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# Vitamin D blood concentrations in relation to regulatory t lymphocytes and other lymphocyte subsets in metastatic and non-metastatic cancer patients

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**Abstract**
Cancer progression tends to be associated with a deficiency in vitamin D blood levels. Vitamin has been proven to inhibit cancer cell proliferation. However, the *in vivo* antitumor activity of vitamin D needs to be still confirmed, since its inhibitory effect on cancer cell proliferation could be vanified by its suppressive activity on the antitumor immunity, due to the stimulatory effect of vitamin D on regulatory T lymphocytes (T reg), which counteracts the antitumor immune response. On these bases, a preliminary study was performed to measure 25 (OH) vitamin D serum levels in relation to the antitumor immune status of cancer patients with early or metastatic diseases. The study included 50 patients suffering from non-metastatic (n=27), or metastatic solid neoplasms (n=23). In each patient, serum levels of vitamin (OH) D, lymphocyte and monocyte counts, lymphocyte-to-monocyte ratio (LMR), TH1 (CD4) and T reg (CD4+CD25+) cells. Abnormally low values of vitamin D were seen in 26/50 (52%) patients, namely in the metastatic ones. Lymphocytopenia occurred in 10/50 (20%) patients. Lymphocyte count, LMR mean values, TH1 number, and TH1-to-T reg ratio were higher in patients with normal vitamin D levels, whereas monocyte and T reg cell counts were higher in patients with low vitamin D concentrations, but none of these differences was statistically significant. The results of this preliminary study may justify vitamin D correction of cancer progression-related vitamin D deficiency, but not its use as an anticancer agent, which will require to be established through longitudinal studies, by monitoring vitamin D levels in relation to the immune status of cancer patients and the clinical course of their neoplastic disease.

**Keywords:** cancer immunity, cancer metabolism, lymphocyte-to-monocyte ratio, regulatory T lymphocytes, vitamin D, vitamin D deficiency

## Introduction
Several recent experimental studies have shown that the human body contains several endogenous protumoral, or antitumoral molecules. The main antitumoral endogenous molecules are constituted of the pineal hormone melatonin (MLT) [1, 2] and other indole hormones [3], the endocannabinoids arachidonyl-ethanol-amide (AEA) and the 2-arachidony-glycerol (2-AG) [4], oxytocin [5], and the antitumor cytokines IL-2 [6], and IL-12 [7]. On the other hand, the main protumoral endogenous substances are represented by the sexual steroids, the various growth factors, and several immunosuppressive molecules, including cortisol [8], the inflammatory cytokines IL-1 beta, IL-6, IL-17, the anti-inflammatory immunosuppressive cytokines, IL-10 and TGF-beta, namely released from regulatory T lymphocytes (T reg), the main cells involved in the suppression of the anticancer immunity [9], and ADH [10], endothelin-1 (ET-1) and VEGF [11], because of their angiogenic properties. Within the great number of endogenous molecules potentially able to influence tumor growth in an antitumor, or in a protumoral way, one of the most controversial compound is vitamin D3 itself, since it has been proven to exert both antitumor [12], or protumoral effects [13]. The antitumor effects of vitamin D are mainly due to its inhibitory effects on cancer cells proliferation [13], in association with an anti-angiogenic activity, an inhibition of growth factor activation, and a stimulation of cystatin secretion, a protein provided by antitumor and anti-metastatic properties [14]. On the other hand, the potential protumoral action would depend on its different modulatory effects on the immune system, mainly consisting of stimulation of T reg cell system [15], with a following suppression of the anticancer immunity, and inhibition of the secretion of the main inflammatory cytokines, including IL-6, IL-1 beta, IL-8, and IL-17 [15, 16]. The inhibition of inflammtory cytokine secretion would enhance the antitumor immunity, whereas the stimulatory action of T reg cell activity may allow a suppression of the anticancer immunity [15], because of its production of TGF-beta [17], which is the main endogenous immunosuppressive molecule of the anticancer immunity. Therefore, the end-result of the immune effects induced by vitamin D would consist of the algebric sum of both pro-tumoral and anti-tumoral effects. On the same way, the potential therapeutic anticancer properties of vitamin D would mainly depend *in vivo* on the immune status of patients, as well as on its effects on the antitumor immunity. Vitamin D-induced stimulation of T reg lymphocytes has been clinically confirmed by the evidence that low serum levels of vitamin D has appeared to predispose to the autoimmune diseases, which are characterized by a diminished T reg cell system activity in association with an enhanced IL-17 secretion [18]. Because of the inhibitory effect of IL-17 on T reg cells [19], autoimmune disease-related Treg cell deficiency could depend on the enhanced production of IL-17 itself. Vitam D deficiency has also been shown to predispose to infections [15, 16]. On the contrary, the immuno modulary effects of vitamin D on the anticancer immunity are still controversial. Then, from a

physiological point of view, the most known biological role of vitamin D in the maintenance of normal calcium blood levels, which is fundamental for cellular biology itself, would be only one of the great number of metabolic effects played by vitamin D. Vitamin D is endogenously produced starting from the 7-dihydro-cholesterol (or ergosterol) at skin level, when exposed to ultraviolet irradiation, with production of cholecalciferol. To be active, it has to undergo two hydroxylation processes, before in the liver with its transfromation into 25 (OH) vitamin D (25 (OH) D), also called ergocalciferol, and after in the kidney with the production of 1, 25 (OH)2 D, the so-called calcitriol (or vitamin D3), through the action of the 1-alpha reductase. Calcitriol is the most biologically active form of vitamin D, and its production depends only on 1-alpha reductase activity, which is stimulated by the parathyroid hormone (PTH) and hypophosphatemia, whereas it is inhibited by calcium, calcitriol itself as a self-regulation and fibroblast growth factor (FGF)-23. Because of its multiple biological effects, vitamin D has been also considered as a cytokine, or a hematopoietic growth factor, which has been proven to be directly secreted by the same immune cells, as well to exert a paracrine regulatory control on immune cell activities [15, 16]. Vitamin D enhances calcium blood levels by stimulating calcium absorption in the small intestine, and calcium reabsorption of bone by promoting osteoblast differentiation [20].Vitamin D into the blood is mainly linked to vitamin D binding protein (VDBP), and in a less maner to albumin. The main circulating form is the 25 (OH) D. Then, the more appropriate parameter to define the human vitamin D status is the measurement of 25 (OH) D blood levels. In any case, to explain vitamin D action on both bone metabolism and cell proliferation, its activity has to be evaluated in relation to that of other endogenous factor also provided by modulating effects on bone metabolism and cell growth and differentiation, including at least PTH itself [21], parathyroid hormone-related peptide or protein (PTH-rP) [22], osteocalcin (OTC) [23], fibroblast growth factor (FGF) [24], namely FGF 23 [25], and calcitonin (CT) [26]. PTH namely influences bone metabolism by stimulating osteoblast activity, with a following demineralization and release of calcium, as well as by promoting calcitriol production by stimulating 1-alpha reductase activity. The main stimulus for PTH secretion is the hypocalcemia, since its major function is the maintenance of normal calcium levels into the blood, whereas its release is inhibited by hypercalcemia, and probabily by calcitriol itself, as well as by the retinoic acid [27]. However, it has to be considered that most PTH-related biological effects are mediated by PTH-rP [22]. While PTH is mainly produced by the parathyroid gland, PTH-rP is synthetized by several cell tissues, including parathyroid cells themselves. In particular, PTH-rP would be responsible for cancer-related malignant hyper calcemia, but it would be also involved in the pathogenesis of hypertension, and in cancer progression, since PTH-rP may direcly stimulate cancer cells proliferation, and the angiogenesis processes [28, 29]. Moreover, PTH-rP secretion has appeared to be stimulatedby TGF-beta [29, 30]. PTH and PTH-rP would act on the same cell receptor. PTH would exert a generalized regulation of human biological functions by modulating the local production of PTH-rP at tissue level, and influencing calcium metabolism, as well as sodium and potassium metabolism is mainly influenced by the adrenal gland. In any case, PTH has appeared to negatively influence the cardiac function by inducing myocardial damage, due to an abnormal increase in intramyocyte calcium concentrations, and interstitial fibrosis, with a following impaired ventricular contractile function. The main hormone with opposite effects with respect to PTH on bone metabolism, is CT, produced by thyroid C cells, since CT may inhibit osteoclast-induced bone resorption, and its secretion is mainly stimulated by the hypercalcemia, and inhibited by the hypocalcemia [26]. The main protein of the mineralized matrix of bone is OTC [23], produced by bone cells, which originate from the monocyte-macrophage cell line. OTC is one of the main osteblast growth and stimulating factor, by promoting osteoblast differentiation, with a following enhanced bone mineralization. Then, both CT and OTC act in an opposite way with respect to PTH-PTH-rP system, but with different effects, since CT would mainly inhibit osteclast activity, whereas OTC would namely stimulate osteoblast activity. OCT ha salso appeared to exert metabolic effects, namely the stimulation of insulin release, with a following enhanced glucose tolerance, the osteclastic action of PTH and the osteoblastic stimulatory activity of OTC. FGF family is a group of 23 forms [24], which are involved in the repair and regeneration of tissues, by stimulating fibrolast differentiation and proliferation, and the angiogenesis. FGF is produced by several tissues, including brain and hypophysis. The main effect of basic FGF (b-FGF) for bone metabolismis the inhibition of osteoclast cell formation [24]. Another molecule, the so-called FGF-23, would constitute the main factor capable of decreasing the phosphatemia by reducing proximal tubular phosphate reabsorption [25]. Moreover, FGF 23 has also appeared to inhibit 1-alpha reductase activity, by reducing calcitriol levels, the activeform of vitamin D. Then, vitamin D, by stimulating both osteoclast activity and intercellular matrix mineralization, could act as an equilibrium-point among the stimulatory action of PTH on the osteoblast activity, the stimulatory effect of OTC and b-FGF on the osteblast activity, and the inhibitory effect of CT on the osteoblast function. Moreover, the parathyroid gland is also under a neuroendocrine control played by pituitary and pineal glands. The parathyroid gland may also produces endothelin-1 (ET-1), a protein provided by vasoconstrictor activity, and probably involved in the pathogenesis of the idiopathich ypertension [31]. The main pituitary hormones involved in bone metabolism is GH itself [32], which would mainly act by modulating bone remodeling through a direct influence on osteoblast-osteclast crosstalk, with potential stimulatory effect on both osteclast and osteblast activities, depending on the different metabolic conditions. On the other side, the pineal gland would inhibit parathyroid gland activity [33], and in particular the pineal indole 5-metoxytryptamine (5-MTT) could reduce PTR-rP-related hyper calcemia [34]. Then, being the pituitary gland under a pineal-pituitary modulation, the parathyroid-pineal-pituitary axis could be considered as a functional trinity involved in the control of calcium and phosphate metabolism, from which depends the same form of the human skeleton and body. Moreover, because of the fundamental role of calcium metabolism for the overall biological functions, as well as the cognitive functions of both vitamin D and PTH, vitamin D deficiency and excessive PTH secretion may predispose to cognitive dysfunctions and cardiovascular diseases. Finally, as far as its immuno modulatory actvity is concerned, vitamin D has appeared to exert a great

variety of controversial immune effects, whose end-result would consist of inhibition of the inflammatory response in association with an enhanced self-tolerance [15]. The anti-inflammatory action of vitamin D is namely due to an inhibition of inflammatory cytokines production, including IL-6, IL-1 beta, and TNF-alpha, in association with a stimulatory action on the secretion of the anti-inflammatory immunsuppressive cytokines TGF-beta and IL-10, both namely released from T reg cells [15]. Within the immune cells, the main effects of vitamin D are consisting of inhibition of TH1 and TH17 lymphocytes, with a following decreased secretion of IL-2, and IL-17, respectively, in association with a stimulatory action of T reg lymphocytes [15, 16]. Then, the effect of vitamin D on T lymphocyte system may change in relation to the various cell subsets. Moreover, vitamin D ha salso been proven to inhibit B cell proliferation, and dendritic cell differentiation, with a following diminished production of IL-12 [15, 16]. Because of the antitumor activity of both IL-2 and IL-12 [6, 7], the inhibitory effect of vitamin D on the secretion of both IL-2 and IL-12 could allow to a suppression of the anticancer immunity, whereas it could contribute to the control of the autoimmune reactions by stimulating T reg cell system and inhibiting IL-17 secretion [15, 19]. All inflammatory cytokines may potentially exert an osteolytic activity, namely TNF-alpha, but the main link between bone metabolism and hematopoietic system would be represented by IL-11, mainly produced by bone stromal cells. IL-11 has been proven to stimulate osteoclast activity [24], which in contrast is inhibited by both b-FGF and OTC, and to influence blood cell differentiation, namely by stimulating megakaryocyte system and exerting throm bopoietic properties [35]. In addition, IL-11 would counteract the antitumor immunity [36], by inhibiting the secretion of the antitumor cytokine IL-12 [7]. Then, because of the importance of the immune status in influencing the prognosis of the neoplastic diseases, as well as the potential immunomodulating activity of vitamin D, this preliminary study was performed in an attempt to evaluate blood levels of vitamin D in relation to some fundamental immune biomarkers of the antitumor immunity in a group of metastatic and non-metastatic cancer patients.

## Materials and Methods

The study included 50 consecutive cancer patients (M/F: 22/28; median age: 68 years, range 34-86), 27 of whom had a locally limited disease, while the remaining 23 patients showed a metastatic disease. Eligibility criteria were, as follows: histologically proven neoplastic disease, no concomitant vitamin D therapy, no previous chemotherapy for at least 3 months prior to study, and no renal failure because of its association with alterations of calcium metabolism. Tumor histo types were, as follows: breast cancer: 14; colorectal carcinoma: 11; non-small cell lung cancer: 10; pancreatic adenocarcinoma: 4; gastric cancer: 4; melanoma: 3; hepato carcinoma: 2: prostate cancer: 2. For vitamin D detection, venous blood samples were collected during the morning after a overnight fast. Vitamin D was measured in its 25 (OH) form. Serum levels of 25 (OH) D were measured by the ELISA method, by using commercially available kits. The results were compared to those observed in a control group of 100 healthy subjects. Normal values of 25 (OH) D observed in our laboratory (95% confidence limits) were greater than 20 ng/ml. Vitamin D values were evaluated in relation to those of total lymphocytes and monocytes, lymphocyte-to-monocyte ratio (LMR), T helper-1 lymphocytes (TH1), regulatory T lymphocytes (T reg), and TH1-to- T reg ratio. Lymphocyte subsets were detected by using specific monoclonal antibodies, supplied by Beckton-Dickinson (Milan, Italy). Data were reported as mean ± SE, and statistically analyzed by the Student's t test, the chi-square test, and coefficient of correlation.

## Results

Abnormally low vitamin D serum levels were seen in 26 /50 (52%) cancer patients. Vitamin D mean serum levels observed in cancer patients were significantly lower than those found in the control group (19 ± 5 vs 38 ± 4 ng/ml, $P < 0.05$). Within the cancer group, metastatic patients showed lower vitamin D mean values with respect to the non-metastatic ones, even though the difference was not statistically significant (17 ± 4 vs 21 ± 3 ng/ml). Lymphocytopenia, with lymphocyte count less than 1,000/mm3, was seen in 10/50 (20%) patients. The percentage of lymphocytopenia observed in patients with low vitamin D levels was higher than that occurring in those with normal values, without, however, statistically significant differences (6/26 (23%) vs 4/24 (17%)). Lymphocyte mean count observed in metastatic patients was significantly lower than that found in the non-metastatic ones, either in patients with low or normal vitamin D concentrations (1,441 ± 121 vs 1,845L ± 82/mm3, $P< 0.05$; 1,483 ± 98 vs 2,096 ± 87/mm3, $P< 0.01$). Lymphocyte and monocyte mean counts, and LMR mean values observed in cancer patients with low or normal vitamin D levels, and in controls are illustrated in Figure 1. Lymphocyte and monocyte mean counts were respectively lower and higher in patients with low vitamin D values than in those with normal concentrations, but none of these differences was statistically significant. Moreover, LMR mean values were higher in patients with normal vitamin D values than in those with abnormally levels, without, however, statistically significant differences. The relation between vitamin D values and T lymphocyte subsets is illustrated in Figure 2. TH1 and T reg mean counts were respectively lower and higher in patients with low vitamin D levels than in those with normal values, without, however, statistically significant differences. On the same way, TH1-to-T reg mean ratio was higher in patients with normal vitamin D concentrations than in those with vitamin D deficiency, without, however, statistically significant differences. In any case, no significant correlation was seen between vitamin D levels and T reg cell count ($r= - 0.09$). Finally, no difference in vitamin D mean levels was seen between patients with lymphocyte count greater or lower than 1,000/mm3 (19 ± 3 vs 19 ± 4 ng/ml).

## Discussion

According to the previous results of other authors [37, 38], this clinical investigation confirms that the frequent occurrence of vitamin D deficiency in cancer patients, namely in those with metastatic disases, even though it has still to be established wether cancer-related vitamin D deficiency may be simply due to disease-related dietary changes, or whether it may play a physio pathological significance because of the potential effects of vitamin D on both cell proliferation and antitumor immunity [12, 13]. In any case, because of the involvement of both vitamin D and

PTH non-only in metabolic, but also in cognitive functions, some cancer progression-related psychological symptoms, including depression and asthenia, could be due at least in part on vitamin D deficiency-induced alterations of brain neurochemical functions [38]. Moreover, despite the stimulatory effect of vitamin D on T reg cell generation observed in experimental conditions [12, 13], the present preliminary study seems to exclude the evidence of a positive correlation betweeen vitamin D levels and T reg cell count, which in contrast was higher in patients with low vitamin D levels than in those with normal values, even though the difference was not significant. The controversial results concerning the immune effects of vitamin D described in experimental conditions with respect to the clinical ones could simply depend at least in part on the great variety of immune actions exerted by its metabolites, which are also provided by immuno modulating properties [38].

Moreover, the immune effects of vitamin D on T reg cells and other T lymphocyte subsets could depend on the basal functional status of cell subsets themselves, and be different in advanced neoplasms and autoimmune diseases, being characterized by an opposite behavior of T reg cell system. Therefore, these preliminary results, by showing that the presence of normal vitamin D levels is not associated with a worse immuno suppressive status, would exclude an eventual immuno suppressive action of vitamin D in cancer patients. Then, these results seem to justify the replacement therapy with vitamin D in the presence of cancer-related vitamin D deficiency, while they are not sufficient to justify an eventual therapy with vitamin D in an attempt to control tumor growth. Therefore, further longitudinal studies, by monitoring the immune status of cancer patients in relation to vitamin D levels not only in basal conditions, but also in response to the various anticancer therapies, including chemotherapy, endocrine therapy and immunotherapy, will be required before proposing vitamin D as a new anticancer agent in humans.

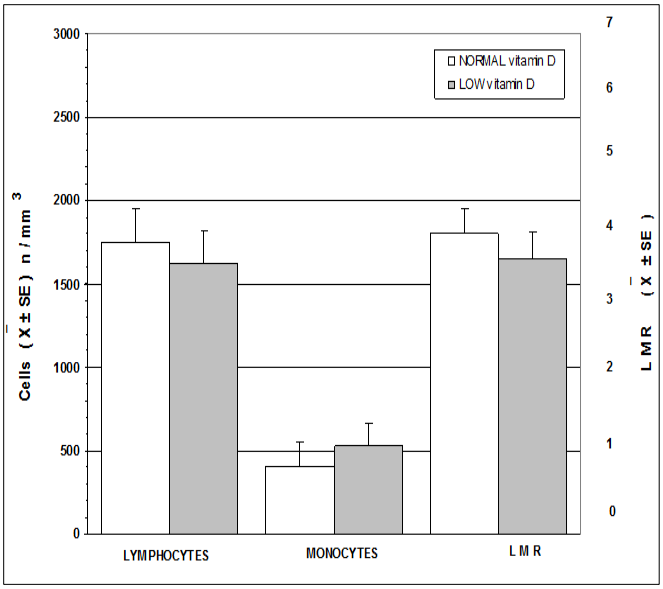


**Fig 1:** Lymphocyte and monocyte mean counts and lymphocyte-to-monocyte mean values *ratio* in cancer patients in relation to vitamin D levels

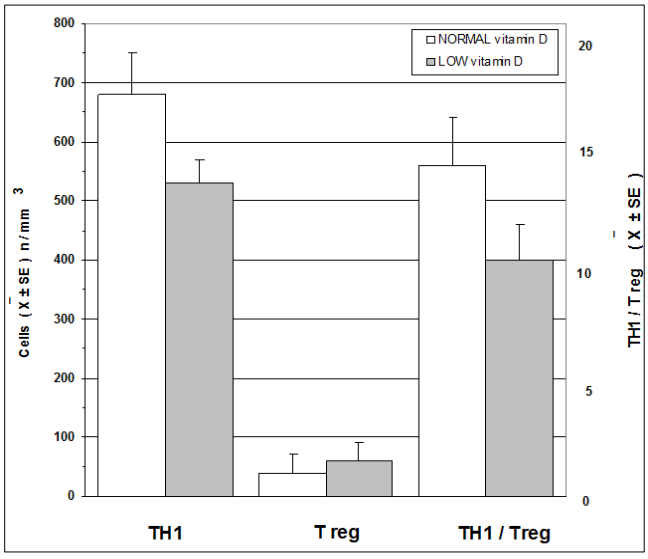

**Fig 2:** TH 1, Treg and TH1 / Treg ratio in cancer patients in relation to vitamin D level

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