Mild Deficiency

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Abstract

Activated vitamin D has a main role in bone metabolism by increasing intestinal calcium absorption and kidney calcium resorption but also by activating both bone formation and resorption. This last effect may be mainly indirect by modulating PTH secretion. In mild forms of vitamin D deficiency, the increase in PTH secretion is probably the main factor determining bone loss.

In diagnosis of vitamin D deficiency, the establishment of a circulating vitamin D (mono or di-hydroxylated vitamin D) cutoff is particularly important but has been difficult because differences in used criteria. Based on PTH circulating levels and femoral neck bone density, a mono-hydroxylated vitamin D cutoff of 25 ng/ml may be the best criterion for distinguishing a mild vitamin D deficiency.

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Vitamin D action is not limited to bone metabolism but involves modulation of immune function, stimulation of insulin, and other hormone secretion and inhibition of cell proliferation. Epidemiological studies have correlated low vitamin D levels to increased prevalence of some forms of cancer (mainly colon cancer but also breast and prostate cancer), type II diabetes, some autoimmune disorders, and cardiovascular diseases. However, in all these conditions, with a few exceptions, trials using high doses of vitamin D have been unsuccessful. The possibility that in nonskeletal diseases, different criteria for determining a vitamin D deficiency should be used is discussed.

Keywords

Vitamin D • Bone metabolism • Osteoporosis colon cancer • Breast cancer • Prostate cancer • Diabetes • Autoimmune diseases • Cardiovascular diseases

List of Abbreviations

Activated vitamin D	$1,25(OH)_2$-vitamin D
PTH	Parathyroid hormone
VDR	Vitamin D receptor
CaM	Calmodulin
BBMI	Brush border myosin I
CaBP	Calbindin
TRPV6	Calcium channel 6
CaSR	Calcium sensing receptor
RIA	Radioimmunoassay
DEXA	Dual-energy X-ray absorptiometry
DC	Dendritic cells
Th cells	T helper cells

Key Facts of Vitamin D

- Vitamin D controls mineral metabolism and the bone homeostasis by increasing calcium availability, by regulating PTH secretion, and by direct effects on bone formation and resorption
- Diagnosis of mild vitamin D deficiency requires circulating levels of 25-OH-vitamin D lower than 25 ng/ml
- Vitamin D has many nonskeletal effects that depend on its ability to reduce cell proliferation, to regulate immune cells, and to stimulate the production of some hormones
- Vitamin D normal levels across the life span may be important for preventing colon cancer and some autoimmune diseases and for reducing the appearance or the progression of breast and prostate cancer, type II diabetes, and cardiovascular diseases
- Long-term administration of vitamin D in at risk populations may be important in reducing the incidence of some cancers and many chronic diseases

Definition of Words and Terms

Rickets	A condition of reduced mineralization of child bone
Osteomalacia	A condition of reduced mineralization of adult bone
Osteoporosis	A condition of increased risk of bone fractures
T helper cells	A lymphocyte cell type that has an important role in the immune system by suppressing or regulating immune responses.
Cutoff	The blood levels of a circulating substance that distinguishes between low and normal levels
Trial	A controlled study involving the administration of a biologic substance compared to the administration of a placebo to a group of patients.
Long-term follow-up	A study in which the studied subjects are controlled for several years to understand the long-term effects of a natural condition or of a therapy

Introduction

A mild deficiency of vitamin D represents the most common cause of altered mineral metabolism and may have important consequences not only on bone quality but also on metabolism and cardiovascular function. In this review, we will present the biological role of vitamin D on mineral metabolism and on extraskeletal tissues, and then we will discuss the way to make diagnosis of mild vitamin D deficiency.

Vitamin D Synthesis and Biological Actions

Activated ($1,25(OH)_2$-vitamin D) vitamin D and parathyroid hormone (PTH) represent the most important regulators of calcium (and phosphate) metabolism (De Luca 2004).

Vitamin D (that includes both ergocalciferol or vitamin D_2 and cholecalciferol or vitamin D_3) is formed in the epidermidis by the effects of ultraviolet rays but to be active needs to be hydroxylated (25OH-vitamin D) in the liver and then in the kidney ($1,25(OH)_2$-vitamin D). 25OH-vitamin D is the principal circulating form of vitamin D, and most assays of vitamin D measure this compound but the biological activity is determined by the $1,25(OH)_2$-vitamin D (activated vitamin D).

Activated vitamin D links to specific receptors that are present in many tissues (Carlberg and Campbell 2013). The main receptor (vitamin D receptor (VDR)) is a nuclear receptor (part of the family of the receptors for steroid hormones, thyroid hormones, and retinoids). Inside the nucleus, VDR heterodimerizes with the receptor of retinoid X (RXR) and the complex VDR/RXR is essential for stabilizing the

binding with vitamin D and producing maximal biological effects. The complex activated vitamin D–VDR/RRX produces a number of different gene transcriptions depending on the interested cell. Activated vitamin D produces also nongenomic effects, such as calcium transportation across cell membranes, that are mediated by different receptors, probably located in the cell membrane, that have not been well characterized.

Vitamin D Role in Mineral Metabolism

That includes activation of intestinal calcium absorption, promotion of bone formation (probably by regulating the expression of several bone growth factors) and resorption, kidney calcium resorption, and inhibition of PTH production (by direct effects on parathyroid cells and indirectly by increasing calcium blood levels).

The most known effect of activated vitamin D is to increase intestinal calcium absorption (Bikle 1990; Wasserman and Fullmer 1995; Hoenderop et al. 2005). Calcium enters the microvillus of the intestinal epithelial cell through TRPV6 calcium channel and then binds to a specific protein, calmodulin (CaM) that is itself bound to brush border myosin I (BBMI). The calcium/CaM complex moves into the terminal web where the calcium is picked up by another specific protein, calbindin (CaBP), and transported through the cytoplasm inside endocytic vesicles. At the basolateral membrane, the calcium is pumped out of the cell by the Ca-ATPase. Activated vitamin D enhances intestinal calcium absorption by inducing most of the mechanisms involved in the microvillus active intestinal calcium transport (TRPV6, CaBP, Ca-ATPase, and the amount of CaM bound to BBMI in the brush border).

Activated vitamin D increases calcium resorption also in kidney with a mechanism that is similar to that found in intestinal microvillus (Friedman and Gesek 1995; Biber et al. 2013). In fact, the molecules critical for calcium reabsorption in the distal tubule appear to be the VDR, calbindin, TRPV5, and the Ca-ATPase.

More difficult has been to determinate whether activated vitamin D has also a role in bone metabolism (Underwood and De Luca 1984; Suda et al. 1992; Takeda et al. 1999; Panda et al. 2004). VDR is found in osteoblasts, and activated vitamin D promotes the differentiation of osteoblasts and increases the production of proteins such as alkaline phosphatase and osteocalcin that are markers of bone formation. Activated vitamin D also increases the production of RANKL so activating the formation of osteoclasts. Patients with vitamin D deficiency present an increase of several bone factors that are linked to bone resorption and formation. However, the rickets resulting from vitamin D deficiency or VDR mutations can be corrected by supplying adequate amounts of calcium and phosphate, and it suggests that the direct vitamin D effect on bone is relatively modest.

Probably, more important for bone metabolism are the indirect effects of vitamin D. In particular, part of the skeletal phenotype in vitamin D deficiency is due to the hyperparathyroidism that develops in the vitamin D deficient state. The relationships

between activated vitamin D availability and PTH secretion are complex (Demay et al. 1992; Liu et al. 1996; Hawa et al. 1996). PTH stimulates the production of 1, 25 $(OH)_2$-vitamin D and in turn 1, 25$(OH)_2$-vitamin D inhibits the production of PTH. This seems to be a direct effect of activated vitamin D on PTH producing cells because within the promoter of the PTH gene is a region that binds the VDR and mediates the suppression of the PTH promoter by 1,25$(OH)_2$-vitamin D. However, calcium alters the ability of activated vitamin D to regulate PTH gene expression. Calcium is a potent inhibitor of PTH production and secretion, acting through the calcium sensing receptor (CaSR) on the plasma membrane of the parathyroid cell. 1, 25$(OH)_2$-Vitamin D induces the CaSR in the parathyroid gland making it more sensitive to calcium.

Figure 1 schematizes the effect of activated vitamin D on bone formation and mineralization.

In states of severe vitamin D deficiency, the reduction of calcium availability has the main effect on bone inducing bone demineralization and, as consequence of it, patients develop rickets if children and osteomalacia if adults.

States of mild vitamin D deficiency increase bone turnover and bone loss determining a condition of osteoporosis. It is probable that the effects of mild vitamin D deficiency on bone are mainly mediated by a direct effect on parathyroid cells with a consequent PTH increase. In fact, in these patients, serum calcium is normal, while circulating PTH is moderately increased and it has been used to monitor the vitamin D deficiency.

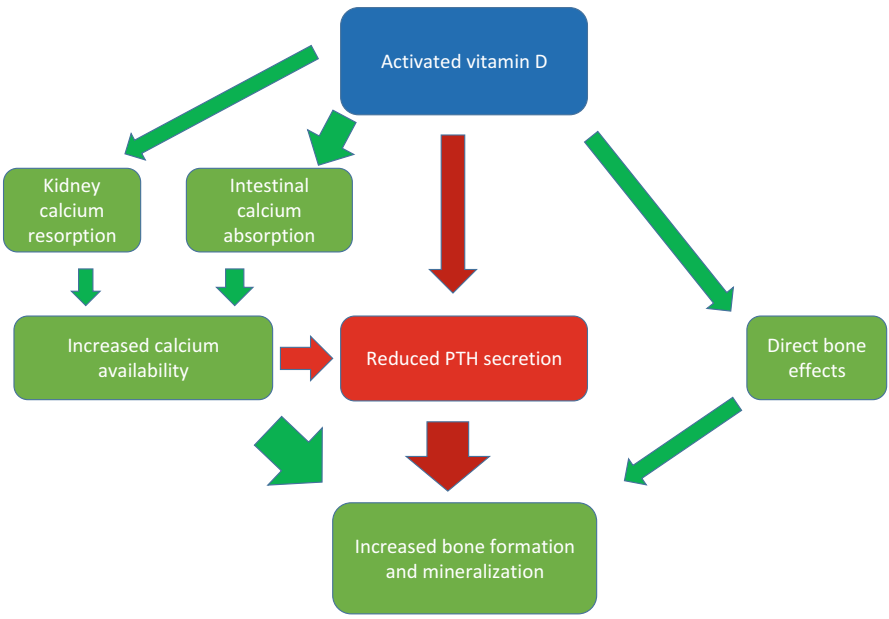

Fig. 1 Effects of activated vitamin D on bone formation and mineralization

Diagnosis of Mild Vitamin D Deficiency

A crucial point for protecting individuals from the consequences of vitamin D deficiency, and at the same time avoiding unneeded therapies is to establish the correct way to make the diagnosis of mild vitamin D deficiency. On this respect, the assay techniques for circulating mono-hydroxylated (25-OH) and di-hydroxylated (1,25-OH) vitamin D have progressed from competitive protein binding assay to radioimmunoassay (RIAs) that utilize both I (125) and chemiluminescent reporters (Carmina et al. 2014). These methods have shown to be very useful in the screening of osteoporotic women for underlying vitamin D deficiency and to be reliable indicators of vitamin D status. Generally 25-OH-vitamin D measurement is a good marker of vitamin D status, while 1, 25-vitamin D assay is needed when kidney function is impaired.

While the assessment of severe vitamin D deficiency is easy, the diagnosis of the much more common mild vitamin D deficiency remains controversial. In fact, completely different threshold values for mild vitamin D deficiency have been suggested. In particular, the American Institute of Medicine has suggested that serum 25-hydroxyvitamin D values lower than 20 ng/ml indicate vitamin D deficiency (Institute of Medicine 2011; Ross et al. 2011), while guidelines of Endocrine Society have indicated that all 25-OH-D values lower than 30 ng/ml are low (Holick et al. 2011). This disagreement depends on different ways to determine vitamin D deficiency, but it has great consequences on clinical decisions. The American Institute of Medicine indicated a threshold value of 20 ng/ml because it corresponded to 2 SD above the median calculated needs of vitamin D. At the contrary, other organizations, in putting the threshold values at 30 ng/ml, used criteria linked to circulating PTH changes. While it is clear that increases in PTH circulating values may reflect bone effect of mild vitamin D deficiency, it may determine an over diagnosis of vitamin D deficiency because small changes of PTH may not have a clinical impact on bone metabolism.

The establishment of a threshold value may also be based on changes of markers of bone resorption (and formation) (Eastell and Hannon 2011) and on bone density. Several biochemical markers of bone turnover are measurable and are widely used for assessing bone formation and resorption. Markers of bone formation include serum bone alkaline phosphatase, total osteocalcin, and the procollagen type I N-terminal propeptide assay. Among the various markers of bone resorption, serum C-terminal cross-linked telopeptides are the most sensitive and specific. In osteoporosis-treatment studies, markers of bone turnover appear even more strongly associated with fracture risk reduction than bone mineral density (BMD).

In a large population study in Southern Italy, we have determined the possible threshold for vitamin D deficiency plotting serum 25OH values against bone t-score values by DEXA plus PTH and bone marker blood levels (Napoli et al. 2014). While the interpretation of the data was complicated by the fact that the studied population was formed by women of postmenopausal age (therefore including also an important component of women having increased bone resorption), our study showed that a

Table 1 Differences in bone t-score by dual X-ray densitometry, bone alkaline phosphatase (*BAP*), collagen telopeptide (*CTX*), and PTH values in a large group of postmenopausal women depending on possible cutoffs for vitamin D deficiency (Modified from Napoli et al. 2014)

	Lumbar T score	Neck femoral T score	BAP	CTX	PTH
20 ng/ml cutoff	NO	YES	NO	YES	YES
25 ng/ml cutoff	NO	YES	NO	YES	YES
30 ng/ml cutoff	NO	NO	NO	YES	NO

threshold value of 25 ng/ml is the best for making diagnosis of mild vitamin D deficiency.

In fact, in our study, using the different cutoffs of 20, 25, and 30 ng/ml of monohydroxylated vitamin D, while no differences in bone markers were noted at any cutoff, a statistically significant decrease in femoral neck t-score and a statistically significant increase in PTH levels were observed when using the 25OHD cutoff of 25 ng/mL but not for 30 ng/ml 25OHD cutoff. The changes in neck femoral bone and PTH were similar using 20 and 25 ng/ml cutoff (Napoli et al. 2014). In Table 1, the results of this study are schematized.

This suggests that a status of vitamin D deficiency exists in women having vitamin D lower than 20 or 25 ng/mL, while the level of 30 ng/mL may be too high. Our data are consistent with the finding of the National Health and Nutrition Survey (NHANES) III where the risk of hip fracture was significantly reduced among participants with 25OHD levels greater than 25 ng/mL compared with those who had lower concentrations (Looker and Mussolino 2008).

In conclusion, it is very difficult to establish in a sure way the threshold for diagnosing mild vitamin D deficiency, but a cutoff of 25 ng/ml seems reasonable and correspondent to the main known effects of vitamin d mild deficiency on bone metabolism.

Vitamin D Effects on Nonskeletal Tissues

While the importance of vitamin D on calcium metabolism and bone maintenance is well proven, in recent years a large debate has occurred regarding possible important extra-skeletal effects of activated vitamin D (Rosen et al. 2012; Cipriani et al. 2015). In fact, vitamin D receptors have been found in most tissues (Rosen et al. 2012; Lee et al. 1994; Bikle 2012), and many studies have shown that activated vitamin D may influence many biological function including cell differentiation and proliferation in many tissues, immune system responses, and some hormone secretions (Carlberg and Campbell 2013; Rosen et al. 2012).

One of the main nonskeletal biologic functions of activated vitamin D is the regulation of immune function. Nuclear receptors for vitamin D (VDR) have been found in many cells of the immune system including macrophages, dendritic cells, and activated T and B lymphocytes (van Etten and Mathieu 2005). In

general, activated vitamin D enhances the innate immune response, whereas it inhibits the adaptive immune response by reducing T cell proliferation, shifting the balance of T cell differentiation from the Th1 and Th17 pathways to Th2 and Treg pathways, and inhibiting the maturation of dendritic cells (DC) important for antigen presentation. Because autoimmune diseases are characterized by excessive Th17 activation, normal availability of activated vitamin D may be essential for preventive excessive inflammatory responses and avoiding the onset of autoimmune diseases (Froicu et al. 2003: van Etten and Mathieu 2005: Adorini and Penna 2008). While vitamin D analogs have shown the ability to improve some disorders like psoriasis, studies in most autoimmune disorders have produced inconclusive results.

It has been suggested that activated vitamin D may protect against insurgence or progression of some cancers by stimulating several inhibitors of cell proliferation. Epidemiologic studies have shown a negative correlation between sun exposure and vitamin D availability and a number of cancers but mainly cancers of the colon, breast, and prostate (Yin et al. 2010; Touvier et al. 2011; Tretli et al. 2012; Maalmi et al. 2014; Wang et al. 2014; Xu et al. 2014; Jacobs et al. 2016). The preventive effect of vitamin D seems particularly important for colon cancer, but activated vitamin D may also reduce the progression and/or the mortality of breast and prostate cancers, too (Jacobs et al. 2016). However, the results of several trials with high doses of vitamin D have been disappointing because no improvement in patients affected by different forms of cancer was observed (Jacobs et al. 2016).

Activated vitamin D may stimulate some hormone secretion. In particular, it may enhance insulin secretion and protect pancreatic beta cells against cytokine-mediated destruction (Lee et al. 1994; Kadowaki and Norman 1985; Both VDR and calbindin are found in pancreatic beta cells. Epidemiological studies have shown that low vitamin D levels are associated to increased risk for type 1 and type 2 diabetes mellitus (Forouhi et al. 2008; Bojesen and Nordestgaard 2013) rising the hope that vitamin D supplementation may reduce the prevalence or the clinical expression of the different forms of diabetes. However, results of randomized controlled trials with vitamin D in patients having type II diabetes have been disappointing because no improvement of the disorder was observed (Avenell et al. 2009; de Boer et al. 2008; Nakashima et al. 2016).

Finally, a deficiency of activated vitamin D has been involved in cardiovascular diseases (Wang et al. 2010; Pittas et al. 2010). Mechanisms related to a better control or a lower prevalence of hypertension have been suggested (Forman et al. 2008), but activation of inflammatory processes and increased atherogenic processes may be also involved. However, as for other disorders that have been linked to vitamin D deficiency, the results of several trials of vitamin D supplementation have been negative, and in some cases the possibility that high doses of vitamin D could be harmful has been raised (Chowdhury et al. 2014; Bjelakovic et al. 2014)

Figure 2 schematizes the effect of activated vitamin D on nonskeletal tissues.

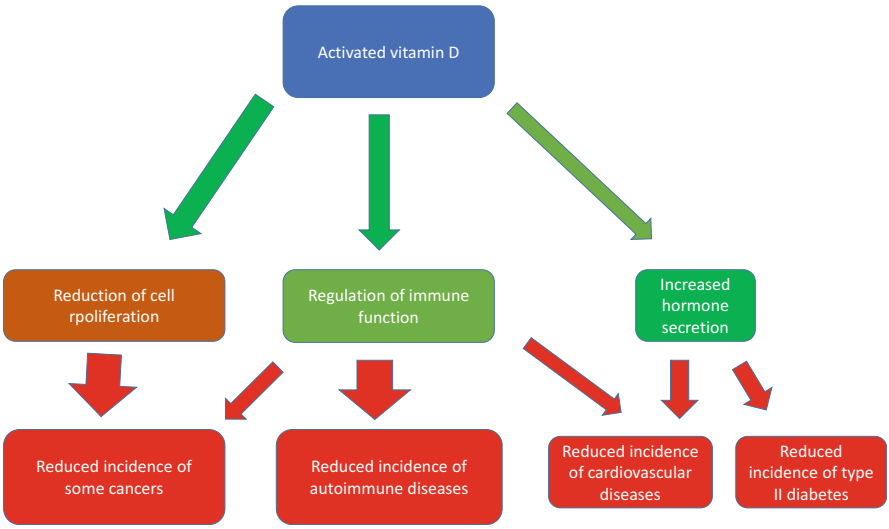

Fig. 2 Effects of activated vitamin D on nonskeletal tissues

Diagnosing Mild Vitamin D Deficiency in Chronic Diseases

Because of the disappointing results of most trials using vitamin D supplementation in chronic diseases, some experts have become convinced that low vitamin D is just a marker of some chronic conditions and not a causal factor.

While it remains a possibility, the quality of many studies has been low and more and better planned studies are needed to understand whether is the role, if any, of vitamin D in these chronic diseases.

It is probable that unsatisfactory results depend on the use of vitamin D in patients who already developed diseases. Most of the nonskeletal effects of activated vitamin D regard the partial inhibition of processes that, if activated, in genetically predisposed individuals, may determine the development of cancers, immune and chronic diseases. It is possible that administration of vitamin D is more effective in preventing than in curing these disorders. Long-term follow-up of populations treated with vitamin D is needed to clarify this possible preventive effect.

Another crucial point is to establish what vitamin D levels should be considered low. Using too high threshold values, while opening the way to a more generalized use of vitamin D supplement, may include in trial studies a too much heterogeneous population so reducing the possibilities of getting correct information. In addition, because vitamin D has a main effect on bone metabolism and other effects on many different tissues, it cannot be excluded that extra-skeletal effects of vitamin D deficiency may require different quantities of vitamin D. If so, it is probable that bone metabolism is sensitive to minor deficiencies of vitamin D, while other tissues

Table 2 Possible cutoffs for vitamin D deficiency in skeletal and extra-skeletal diseases

	Cutoff for vitamin D deficiency
Skeletal diseases	<25 ng/ml
Extra-skeletal chronic diseases	<20 ng/ml

may be affected only when more severe deficiency is present. Using parameters related to mineral metabolism may be incorrect and determine the false conclusion that, in chronic nonskeletal diseases, vitamin D supplementation is not useful. Maybe, until new parameters related to extra-skeletal vitamin D effects are found, trials of vitamin supplementation in chronic diseases should be directed only to patients presenting severe vitamin deficiency or at least lower threshold values as the 20 ng/ml cutoff.

In Table 2, the possible cutoffs for establishing vitamin D deficiency in skeletal and nonskeletal tissues are shown.

Potential Applications

Activated vitamin D has widespread effects that are not limited to mineral metabolism but also to the regulation of the function of different tissues. The consequences of vitamin D deficiency may be rickets, osteomalacia, or osteoporosis but also the appearance or the worsening of chronic diseases like autoimmune diseases, diabetes, cardiovascular disorders, and some forms of cancer, in particular colon cancer.

Vitamin D administration may have an important role in preventing not only classic bone diseases but also colon cancer and some chronic diseases and should be directed to all individuals bearing low circulating 25OH-vitamin D levels. Screening general population for mild vitamin D deficiency may be needed.

Summary Points

- Vitamin D is formed by the effect of ultraviolet exposure but requires two hydroxylations (in liver and kidney) to be activated and be able to determine biologic effects.
- Biologic effects require the link of activated vitamin D with specific receptors that are present in a large number of tissues.
- The main receptor (VDR) is a nuclear receptor and the complex activated vitamin D – VDR activates multiple gene transcriptions. Other receptors may be found in cell membrane and may determine non genomic effects like calcium transportation across cell membrane.
- Activated vitamin D is one of the main regulators of mineral metabolism and bone homeostasis by increasing active calcium absorption in intestinal microvilli and in kidney. These effects are mainly mediated by the link to VDR because require synthesis of multiple proteins.

- Activated vitamin D may also directly increase bone formation and reduces bone resorption but these effects are mainly mediated by its effect on reducing PTH secretion.
- In diagnosis of mild vitamin D deficiency, measurement of 25OH-vitamin D is generally used unless kidney altered function is present.
- Different cutoffs of 25OH vitamin D have been suggested, but a cutoff of 25 ng/ml seems the most reasonable and corresponds to initial bone damage.
- Activated vitamin D has many extra-skeletal actions and in particular may regulate cell proliferation, immune function, and some hormone production.
- Epidemiologic studies have shown a negative correlation between vitamin D availability or levels and several chronic diseases including cancer of the colon, type II diabetes, some autoimmune and cardiovascular diseases. However, in these disorders, trials with vitamin D administration have been disappointing.
- Unsatisfactory results with vitamin D trials in chronic diseases may depend on the use of too high cutoffs and therefore of unselected population but probably depend mainly on the use of this substance in subjects who already developed diseases. Long-term preventive administration of vitamin D in general population may be needed.

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