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From molecular basis to clinical insights: a challenging future for the vitamin D endocrine system in colorectal cancer

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Abbreviations: $1,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 25(OH)D, 25-hydroxyvitamin D; CAFs, cancer-associated fibroblasts; CDK, cyclin-dependent kinase; CK1, casein kinase 1; COX-2, cyclooxygenase-2; CRC, colorectal cancer; CSCs, cancer stem cells; DBP, vitamin D-binding protein; DKK, Dickkopf; EGF, epidermal growth factor; EGFR, EGF receptor; EMT, epithelial-to-mesenchymal transition; GSEA, gene set enrichment analysis; GSK3β, glycogen synthase kinase 3β; GWAS, genome-wide association studies; HIF-1α, hypoxia inducible factor 1α; HR, hazard ratio; IBD, inflammatory bowel disease; IGF, insulin-like growth factor; IKKβ, IκB kinase β; IL, interleukin; miRs, microRNAs; MR, mendelian randomization; MRP, multi-drug resistant-associated protein; NAT2, N-acetyltransferase 2; NF-κB, nuclear factor κB; NFs, normal fibroblasts; NSAID, nonsteroidal anti-inflammatory drug; OR, odds ratio; OS, overall survival; PARP, poly(ADP-ribose) polymerase; PD-L1, programmed cell death ligand 1; PG, prostaglandin; PFS, progression-free survival; RCTs, randomized controlled trials; RFS, relapse-free survival; RR, relative risk; RSPO, R-Spondin; SCs, stem cells; SNPs, single nucleotide polymorphisms; TCF, T-cell factor; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor α; UV, ultraviolet;

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VDES, vitamin D endocrine system; VDR, vitamin D receptor; VEGF, vascular endothelial growth factor; ZO, zonula occludens.

Keywords: vitamin D, colorectal cancer, mechanisms of action, randomized controlled trials, epidemiology

Conflict of interest

All authors declare no conflict of interest.

Abstract

Colorectal cancer (CRC) is one of the most life-threatening neoplasias in terms of incidence and mortality worldwide. Vitamin D deficiency has been associated with an increased risk of CRC. 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], the most active vitamin D metabolite, is a pleiotropic hormone that, through its binding to a transcription factor of the nuclear receptor superfamily, is a major regulator of the human genome. $1,25(OH)_2D_3$ acts on colon carcinoma and stromal cells and displays tumor protective actions. Here, we review the variety of molecular mechanisms underlying the effects of $1,25(OH)_2D_3$ in CRC, which affect multiple processes that are dysregulated during tumor initiation and progression. Additionally, we discuss the epidemiological data that associate vitamin D deficiency and CRC, and the most relevant randomized controlled trials of vitamin D_3 supplementation conducted in both healthy individuals and CRC patients.

Introduction – A historical perspective

It is likely that vitamin D was initially originated as an inert molecule before the apparition of life billions of years ago, and although its physiological function in the early organisms and primal evolution is unknown, it might have acquired an initial vital function in the protection of life in early marine organisms against ultraviolet (UV) radiation-induced DNA damage before the existence of protective ozone layers in the atmosphere. Indeed, it was demonstrated that plankton species unchanged for at least 750 million years hold the capacity of synthesizing previtamin D from its precursors [1-3]. This may have had an ultimate importance in the dawdling evolutionary jump from sea to earth life when confronting the characteristics of a new hostile environment and the advantage of calcium homeostasis and eventually, a skeleton. It is presumable indeed, that during this evolution, the photochemical reaction leading to vitamin D production was transferred, in the long run, to the skin of animals [2]. The "skin-lightening hypothesis" proposed by Jablonski & Chaplin would explain the role of vitamin D in human dispersion from Africa and its presumable responsibility in skin depigmentation, since darker skin in primitive hominids avoided excessive production of vitamin D as minimal storage was required in a tropical climate with high and direct sun exposure [4]. Although whiter skin is better adapted to vitamin D synthesis, the migration of modern humans from eastern Africa in the first major demographic expansion would have resulted in unexpected scenarios of vitamin D deficiency, as documented by osteological examinations in excavated prehistoric skeletons found in northern Europe [5, 6]. This hypothesis has been challenged recently as new archeogenomic data on population genetics arise and alternative explanations for the adaption of the vitamin D endocrine system (VDES) are under debate [7]. Notwithstanding, it is the beginning of writing and the narration of human past in Ancient History that renders the earliest references of the physiological effect of sunlight on bone composition, initially by the ancient Greek historian Herodotus (5th century BC) when examining the softer skulls of turban-wearing dead warriors and later by the Greco-Roman physician Sorano of Ephesus (1st-2nd century AC) in the observation of bone deformities among infants residing in Rome [2]. It would take centuries though until the first publication identifying and recognizing a specific clinical disease termed, so far popularly, rickets.

Two renowned physicians educated in England initiated the scientific literature on rickets, which was first clearly described and concisely documented in Daniel Whistler thesis

presented in the Netherlands in 1645 and shortly after by Francis Glisson treatise published in England in 1650 [2, 8]. In the early 1800s, Jedrzej Sniadecki, a polish physician, documented the differential incidence of rickets in sunless city-dwelling children vs. rural-dwellers and hypothesized that exposure to sunlight was involved. By the end of the 19th century rickets appeared in epidemic proportions in large, polluted cities, as people began to stay indoors with reduced exposure to sunlight. The incidence of the disease continued to increase during the Industrial Revolution, especially in children who lived in the industrialized cities of northern Europe and north-eastern United States. In 1890, a British medical epidemiologist named Theodore Palm studied the relationship between incidence of rickets and its geographical distribution and concluded that rickets in Britain-resident infants, although having a superior diet and better sanitation, was caused by lack of exposure to sunlight when compared to those living in the tropics [8-10]. In fact, Palm recognized the role of sunlight in the prevention and treatment of rickets but unfortunately these seminal observations supporting an environmental perspective on the nature of rickets remained unnoticed until the early 20th century, when a debate in the scientific community focused on whether the disease was a result of some dietary substance deficit or an environmental factor. Several scientists performed experiments in the following decades in which laboratory animals and affected children were cured when exposed to sunlight or mercury lamps [11, 12]. On the other hand, at that time, scientists realized that there were micronutrients present in food necessary for normal growth and reproduction. A number of disorders, such as xerophthalmia and scurvy, were defined to be related to the lack of nutritional substances of water/fat-soluble origin. The use of purified diets in experimental animals and deprivation studies led to the breakthrough discovery of these "vital-amines", i.e., vitamins [13]. Based on this previous knowledge, the search for specific foods or substances within that could prevent rickets was on the run [14].

Classic animal experiments by Edward Mellanby and Elmer McCollum irrevocably established the antirachitic properties of cod liver oil [8, 15]. Mellanby performed a series of experiments keeping Beagle dogs indoors, away from sunlight, and feeding them diets that, together with the lack of UV radiation, were capable of inducing rickets. He then fed the rachitic dogs with cod liver oil, among others, and proved that these puppies could be cured by administering this oil. Through these experiments, cod liver oil was confirmed as a scientific model for an essential micronutrient. They attributed the anti-rachitic function of cod liver oil to "fat soluble A" (or vitamin A, which is present in high

concentrations in cod liver oil) or a similar substance [16]. In the following years, Hariette Chick and collaborators were able to reproduce these results and demonstrated that rickets in post-World War I malnourished children could be overturned by the ingestion of whole milk or cod liver oil [17]. The breakthrough discovery that the anti-rachitic substance in cod liver oil was distinct from vitamin A came up with Elmer McCollum, a chemist at the University of Wisconsin (USA). In a series of experiments, McCollum and his co-workers demonstrated that heated and oxygenated cod-liver oil lost its protectiveness against vitamin A deficiency (xerophthalmia) but still retained its anti-rachitic function, leading to the conclusion that there were two different active compounds [18]. McCollum coined the term "vitamin D" to refer to the anti-rachitic substance in cod liver oil as it was fourth in the sequence of vitamins discovered [11].

In the meantime, different laboratories found that UV irradiation of inert food was able to provide it with anti-rachitic properties, which would lead the way to find the substance that could be activated by irradiation [15]. Although initially thought to be cholesterol according to experiments conducted by Hess and collaborators, spectroscopic studies generated some doubts on the purity of the sample stated to be activated by UV-radiation [19]. At this point, in 1926, Hess asked the famous German steroid chemist Adolf Windaus to collaborate on the clarification of the chemical structure of the anti-rachitic product activated by UV-radiation. A third investigator in England, Otto Rosenheim, also joined this collaboration. In fact, Rosenheim's team performed the key experiment and provided the essential clue with the demonstration that the immediate precursor of vitamin D was not cholesterol [20]. The following work on the identification of the provitamin by Windaus and Hess was greatly influenced by the previous knowledge of the absorption spectrum of cholesterol, and finally led to the determination that a fungal steroid from ergot (a parasite that infects cereals), named ergosterol, was the UV-radiation convertible provitamin D. This finding was finally corroborated by Rosenheim's group.

These achievements, which contributed to the finale and culmination of an era in the isolation and identification of the precursors of vitamin D, together with previous intensive work on sterols/cholesterol, rendered Adolf Windaus the Nobel Prize of Chemistry in 1928 "for his studies on the constitution of the sterols and their connection with vitamins" [13, 15]. But aside from earning the upmost honoured recognition in Science, Windaus and others continued on the pursue of new achievements and answers to key questions. The irradiation product of ergosterol, named vitamin D_2 or calciferol (the initial isolation of vitamin D_1 by the group of Windaus was proved to be an adduct

and an error in identification), was purified and crystallized shortly after in 1931 by 3 independent teams, including Windaus' who determined its chemical properties and structure, which would be corrected by himself later on in 1936. One year later Windaus and Bock finally unveiled how animals obtain active vitamin D from UV-light, hitting and identifying 7-dehydrocholesterol and the structure of its irradiation product named vitamin D_3 or cholecalciferol [15].

It would take almost another 50 years after the discovery of vitamin D to finally unveil the exact sequence of steps leading to the photoproduction of vitamin D_3 in the skin, the activation steps in the liver to generate the intermediate 25-hydroxyvitamin D_3 ($25(OH)D_3$) or calcifediol and subsequently in the kidney and other tissues to render the active 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) or calcitriol [21-23] (Fig. 1).

In this review we will overhaul thoroughly the latest scientific evidence on the VDES and colorectal cancer (CRC) with special consideration on the molecular mechanisms and its clinical applications.

The vitamin D endocrine system

Vitamin D in humans is either obtained from the diet or synthesized in the skin. Dietary intake of vitamin D (mostly D_3 and only minimal amounts of D_2) is usually low, and therefore the main source of vitamin D is the non-enzymatic skin production of vitamin D_3 from UVB exposed 7-dehydrocholesterol [21, 24]. In the liver, vitamin D_3 is hydroxylated to render $25(OH)D_3$ by the CYP2R1 hydroxylase. CYP2R1 is also responsible for vitamin D_2 hydroxylation into $25(OH)D_2$ [25]. These 25-hydroxylated forms of vitamin D, together known as 25(OH)D, are the most stable vitamin D metabolites. Thus, serum 25(OH)D concentration is widely used as a biomarker for the vitamin D status of a person and to establish vitamin D deficiency [24, 26, 27]. Defining vitamin D deficiency is still problematic and there is so far no unanimity. Most guidelines define it as serum 25(OH)D levels below 50 nmol/l (20 ng/ml), whereas some experts propose the terminology of vitamin D insufficiency for subjects with serum 25(OH)D between 50 and 75 nmol/l (20 and 30 ng/ml) [28]. In the blood, 25(OH)D is bound to vitamin D-binding protein (DBP), a member of the albumin family, encoded by the *GC* gene, that can transport various forms of vitamin D between skin, liver, and kidney, and then on to other target tissues [29]. $25(OH)D_3$ can be subsequently hydroxylated into

$1,25(OH)_2D_3$, the active hormone, by the CYP27B1 hydroxylase. This reaction occurs mainly in the kidney but also in several types of epithelial and immune cells, although only kidney-produced $1,25(OH)_2D_3$ can be exported to the bloodstream. Inactivation of both $25(OH)D_3$ and $1,25(OH)_2D_3$ is mediated by the CYP24A1 hydroxylase which generates a series of 24- and 23-hydroxylated products (e.g., $24R,25(OH)_2D_3$) that are targeted for excretion along well-established pathways [30] (Fig. 1).

In target cells, $1,25(OH)_2D_3$ binds with high affinity to the vitamin D receptor (VDR), which mediates all its actions. VDR was discovered in 1969 [31] and the human cDNA cloned in 1988 [32]. It is a member of the nuclear receptor superfamily which also includes receptors for thyroid hormones, retinoid acid, glucocorticoids, estrogen, or progesterone, among others. These receptors are transcription factors with a DNA-binding domain and a ligand-binding domain. In the case of VDR, the ligand-binding domain binds $1,25(OH)_2D_3$ and its synthetic analogues with high affinity [33, 34]. VDR forms heterodimers with RXR, another member of the superfamily and the receptor for 9-*cis*-retinoic acid, and upon $1,25(OH)_2D_3$ binding regulates the expression of a large number of target genes involved in most cellular processes, including proliferation, survival, and differentiation [34]. Besides its usual nuclear location, in some cell types VDR also locates in the cytoplasm or in caveolae at the plasma membrane and upon ligand binding elicits rapid responses by acting on kinases, phosphatases and ion channels [35, 36].

It is worth noting that the VDES presents many similarities with the thyroid hormone endocrine system as cleverly pointed out by Bouillon and collaborators [37, 38].

The vitamin D endocrine system and colorectal cancer: Observational studies

The first hint suggesting a relationship between the VDES and human cancer comes from an ecological study published by Sigismund Peller in 1936. He reported that people who developed skin cancer from sun exposure had lower incidence of other cancers [39]. A year later, Peller also showed that US Navy personnel with high exposure to sunlight had eight times the expected rate of skin cancer but only 40% of the expected rate of internal cancers [40, 41]. In 1941, Frank Apperly reported that total cancer mortalities in various

US states and Canadian provinces decreased with increasing solar radiation [42]. However, neither Peller nor Apperly related these effects to vitamin D_3 skin production. In 1980, the seminal work of Cedric and Frank Garland revealed that CRC mortality rates where highest in US areas where people were exposed to the least amounts of natural light. The Garland brothers were the first to propose that this was probably due to lower amounts of vitamin D_3 skin production in populations living in higher latitudes with lesser sun exposure [43]. Later on, an eight-year prospective study concluded that there was an inverse correlation between serum levels of 25(OH)D and risk of CRC, suggesting a protective effect of the VDES against this neoplasia [44]. Subsequent epidemiological studies have in general confirmed this initial observation [41, 45-52].

Generally, observational studies using serum 25(OH)D concentration from blood drawn before cancer diagnosis are considered more accurate than those in which blood is drawn near the time of diagnosis [41], possibly because having the disease may reduce 25(OH)D concentrations [53, 54]. Thus, higher prediagnosis plasma 25(OH)D levels have been associated with a significant improvement in overall survival (OS) in CRC patients [55] and a recent meta-analysis supports the inverse association between circulating 25(OH)D levels and CRC risk [56]. Moreover, in a large cohort of patients with advanced or metastatic CRC, higher plasma 25(OH)D levels have been associated with improved OS and progression-free survival (PFS) [57]. Interestingly, in CRC patients that undergo surgery, higher post-operative (but not pre-operative) 25(OH)D levels were associated with better survival outcome [58, 59] and this association was independent of post-surgery systemic inflammatory response that could affect 25(OH)D levels [60]. Interestingly, the protective effect of serum 25(OH)D was stronger in patients with the Cdx2 polymorphism (rs11568820 GG) of VDR [60]. In this regard, a number of epidemiological studies searching for an association between *VDR* polymorphisms (SNPs) and CRC risk in different populations/countries have been reported. Some studies showed contradictory or no association between *VDR* genetic variants and CRC risk or survival [61-64], while many others presented significant associations [61, 65-68]. SNPs in additional genes of the VDES have also been related to CRC. Polymorphisms in *CYP27B1* (rs10877012) and *CYP24A1* (rs6013897, rs158552, rs17217119) have been associated with a higher risk of CRC [69]. Likewise, the association of prediagnostic 25(OH)D levels with mortality among CRC patients may differ depending on the functional DBP isoforms. Patients who inherit the DBP2 isoform (GC rs4588-A, T420K) have lower 25(OH)D blood concentrations than those with DBP1 isoforms (GC rs7041-

T; GC rs7041-G, D416E) and may particularly benefit from higher 25(OH)D levels for CRC prevention because these concentrations may lead to stronger $1,25(OH)_2D_3$ pathway activation needed to compensate for DBP2 individuals' reduced capacity to otherwise maintain adequate 25(OH)D levels [70].

Thus, although epidemiological studies have inherent limitations, data available clearly point to a protective role of the VDES on CRC and these observations, particularly early ones, prompted us and others to study the mechanisms of $1,25(OH)_2D_3$ anticancer action in the laboratory.

Vitamin D receptor expression and $1,25(OH)_2D_3$ levels – Enabling characteristics

There are two important characteristics to consider in the study of the mechanisms of action of $1,25(OH)_2D_3$ and its synthetic analogues in experimental CRC systems and also in the design of their potential clinical use, namely i) the expression of VDR in colon carcinoma and stromal cells, and ii) the availability of the adequate doses of the ligands that are required to observe effects. We have called them "enabling characteristics" as an analogy of the term used by Hanahan and Weinberg in their seminal review "The hallmarks of cancer" to denote the means necessary for premalignant cells to reach the hallmark capabilities of cancer [71]. In our case, these enabling characteristics are required for colon carcinoma and stromal cells to respond to $1,25(OH)_2D_3$, and thus for $1,25(OH)_2D_3$ to be able to deploy its cancer-preventive actions.

Vitamin D receptor expression in colorectal cancer

The intestine is one of the main target tissues for VDES action, so it is not surprising that colon epithelial and stromal cells express VDR [72-75]. Likewise, a number of colon carcinoma cell lines have retained VDR expression during tumor progression while others have lost it and become resistant to $1,25(OH)_2D_3$ [73, 76, 77]. Studies in human CRC biopsies suggest that expression of VDR tends to increase in precancerous lesions and early stages of colorectal carcinogenesis but decreases or is lost in advanced stages [72, 78, 79], which become resistant to endogenous $1,25(OH)_2D_3$ antitumor activity and to a potential therapy with VDR agonists. Interestingly, our group has recently reported that high VDR expression in stromal cancer-associated fibroblasts (CAFs) is associated with

better OS and PFS in CRC, independently of VDR expression in carcinoma cells [74]. Therefore, CRC patients with low VDR-expressing tumor cells could still benefit from treatment with VDR agonists if their CAFs express adequate levels of VDR, which highlights the importance of the tumor stroma for cancer progression and therapy.

Several mechanisms may account for the downregulation of VDR in advanced CRC. Afshan and colleagues have recently reported epigenetic DNA hypermethylation at the *VDR* gene promoter in 37% colorectal tumor samples (28/75) as compared to 9% in matched non-cancerous adjacent tissue (7/75). This promoter hypermethylation is significantly associated with lower VDR expression, poorly differentiated and advanced/metastatic tumors, and reduced patient OS [80]. In addition, we have shown that the transcription factors SNAIL1 and 2 (formerly called SNAIL and SLUG, respectively), master regulators of epithelial-to-mesenchymal transition (EMT), repress the expression of VDR in colon carcinoma cells, through a mechanism that involves SNAIL1 and 2 binding to three E-boxes located in the proximal promoter of the human *VDR* gene [81, 82]. Moreover, we found that around 75% of human colorectal tumors express higher SNAIL1 and/or 2 levels than the adjacent healthy tissue, and this increase is associated with a reduced expression of VDR, which is lower when both SNAIL1 and 2 are overexpressed [81-83]. Interestingly, we have also reported reduced VDR expression in histologically normal tissue adjacent to a tumor with high levels of SNAIL1, suggesting that SNAIL1-expressing colon carcinoma cells secrete molecules that can inhibit expression of VDR in neighboring normal cells [84]. In support of our data, other groups have also demonstrated an inverse correlation between SNAIL transcription factors and VDR expression in CRC or acute colitis [85-87]. Therefore, CRC patients with high expression of SNAIL1 and/or 2 in their carcinoma cells should be poor responders to VDR agonists.

A number of microRNAs (miRs) including *miR-27b*, *miR-298*, *miR-346*, and the *miR-372/373* cluster have been reported to post-transcriptionally downregulate VDR expression in colon carcinoma cells [88-91]. Additionally, *miR-675-5p* mediates long non-coding *H19* RNA repression of VDR through a site in the 3' UTR of the *VDR* mRNA [92]. *MiR-125b*, which decreases VDR expression in MCF-7 breast cancer cells [93], has also been shown to be overexpressed in CRC metastasis [94] which might result in VDR downregulation and resistance to $1,25(OH)_2D_3$ antitumor action.

It is worth noting that mutations commonly found in CRC patients (e.g., *APC*, *TP53*, *KRAS*, *PIK3CA*) can influence or modulate VDR activity and $1,25(OH)_2D_3$ responses.

Thus, VDR overexpression in colorectal tumors is independently associated with *PIK3CA* and *KRAS* gene mutations, which supports a potential interaction between the VDES and RAS-MAPK and PI3K-AKT pathways [95]. Moreover, Maruyama and colleagues have shown that p53 as well as several other p53 family members induce VDR expression in CRC cell lines and potentiate VDR target gene expression in a $1,25(OH)_2D_3$-dependent manner. Reciprocally, ectopic expression of VDR in HCT116 CRC cells resulted in induction of several genes known to be p53 targets and in suppression of cell growth [96]. Supporting this crosstalk, Stambolsky and colleagues showed that mutated p53 can interact functionally and physically with VDR at VDREs and regulate the expression of $1,25(OH)_2D_3$ target genes. Furthermore, mutant p53 increases the nuclear accumulation of VDR and alters some of its antitumor activities (e.g., proapoptotic effects), which suggests that p53 status can determine the biological impact of $1,25(OH)_2D_3$ on carcinoma cells [97]. Recently, Wang and colleagues have reported a positive correlation between VDR and the homeobox transcription factor CDX2 in CRC cell lines and have shown that low VDR and CDX2 expression associates with higher sensitivity to adjuvant chemotherapy (cisplatin, docetaxel) and to BRAF and PI3K-mTOR inhibitors [98]. Therefore, data available suggest that the mutational status of CRC patients will determine their response to therapy or chemoprevention with vitamin D compounds.

Some natural products have proven effective in overcoming VDR downregulation in CRC. Thus, silibinin, a flavonolignan that inhibits tumor necrosis factor-α (TNF-α)-induced upregulation of SNAIL1 and 2 in HT-29 cells, increases VDR expression and restores $1,25(OH)_2D_3$ antitumor action [87]. Likewise, 17β-estradiol and several phytoestrogens induce VDR expression in CRC cells and animal models, increasing $1,25(OH)_2D_3$ responsiveness [99-102]. Moreover, 17β-estradiol-based postmenopausal hormone replacement therapy aimed at raising serum estradiol to premenopausal levels results in upregulation of VDR and E-cadherin, a downstream target of $1,25(OH)_2D_3$ action, in the human rectal mucosa [103]. Finally, the short-chain fatty acid butyrate and its prodrug tributyrin induce the expression of VDR in human CRC cells and subsequently promotes differentiation and cell cycle arrest in response to $1,25(OH)_2D_3$ [104, 105].

Expression of vitamin D hydroxylases in colorectal cancer

Besides VDR expression, the level of $1,25(OH)_2D_3$ within the cell will also determine the response of CRC cells to its antitumor action. Intracellular concentration of $1,25(OH)_2D_3$ depends on circulating levels of $25(OH)D_3$ and $1,25(OH)_2D_3$, but also on the net balance

between its synthesis and degradation inside the cell due to the activity of CYP27B1 and CYP24A1 hydroxylases. Both *CYP27B1* and *CYP24A1* genes can be dysregulated in cancer, although low responsiveness to vitamin D compounds is most commonly associated to upregulation of *CYP24A1*. CYP24A1 is expressed at low levels in healthy colon mucosa but overexpressed in colorectal tumors [106-109]. Its upregulation correlates with an increased expression of the proliferation marker Ki-67 and pre-replication complex proteins CDC6, MCM2, 4 and 7 [108, 109]. Moreover, xenografts generated in mice by the injection of HT-29 CRC cells overexpressing CYP24A1 grow faster and are more aggressive than those generated by control cells [110]. All these data suggest that overexpression of CYP24A1 confers proliferative advantages to colon carcinoma cells through the reduction of $1,25(OH)_2D_3$ intracellular levels.

Höbaus and colleagues have studied the mechanism responsible for CYP24A1 overexpression in CRC and have shown that 60% of tumors show increased *CYP24A1* gene copy-number and that more than 6 copies of the gene correlate positively with *CYP24A1* RNA expression suggesting a causal relationship. They also investigated but discarded other possible mechanisms such as *CYP24A1* promoter methylation and VDR or RXR upregulation [109]. Chronic inflammation may also result in increased expression of CYP24A1. Chen and colleagues have recently reported that inflammatory factors such as interleukin (IL)-6 and TNF-α may induce CYP24A1 expression in CRC cell lines via nuclear factor κB (NF-κB) pathway activation, which in turn triggers activation of the Wnt/β-catenin pathway [111]. In contrast, Lin and colleagues have found that the expression of CYP24A1 is inhibited by *miR-1278* in CRC [112], which opens the possibility to increase *miR-1278* expression to sensitize colon carcinoma cells to vitamin D compounds. Since augmented expression of CYP24A1 in CRC cells probably leads to depletion of intracellular $1,25(OH)_2D_3$ and therefore to the abolishment of its antitumor actions, combination therapy of vitamin D compounds with CYP24A1 inhibitors is worth exploring in CYP24A1-overexpressing tumors [113]. In this regard, Höbaus and colleagues showed that $1,25(OH)_2D_3$ reduces proliferation of CRC cells overexpressing CYP24A1 only in the presence of the CYP24A1 inhibitor VID400 [110].

Expression of CYP27B1 in CRC parallels that of VDR. Some authors have shown that CYP27B1 levels increase in well to medium-differentiated tumors when compared to normal mucosa, but its expression decreases dramatically or is lost in advanced, high grade, undifferentiated carcinomas [78, 106, 107, 114]. In slight contrast, Matusiak and colleagues have reported that CYP27B1 is present at equally high levels in normal colon

epithelium as in aberrant crypt foci, polyps, and CRC irrespective of tumor cell differentiation. However, its expression as well as that of VDR is negligible in CRC cells metastasizing to regional lymph nodes [79]. Recently, Sadeghi and colleagues have shown an increase in *CYP27B1* RNA levels in CRC samples compared to those of adjacent normal tissue [115]. Altogether, these data suggest that the upregulation of CYP27B1 in precancerous lesions and early CRC and, therefore, the associated increase in $1,25(OH)_2D_3$ production, might be an autocrine/paracrine mechanism to prevent intestinal tumor formation and progression that is lost in advanced and metastatic CRC [78, 107, 114].

In summary, reduced expression of CYP27B1 and VDR and increased expression of CYP24A1 in colon carcinoma cells along CRC progression and particularly in advanced stages result in a net decrease in intracellular $1,25(OH)_2D_3$ levels and in partial or total attenuation of its antitumor effects. Interestingly, similar to VDR downregulation, there are strategies to try to overcome this situation. Thus, 17β-estradiol and the phytoestrogen genistein induce CYP27B1 and reduce CYP24A1 activity in CRC cells and in the mouse colon [116, 117]. Moreover, genistein counteracts the increase in CYP24A1 expression promoted by low dietary calcium in mice [118]. Accordingly, calcium supplementation reduces CYP24A1 expression in the human rectal mucosa [119]. Therefore, these compounds can modulate the VDES and restore appropriate levels of $1,25(OH)_2D_3$ in CRC cells.

Mechanisms of $1,25(OH)_2D_3$ action in colorectal cancer

In 1981, two research groups reported the first evidences of the antitumoral effects of $1,25(OH)_2D_3$ on cultured cancer cells. Colston and colleagues showed that $1,25(OH)_2D_3$ inhibits melanoma cell proliferation [120], whereas Abe and collaborators found that it promotes the differentiation of mouse myeloid leukemia cells into macrophages [121]. Since then, numerous studies have demonstrated the anticancer effects of $1,25(OH)_2D_3$ in other tumor cell types, including CRC cells, and have uncovered new mechanisms of action of this hormone.

Inhibition of colon carcinoma cell proliferation

Inhibition of proliferation is possibly the most reported mechanism of $1,25(OH)_2D_3$ action in cancer cells (Fig. 2). In colorectal tumor cells, $1,25(OH)_2D_3$ induces cell cycle arrest by promoting transcription of the cyclin-dependent kinase (CDK) inhibitor p27 (encoded by the *CDKN1B* gene) via SP1 and NF-Y binding sites in the *CDKN1B* gene promoter that lacks a proper VDRE sequence. $1,25(OH)_2D_3$ stimulates binding of VDR to the SP1 transcription factor and subsequently the complex binds to SP1 consensus sequences in the *CDKN1B* promoter to trigger gene expression [122-124]. Additionally, Scaglione-Sewell and colleagues have reported that a fluorinated $1,25(OH)_2D_3$ analogue induces cell cycle arrest at G1 in Caco-2 CRC cells. This arrest is accompanied by an increase of CDK inhibitors p21 and p27, which resulted in a decreased activity of CDK2 and CDK6, whereas expression and phosphorylation of pRB is unaffected [125].

The *MYC* proto-oncogene is probably one of the most relevant targets of $1,25(OH)_2D_3$ in CRC cells. The importance of this gene for colorectal carcinogenesis is highlighted by the finding that nearly 100% of colorectal tumors have changes in MYC transcriptional targets [126]. MYC promotes cell cycle progression and thus CRC cell proliferation through at least three mechanisms: (i) the transcriptional activation of cyclins D2, A, and E; (ii) the repression of *CDKN2B* and *CDKN1A* genes encoding the CDK inhibitors p15 and p21, respectively; and (iii) the degradation of p27 cell cycle inhibitor [127, 128]. $1,25(OH)_2D_3$ was first shown to inhibit MYC expression in a promyelocytic leukemia cell line [129] and later to repress *MYC* transcription in RWPE-1 prostate epithelial cells by direct binding of VDR to two VDREs located in the gene promoter region [130]. Additionally, $1,25(OH)_2D_3$ inhibits MYC expression through a number of indirect mechanisms, of which antagonism of Wnt/β-catenin signaling is possibly the most relevant in CRC, given the importance of the pathway in this neoplasia. Related to this, $1,25(OH)_2D_3$ induces transcription of the long non-coding RNA maternally expressed gene (MEG) 3 which inhibits colon carcinoma cell proliferation and is commonly downregulated in CRC [131]. Induction of MEG3 results in a reduction in clusterin levels, but also in the ubiquitin-dependent degradation of MYC and thus inhibition of MYC target genes, including those involved in aerobic glycolysis (Warburg effect), a metabolic hallmark of tumor cells [131, 132].

$1,25(OH)_2D_3$ may also inhibit CRC cell proliferation by modulating key mitogenic pathways. One of them is the epidermal growth factor (EGF) pathway, particularly important in CRC where it constitutes a target for anti-EGF receptor (EGFR) therapies [133]. EGFR signaling rapidly induces elevation of MYC and cyclin D1 levels in colon

carcinoma cells, whereas $1,25(OH)_2D_3$ treatment inhibits EGFR expression and promotes EGF-induced EGFR internalization [134, 135]. Moreover, $1,25(OH)_2D_3$ reduces basal and EGF-stimulated expression of cyclin D1 [135]. $1,25(OH)_2D_3$ may also antagonize EGFR signaling indirectly through the induction of the cell-cell adhesion molecule E-cadherin [77], which is a negative regulator of EGFR [136, 137]. More recently, Dougherty and colleagues have shown that VDR suppresses EGFR/RAS signaling and inhibits colitis-associated tumorigenesis in mice models, whereas EGFR activation increases SNAIL1 and downregulates VDR in colon tumors [86]. Additionaly, $1,25(OH)_2D_3$ and its analogues EB1089 and CB1093 have been reported to antagonize the insulin growth factor (IGF) mitogenic pathway in CRC cells by inhibiting secretion of IGF-II and increasing the expression of IGF binding protein 6 [138], which is a negative regulator of IGF-II-induced proliferation [139].

Transforming growth factor (TGF)-β signaling during cancer progression is complex. In early stages of tumorigenesis, TGF-β inhibits cell proliferation, whereas in advanced stages it promotes EMT, dissemination, dormancy, and metastasis [140, 141]. Chen and colleagues have shown that $1,25(OH)_2D_3$ increases the amount of active TGF-β1 in CRC cells, sensitizing them to TGF-β1 growth inhibitory effects [142]. Therefore, this factor is a mediator of colon carcinoma cell growth inhibition by $1,25(OH)_2D_3$. In contrast, $1,25(OH)_2D_3$ has been reported to antagonize TGF-β1/2-induced migration, invasion, and expression of EMT-related transcription factors in SW480 and HT-29 CRC cells [143].

Our group has identified *miR-22* as a mediator of $1,25(OH)_2D_3$ antiproliferative activity in colon tumor cells [144]. Interestingly, *miR-22* can inhibit SP1-mediated activation of the PTEN/AKT pathway [145], suggesting that antagonizing AKT signaling might be another mechanism of growth suppression by $1,25(OH)_2D_3$. In addition, Zhu and colleagues have reported N-acetyltransferase (NAT) 2 as a new target of $1,25(OH)_2D_3$ that may contribute to its antiproliferative effects [146]. NAT2 is downregulated in CRC patients and low expression of NAT2 is correlated with high metastatic risk and poor survival. Moreover, NAT2 suppresses proliferation and migration of CRC cells, possibly through the regulation of the JAK1/STAT3 signaling pathway [146]. Finally, García-Martínez and colleagues have recently described that $1,25(OH)_2D_3$ activates the epigenetic modifier SIRT1 which, in turn, is required for the inhibitory effect of $1,25(OH)_2D_3$ on CRC cell growth. Remarkably, they have also shown that SIRT1 activators may be used to exert an antiproliferative action in CRC cells unresponsive to $1,25(OH)_2D_3$ due to VDR downregulation [147]. In summary, the antiproliferative

activity of $1,25(OH)_2D_3$ in CRC is well-documented and a variety of underlying mechanisms have been demonstrated.

Promotion of colon carcinoma cell differentiation and sensitization to cell death

The inhibitory effect of $1,25(OH)_2D_3$ on CRC cell proliferation is frequently concomitant with a promotion of, at least, partial epithelial differentiation [77, 148-153] (Fig. 2). Our group and others have demonstrated that $1,25(OH)_2D_3$ promotes differentiation of colorectal carcinoma cells by inducing the expression of proteins involved in cell-cell adhesion such as occludin, zonula occludens (ZO)-1 and 2, claudins 1, 2, 7 and 12, and E-cadherin [77, 152, 154, 155]. Of those, induction of the invasion suppressor E-cadherin is probably one of the most relevant. We have shown that $1,25(OH)_2D_3$ increases the expression of E-cadherin in VDR-positive SW480-ADH cells through a rapid mechanism that requires VDR and calcium and involves activation of RhoA, ROCK1, p38MAPK and MSK1 and finally leads to the induction of E-cadherin transcription [77, 152]. E-cadherin downregulation by EMT transcription factors (SNAIL, ZEB, etc.) is necessary for epithelial cell dedifferentiation and acquisition of mesenchymal features, so by inducing E-cadherin expression $1,25(OH)_2D_3$ is opposing EMT and favoring a more differentiated phenotype [153, 156]. Additionally, $1,25(OH)_2D_3$ can also antagonize EMT in CRC cells by (i) inducing the expression of KDM6B, a histone H3 lysine 27 demethylase that indirectly downregulates SNAIL1, ZEB1, and ZEB2; (ii) increasing the expression of cystatin D, an inhibitor of cysteine proteases of the cathepsin family that represses SNAIL1, SNAIL2, ZEB1, and ZEB2 and upregulates E-cadherin, occludin, and p120-catenin; and (iii) inhibiting the expression of Sprouty-2, an intracellular modulator of growth factor tyrosine kinase receptor signaling that increases ZEB1 expression [156]. Likewise, $1,25(OH)_2D_3$ treatment or VDR overexpression interferes TGF-β induction of EMT in colon carcinoma cells, while VDR knock-down potentiates it [143, 157].

Some authors have reported the occurrence of apoptosis subsequently to the induction of differentiation by $1,25(OH)_2D_3$, which suggests a possible link between both processes [150]. $1,25(OH)_2D_3$ and some of its analogues have been shown to promote apoptosis of colon carcinoma cells through several mechanisms: i) increasing the levels of the proapoptotic protein BAK1 [150]; ii) reducing the nuclear levels of the antiapoptotic protein BAG-1 [158]; iii) promoting the proteolytic cleavage of poly(ADP-ribose) polymerase (PARP) suggesting a possible activation of the ICE/CED-3 proteolytic pathway [159]; and iv) inducing the expression of the G0-G1 switch gene 2 (G0S2),

which encodes a mitochondrial protein that specifically interacts with BCL-2 and promotes apoptosis by preventing the formation of protective BCL-2/BAX heterodimers [151, 160]. Interestingly, $1,25(OH)_2D_3$ and some of its analogues have been shown to sensitize CRC cells to chemotherapy-induced cell death [161-163], which opens the possibility to combination therapies.

It is worth noting that the *TP53* tumor suppressor gene, which is mutated in approximately 50% of cancers, including CRC, is a key player in the control of apoptosis [164]. This is relevant since as mentioned above, mutated p53 can interact with VDR and reverse the proapoptotic effects of $1,25(OH)_2D_3$ [97], suggesting that tumors with mutated p53 might escape $1,25(OH)_2D_3$-mediated apoptosis. However, some authors have reported proapoptotic actions of $1,25(OH)_2D_3$ and its analogues which are p53-independent [150, 165].

More recently, $1,25(OH)_2D_3$ has also been shown to induce autophagy-dependent cell death as a protective mechanism against tumor progression, although evidences in CRC are still scarce [166]. In line with this, Abu El Maaty and colleagues have shown that combined treatment with $1,25(OH)_2D_3$ and metformin promotes apoptosis in CRC cell lines expressing mutant p53, whereas it induces autophagy through the AMPK-mTOR-dependent pathway in p53 wt cells [167]. These data as well as those in other cancer cell types should encourage further research on the role of autophagy in the antitumor effects of $1,25(OH)_2D_3$.

Antagonism of Wnt/β-catenin signaling

The Wnt/β-catenin pathway is essential for the maintenance of intestinal homeostasis. However, its aberrant activation is frequently observed in CRC [168, 169]. In fact, the Cancer Genome Atlas Network has reported that over 94% colorectal tumors have a mutation in one or more members of the Wnt signaling pathway, predominantly in the *APC* gene, which encodes a negative regulator of β-catenin [126]. The APC protein, together with the tumor suppressor AXIN and the Ser/Thr kinases casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK3β) are part of a multiprotein complex known as the β-catenin destruction complex because it phosphorylates β-catenin promoting its ubiquitination and subsequent degradation by the proteasome. Wnt/β-catenin signaling is triggered by binding of Wnt factors to heterodimeric transmembrane receptors composed of a member of the Frizzled family and LRP5 or 6. Wnt binding results in inactivation of the β-catenin destruction complex which leads to the accumulation of β-catenin in the

cytoplasm, a part of which enters the nucleus and behaves as a coactivator for the T-cell factor (TCF) transcription factor family. The complex β-catenin/TCF regulates the expression of many genes involved in several cellular processes including cell proliferation, differentiation, survival, and migration (e.g., *MYC*, *CCND1*) [169, 170].

Our group has shown that $1,25(OH)_2D_3$ can antagonize Wnt/β-catenin signaling in CRC cells through at least three mechanisms (Fig. 2). First, $1,25(OH)_2D_3$ potentiates a direct physical interaction between VDR and β-catenin which hampers β-catenin binding to TCF and thus β-catenin/TCF transcription of target genes [77]. This interaction involves the C-terminal region of β-catenin and the C-terminal activation function-2 domain of VDR [171] and is potentiated by wt APC [172]. Second, $1,25(OH)_2D_3$ induces E-cadherin expression which leads to a redistribution of β-catenin from the nucleus to the cell membrane adherent junctions where it binds the cytoplasmic tail of E-cadherin. The reduction in β-catenin nuclear levels results in a decreased β-catenin/TCF transcriptional activity [77]. And third, $1,25(OH)_2D_3$ regulates the expression of the extracellular Wnt inhibitors Dickkopf (DKK) 1 and DKK4, which bind LRP5 and 6 and inhibit Wnt/β-catenin signaling [173-175]. Specifically, $1,25(OH)_2D_3$ induces DKK1 expression in colon carcinoma cells and there is a positive correlation between *VDR* and *DKK1* RNA levels in CRC human biopsies [175]. Since most colorectal tumors have intracellular activating mutations in components of the Wnt/β-catenin pathway (mainly APC, but also β-catenin or AXIN), the relevance of DKK1 upregulation, which acts at receptor level, might seem dubious. However, Voloshanenko and colleagues have shown that extracellular Wnt proteins can reinforce β-catenin nuclear accumulation even in cells with an intracellularly activated pathway [176], which suggests that DKK1 augmented levels might antagonize autocrine or paracrine Wnt stimulation. Moreover, DKK1 upregulation is likely to be relevant in a subset of colorectal tumors which are Wnt ligand-dependent because they present alterations in components of the R-Spondin (RSPO) pathway (RNF43 mutations, RSPO2 and 3 fusions) which modulates the intensity and duration of Wnt activation by controlling Frizzled ubiquitination and degradation [177, 178]. Additionally, we and others have proposed antitumoral effects of DKK1 that are independent of Wnt/β-catenin pathway inhibition [179-184]. Thus, our group has shown that a proportion of DKK1 is located in the nucleus of CRC cells, where it is involved in the transcription of genes related to detoxification of chemotherapeutic drugs [180]. Interestingly, DKK1 expression is lost during colorectal tumor progression [180, 185] in part due to promoter hypermethylation in advanced stages [179, 186-188]. However,

around 15% of CRC patients present high levels of nuclear DKK1 associated with reduced PFS after chemotherapy and shortened OS [180].

In contrast to DKK1, expression of DKK4 is inhibited by $1,25(OH)_2D_3$ in CRC cells [174]. Moreover, we and others have shown that DKK4 levels are increased in CRC and in inflammatory bowel disease (IBD) [173, 174, 189-191] and there is an inverse correlation between *VDR* and *DKK4* RNA levels in CRC human biopsies [174]. Overexpression of DKK4 in CRC cell lines enhances its migratory, invasive, and proangiogenic capacities [174, 192] and induces chemotherapy resistance to 5-fluorouracil and to the VEGF receptor inhibitor YN968D1, but not to irinotecan or oxaliplatin [192, 193]. Additionally, Ebert and colleagues have reported that DKK4 mediates CRC chemotherapy resistance induced by silencing of the transcription factor AP-2 epsilon [193, 194]. Therefore, downregulation of DKK4, as well as induction of DKK1, may contribute to the antitumor actions of $1,25(OH)_2D_3$ in CRC, and targeting DKK4 may be an option to overcome drug resistance.

Other authors have proposed additional mechanisms for $1,25(OH)_2D_3$ antagonism of Wnt/β-catenin signaling in CRC. Beildeck and colleagues have shown that $1,25(OH)_2D_3$ induces the expression of TCF-4 [195], which in the absence of β-catenin behaves as a transcriptional repressor that restricts CRC cell growth [196]. Jin and colleagues have reported that $1,25(OH)_2D_3$ induces *AXIN1* gene expression in HCT116 CRC cells, whereas its levels are reduced in a conditional knock-out mouse model ($VDR^{\Delta IEC}$) that lacks VDR expression in the gut epithelium [197]. Upregulation of AXIN1 promotes β-catenin degradation and inhibition of Wnt/β-catenin signaling. Interestingly, Kaler and colleagues have shown that colon tumor cells can induce the release of IL-1β from stromal macrophages, which subsequently induces inhibition of GSK3β, β-catenin stabilization and β-catenin/TCF transcriptional activation in the tumor cells [198]. Additionally, IL-1β stabilizes SNAIL1 in a NF-κB/Wnt-dependent manner, which protects tumor cells from TRAIL-induced apoptosis [199]. $1,25(OH)_2D_3$ blocks this crosstalk tumor-stroma by inhibiting macrophage IL-1β synthesis, which hampers activation of Wnt/β-catenin signaling in the tumor cells and sensitizes them to TRAIL-induced cell death [198]. Finally, Meyer and colleagues have studied the overlap between VDR/RXR and TCF4/β-catenin cistromes in a CRC cell line and have shown that both heterodimers colocalize at 74 sites near a limited set of genes that included *FOS* and *MYC*, suggesting a transcriptional antagonism between both complexes at certain gene loci [200].

Further supporting the antagonism exerted by $1,25(OH)_2D_3$ on this pathway, germline deletion of VDR in the APC^{min} CRC mouse model, which harbors constitutively active Wnt/β-catenin signaling, results in increased intestinal tumor burden accompanied by enhanced tumor β-catenin nuclear levels and elevated expression of its targets genes [201, 202]. In summary, antagonizing Wnt/β-catenin signaling is an important mechanism of tumor protection by $1,25(OH)_2D_3$ in CRC and represents an attractive target for therapeutic intervention.

Role in inflammation, immunomodulation, and angiogenesis

Chronic inflammation predisposes to cancer and, specifically, chronic IBD is associated to increased risk of CRC [203, 204]. One of the best studied actions of the VDES is its immunomodulatory activity and, in particular, its potent anti-inflammatory effects. Consequently, vitamin D deficiency has been associated with IBD [205, 206]. The anti-inflammatory actions of $1,25(OH)_2D_3$ in CRC include: (i) inhibition of NF-κB, (ii) inhibition of cyclooxygenase-2 (COX-2), and (iii) modulation of the expression of several cytokines (Fig. 2).

The NF-κB transcription factors are a family of five different DNA-binding proteins that form a variety of homodimers and heterodimers [207]. They are key regulators of innate and adaptive immune responses and can accelerate cell proliferation, inhibit apoptosis, promote cell migration and invasion, and stimulate angiogenesis and metastasis [208]. The classical NF-κB pathway mainly involves p50/p65 (encoded by *NFKB1* and *RELA* genes, respectively) heterodimers and is activated by pro-inflammatory cytokines. The activation of NF-κB depends on the phosphorylation and subsequent degradation of its specific inhibitors (IκB) in the cytoplasm, which allows NF-κB to translocate into the nucleus and stimulate transcription of pro-inflammatory cytokines (e.g., TNF-α, IL-1, IL-6) and enzymes (e.g., COX-2), matrix metalloproteinases (e.g., MMP9), etc. [208]. The inhibitory effect of $1,25(OH)_2D_3$ on NF-κB was first reported by Yu and collaborators in human lymphocytes [209]. Later on, Sun and colleagues showed that $1,25(OH)_2D_3$ and lithocholic acid, which also binds VDR, inhibit the activation of NF-κB in CRC cells by increasing the expression of IκBα and preventing IL-1β-mediated phosphorylation and activation of p65 [210]. Moreover, Chen and colleagues have demonstrated that $1,25(OH)_2D_3$ enhances the direct interaction between VDR and the IκB kinase β (IKKβ), which abolishes IKKβ activity to phosphorylate IκBα. Consequently, stabilization of IκBα inhibits p50/p65 nuclear translocation [211]. In line with this, the VDR antagonist

ZK191732 upregulates NF-κB basal activity in CRC cells by decreasing IκBα levels [212], and *in vivo* experiments showed that an enriched vitamin D diet decreases NF-κB activation in the colonic epithelial cells of a mouse model of bacteria-driven colitis and CRC [213].

COX-2 (also called prostaglandin-endoperoxide synthase 2) converts arachidonic acid to prostaglandins (PG). It is overexpressed in most CRCs [214, 215], and the biological effects of upregulating this enzyme are mediated predominantly through increased PGE_2 production [216, 217]. Accordingly, epidemiological evidence has suggested that aspirin and other nonsteroidal anti-inflammatory drugs (NSAID) that inhibit COX-2 may reduce the risk of CRC [216, 217]. $1,25(OH)_2D_3$ and its analogue Ro26-2198 decrease COX-2 expression in chemically-induced CRC mouse models [218, 219]. Additionally, Fichera and colleagues also showed that Ro26-2198 inhibits IL-1β-induced COX-2 increase in CRC cells [218]. Moreover, the ratio COX-2/15-hydroxyprostaglandin dehydrogenase (15-HPGD), an enzyme responsible for PG inactivation, is reduced in the normal rectal mucosa of colorectal adenoma patients after 1-year supplementation with vitamin D_3 [220].

$1,25(OH)_2D_3$ has also been shown to decrease the expression of pro-inflammatory cytokines such as IL-6, IL-8, IL-1β or TNF-α, which are overexpressed in CRC [221-223], at least in part through inhibiting NF-κB, as described previously. Interestingly, a recent study by Wesselink and colleagues reported that higher circulating 25(OH)D levels are associated with lower plasma IL-6 concentration at CRC diagnosis [224], which may be relevant as IL-6 plays an important role in chronic inflammation and thus in cancer progression. It is worth noting that some authors have suggested that low 25(OH)D levels are a consequence of chronic inflammation rather than its cause [225]. Supporting this, Hummel and colleagues showed that TNF-α and IL-6 inhibit the expression of CYP27B1 in CRC cells and consequently the synthesis of $1,25(OH)_2D_3$ [226]. Therefore, these data suggest that there is a reciprocal inhibition between the VDES and inflammation.

The effects of $1,25(OH)_2D_3$ on angiogenesis are linked to its capacity to inhibit the hypoxia inducible factor 1α (HIF-1α), which is a mediator of angiogenesis. $1,25(OH)_2D_3$ reduces HIF-1α protein expression in several cancer cell lines, including CRC cells [227]. Likewise, in NCM460 colon epithelial cells and in colitis experimental mouse models, $1,25(OH)_2D_3$ suppresses HIF-1α overexpression by inhibiting NF-κB signaling [228]. The effect of $1,25(OH)_2D_3$ on the expression of vascular endothelial growth factor (VEGF) is contradictory. Ben-Shoshan and colleagues have shown that $1,25(OH)_2D_3$

inhibits hypoxia-induced VEGF expression in CRC cells through a HIF-dependent pathway [227] whereas other authors have reported that $1,25(OH)_2D_3$ increases VEGF expression in colon carcinoma cells, osteoblasts, and vascular smooth muscle cells [229-231], possibly through two VDREs in the *VEGF* promoter [231]. $1,25(OH)_2D_3$ has also been shown to induce the expression of thrombospondin 1, one of the major inhibitors of angiogenesis, in CRC cancer cells [229] and, as mentioned before, we have also found that $1,25(OH)_2D_3$ represses the expression of the extracellular Wnt inhibitor DKK4, that promotes angiogenesis in colon carcinoma cells [174].

Regulation of gut microbiome and detoxification

There is strong evidence suggesting that gut microbiome imbalance, called dysbiosis, can promote CRC [232-234]. It has also been shown that the VDES regulates the composition of the gastrointestinal microbiome and that this interaction with microbiota is relevant for maintenance of immune homeostasis [235-237]. In this regard, knock-out mice for either CYP27B1 or VDR present dysbiosis with lower expression of E-cadherin on gut epithelial and immune cells and fewer tolerogenic dendritic cells that results in gut inflammation. The effects on CYP27B1 knock-out mice can be reversed by $1,25(OH)_2D_3$ treatment, suggesting that vitamin D or VDR deficiency results in dysbiosis [238] (Fig. 2). Likewise, a randomized, placebo-controlled, double-blind study in a cohort of vitamin D-deficient overweight and obese but otherwise healthy individuals has demonstrated an effect of vitamin D_3 supplementation on fecal microbiota composition [239].

Using a model of chemically-induced CRC in a $VDR^{\Delta IEC}$ mice backgound, Zhang and colleagues have recently shown that VDR deletion leads to a bacterial profile shift from normal to higher risk of CRC [240]. Furthermore, fecal samples from $VDR^{\Delta IEC}$ mice enhance the expression of STAT3 in human and mice organoids. This effect is mediated by activation of the JAK2 protein kinase and is abolished by an inhibitor of the JAK/STAT pathway [240], suggesting that this pathway may be involved in vitamin D deficiency-induced dysbiosis. Interestingly, treatment of HCT116 CRC cells with conditioned medium from probiotic lactic acid bacteria induces the expression of VDR [241], which suggest that increasing the levels of VDR can, at least in part, mediate the protective effects of probiotics on IBD and CRC.

The intestinal mucosa is frequently exposed to external stimuli including those from food, xenobiotics, and commensal microorganisms that can contribute to inflammation and cancer [242]. These compounds are metabolized by a large number of detoxifying

enzymes, many of them belonging to the cytochrome P450 family [243]. $1,25(OH)_2D_3$ induces the expression of some of these enzymes, including CYP3A4 and SULT2A1, and also that of members of the multi-drug resistance-associated protein (MRP) family of drug efflux pumps [244-247], thus contributing to detoxification (Fig. 2).

Bile acid synthesis occurs mainly in the liver yielding primary bile acids that are transported to bile and then secreted into the gut where they are essential for fat metabolism. In the colon, a fraction of primary bile acids is converted to secondary bile acids by gut microbiota. A high-fat diet promotes the synthesis of bile acids increasing their delivery to the colonic lumen and numerous reports have associated bile acids, especially secondary bile acids, with CRC incidence [248-251]. High concentrations of bile acids can generate reactive oxygen and nitrogen species, induce cell membrane and DNA damage, and promote apoptosis in the short term, but apoptosis resistance in the long term. All these processes are likely related to carcinogenesis [248]. Several nuclear receptors, including VDR, act as sensors for bile acids and play an important role in protecting against their carcinogenic effects by activating transcriptional programs aimed at coordinating intestinal epithelium bile acid uptake, detoxification, and basolateral secretion [248]. Accordingly, mice lacking Vdr expression in the gut show increased levels of secondary bile acids [250] and in humans there is an inverse correlation between circulating 25(OH)D levels and fecal primary bile acid concentration [252]. Makishima and colleagues showed that VDR functions as a receptor for lithocholic acid (a toxic secondary bile acid) and its metabolites with higher sensitivity than other nuclear receptors [253]. Interestingly, VDR target genes *CYP3A4*, *SULT2A1* and *MRP3* are involved in the elimination of lithocholic acid [246, 247, 253, 254], and since this bile acid is a VDR ligand [253, 254], the induction of these detoxifying proteins constitutes an inhibitory feedback mechanism through which VDR reduces the levels of its agonist by promoting its elimination.

Modulation of colorectal cancer-associated fibroblasts

The tumor microenvironment includes diverse immune cell types, CAFs, endothelial cells, pericytes, and various additional heterogeneous tissue-resident cells. These host cells were once considered bystanders of tumorigenesis but are now known to play critical roles in the pathogenesis of cancer [255, 256]. CAFs, a key component of the tumor microenvironment, exert diverse actions, including matrix deposition and remodeling, and have extensive reciprocal signaling interactions with cancer cells and infiltrating

leukocytes. For that reason, they represent a potential target for optimizing therapeutic strategies against cancer [257].

Our group studied the effects of $1,25(OH)_2D_3$ on colon normal fibroblasts (NFs) and CAFs and showed that $1,25(OH)_2D_3$ antagonizes the protumoral activity of CAFs through at least two mechanisms: i) by inhibiting their capacity to alter the extracellular matrix; and ii) by hampering their ability to promote migration of colon carcinoma cells [74] (Fig. 2). We also found that $1,25(OH)_2D_3$ regulates the expression of 958 genes in NFs and 1,489 genes in CAFs, including some that encode proteins involved in cell adhesion and migration, extracellular matrix organization, wound healing, blood vessel development and tissue remodelling. Interestingly, there is only a 21% overlap between both sets of genes, suggesting that $1,25(OH)_2D_3$ modulates common but also specific gene expression programmes in colon NFs and CAFs. Moreover, we defined a $1,25(OH)_2D_3$-associated signature with those genes most differentially regulated by $1,25(OH)_2D_3$ in CAFs that proved to be associated with a better clinical behavior of CRC patients [74]. Thus, our work reveals that the antitumor action of $1,25(OH)_2D_3$ in CRC is not exclusively mediated by its effects on colon carcinoma cells, but also by the inhibition of the protumoral properties of CRC-associated fibroblasts.

In line with our findings, Yu and colleagues have shown that $1,25(OH)_2D_3$ treatment or VDR overexpression inhibits TGF-β-induced activation (measured as α-SMA, fibronectin, collagen I, and other fibrosis marker expression) of primary human colon fibroblasts and CCD-18Co human colon myofibroblasts, whereas VDR knock-down has the opposite effect [157]. Similarly, Tao and collaborators found that $1,25(OH)_2D_3$ reduces the activation of mouse colon subepithelial myofibroblasts promoted by TGF-β [258] .

Our group also studied the crosstalk between Wnt signaling and $1,25(OH)_2D_3$ in colon myofibroblasts. Both Wnt3A and $1,25(OH)_2D_3$ reduce the proliferation and migration capacity of CCD-18Co myofibroblasts and the combined treatment have an additive inhibitory effect on proliferation but not on migration [259]. Likewise, $1,25(OH)_2D_3$ increases VDR expression and reduces migration of Crohn's disease fibroblasts [260]. In contrast, $1,25(OH)_2D_3$ inhibits the remodeling of the extracellular matrix induced by Wnt3A [259]. Additionally, global transcriptomic analyses showed that most genes regulated by the single treatment with $1,25(OH)_2D_3$ (74%; 2,329/3,129) or Wnt3A (55%; 994/1,794) in CCD-18Co myofibroblasts are unshared, while 800 genes are common targets, suggesting that the gene regulatory action of both agents is mainly exclusive. Of

the common targets, 55% are up or downregulated by both agents whereas 45% are regulated in opposite directions, which is consistent with the common and opposite effects exerted by $1,25(OH)_2D_3$ and Wnt3A on CCD-18Co proliferation, migration, and extracellular matrix remodeling capacity. Moreover, the results obtained with the combined treatment reveal a predominantly additive effect of $1,25(OH)_2D_3$ and Wnt3A on CCD-18Co gene expression [259]. As previously commented, $1,25(OH)_2D_3$ antagonizes Wnt/β-catenin pathway in colon carcinoma cells [261, 262]. However, our data in CCD-18Co myofibroblasts show that $1,25(OH)_2D_3$ does not globally change the expression of Wnt3A target genes, although it significantly reduces the effect of Wnt3A on 23% (410/1,794) of them, including the known Wnt targets *CCND1*, *DKK1*, *MMP14*, *TNFRSF19*, and *CYR61*. In addition, $1,25(OH)_2D_3$ induces the expression of the Wnt inhibitors NKD1, NKD2 and APCDD1 [259]. In summary, Wnt3A and $1,25(OH)_2D_3$ have profound, mostly additive, and partially overlapping effects on the gene expression profile and phenotype of human colon myofibroblasts.

Regulation of colorectal cancer stem cells

Cancer stem cells (CSCs) are a small subpopulation of cells within tumors that show self-renewal and pluripotency and are capable of initiating and sustaining tumor growth [263-265] . Colon CSCs arise as a consequence of malignant transformation of normal intestinal stem cells (SCs) located in the lower part of colonic crypts and have been shown to play a crucial role in CRC initiation, progression, and chemotherapy resistance [266-270].

Our group has used patient-derived organoids to study the effect of $1,25(OH)_2D_3$ on colorectal normal SCs and CSCs. Organoids are 3D self-organized multicellular structures generated by SCs, embedded in an extracellular matrix, and grown in specific niche-like medium. They have long-term proliferation and differentiation capacities and recapitulate several features of the tissue or tumor of origin [271] We have shown that $1,25(OH)_2D_3$ has a strong and differential effect on gene expression and phenotype in patient-derived colorectal normal and tumor organoids (Fig. 2). In normal organoids, $1,25(OH)_2D_3$ induces stemness-related genes (e.g., *LGR5*, *SMOC2*, *MSI1*, *LRIG1*, etc.), some of them through direct VDR binding to their regulatory regions (*SMOC2*, *MSI1*), and inhibits cell proliferation, suggesting a role of $1,25(OH)_2D_3$ in the maintenance and renewal of the colonic epithelium [75, 272]. In line with our results, Peregrina and collaborators had previously found that SC properties are compromised in the small

intestine and colon of mice fed with a low vitamin D_3 and calcium diet or harboring VDR deletion in intestinal SCs [273]. However, in striking contrast, Sittipo and colleagues have recently reported that $1,25(OH)_2D_3$ inhibits stemness and promotes differentiation and apoptosis in mouse small intestine organoids [274]. The reasons for this discrepancy are unclear.

Regarding tumor organoids, we have found that $1,25(OH)_2D_3$ inhibits the expression of cell proliferation and tumorigenesis genes (e.g., *ALDH3A1*, *TNS4*, *S100P*, etc.) and variably reduces their proliferation [75, 272]. Moreover, Gene Set Enrichment Analysis (GSEA) confirms the inverse correlation between the gene expression profile imposed by $1,25(OH)_2D_3$ in organoids and several proliferative signatures (e.g., E2F, mTOR, MYC), and unveils a direct correlation with a differentiation signature only in tumor organoids, which was confirmed by electron microscopy ultrastructural studies [75]. These results support an antitumor activity of $1,25(OH)_2D_3$ on CRC by regulating CSCs.

In a similar study, Li and colleagues have studied $1,25(OH)_2D_3$ effects on genome-wide gene expression and chromatin accessibility in human colon normal organoids. A number of genes such as *CYP24A1, FGF19, MYC, FOS*, and *TGFBR2* show significant transcriptional and chromatin accessibility responses to $1,25(OH)_2D_3$ treatment with accessible chromatin located distant from the promoters in some cases [275]. More recently, Vaughan-Shaw and colleagues have also performed a whole genome expression analysis of CRC patient-derived tumor organoids after $1,25(OH)_2D_3$ treatment and have found an enrichment in genes involved in several cellular processes, including negative regulation of cell proliferation, and regulation of cell migration and differentiation [276]. Interestingly, these last two studies show a substantial concordance with our data [75].

Human intestinal organoids have also been used to confirm *ex vivo* the regulation of $1,25(OH)_2D_3$ target genes previously identified in cell lines or animal models [277, 278]. Likewise, mouse intestinal organoids have been employed as a model system to study VDES actions in the intestine [279, 280].

Human clinical trials of vitamin D_3 supplementation

As we stated previously, epidemiological studies provide consistent evidence towards an inverse association between 25(OH)D serum levels and CRC risk and mortality. Indeed, the antitumor activity of $1,25(OH)_2D_3$ against CRC is supported by strong biological

plausibility and abundant preclinical data. However, the evidence from randomized controlled trials (RCTs) remains inconsistent and inconclusive. In this section, we will review and examine the major human RCTs involving vitamin D_3 supplementation in three different scenarios: healthy individuals, colorectal adenoma high-risk population, and CRC patients.

Vitamin D_3 supplementation in healthy individuals

In order to determine whether the VDES is involved in the prevention of CRC, Wactawski-Wende and colleagues published in 2006 the results of a RCT involving a large series of participants (36,282 postmenopausal women aged 50 to 79 years) split to receive daily either 400 IU vitamin D_3 plus 1 g elemental calcium or placebo for an average of 7 years. The incidence of CRC did not differ significantly between groups [281]. Interestingly, Vaughan-Shaw and collaborators showed that supplementation with 3,200 IU/day vitamin D_3 for 12 weeks in 50 individuals promotes gene expression patterns consistent with antitumor effects in the rectal normal mucosa [282].

Regarding the VDES and global cancer risk, Lappe and colleagues examined the effect of 1,100 IU/day vitamin D_3 plus 1,500 mg calcium in a 4-year RCT in which overall cancer incidence was a secondary endpoint. 1,179 post-menopausal women aged older than 55 years were enrolled. Cancer incidence was found to be lower in the treatment group when compared to placebo controls (RR 0.40; 95% CI, 0.20 to 0.82; P=0.013), especially if tumors developed in the first 12 months were excluded (RR 0.23; 95% CI, 0.09 to 0.60; P<0.005). Additionally, both treatment and serum 25(OH)D concentrations were found to be significant independent predictors of cancer risk [283]. A decade later, Lappe and colleagues reported a similar RCT but with 2,303 participants, 2,000 IU/day vitamin D_3 and the incidence of all-type cancer as primary outcome. Intention-to-treat analysis showed that supplementation with vitamin D_3 plus calcium compared with placebo did not result in a significantly lower risk of all-type cancer at 4 years. However, in a post hoc analysis, in which participants who withdrew, died, or developed cancer in the first 12 months were excluded, the hazard ratio (HR) was 0.65 (95% CI, 0.42 to 0.99; P=0.03) [284].

Contrarily, two other mega trials reported no significant correlations between vitamin D_3 supplementation and overall cancer incidence and mortality. (i) The RECORD trial included 5,292 individuals aged at least 70 years (85% women) supplemented daily with vitamin D_3 (800 IU) and/or calcium (1,000 mg) for 24–62 months and was aimed at

preventing secondary fragility fractures [285]. The trial also prespecified a long-term follow-up for secondary outcomes of mortality such as cancer. Cancer mortality (HR 0.85; 95% CI, 0.68 to 1.06; P=0.157) and incidence (HR 1.07; 95% CI, 0.92 to 1.25; P=0.376) did not differ significantly between participants allocated vitamin D_3 and those with placebo. A post hoc statistical analysis adjusting for compliance showed an accentuated trend for reduced mortality in response to vitamin D_3 (HR 0.61; 95% CI, 0.37 to 1.30), although all results remained non-significant [286]. (ii) The Vitamin D Assessment (ViDA) trial recruited over 5,000 participants in New Zealand aged 50 to 84 years for a mean duration of 3.3 years to assess the effect of vitamin D_3 supplementation (initial bolus dose of 200,000 IU followed by monthly doses of 100,000 IU) on the incidence of cardiovascular disease [287]. A post hoc analysis focusing on cancer mortality as a primary outcome showed that vitamin D_3 did not modify incidence of all primary invasive and in situ malignant neoplasms (HR 1.01; 95% CI, 0.81 to 1.25; P=0.95), even after exclusion of cancer deaths registered in the first year after randomization (HR 0.95; 95% CI, 0.74 to 1.23; P=0.69) [288].

The largest RCT to date, the Vitamin D and Omega-3 Trial (VITAL), evaluated the impact of 2,000 IU/day vitamin D_3 on primary prevention of cancer, enrolling 25,871 participants without a history of cancer aged over 50 years from 44 centres in the USA for a mean duration of 5.3 years. This study failed to find any effect of vitamin D_3 in reducing total invasive cancer (HR 0.96; 95% CI, 0.88 to 1.06; P=0.47) or CRC (HR 1.09; 95% CI, 0.73 to 1.62) incidence, but found a non-significant trend of reduction in total cancer mortality in the vitamin D_3 group (HR 0.83; 95% CI, 0.67 to 1.02) [289]. In addition, a post hoc sub-analysis further suggested a benefit of vitamin D_3 supplementation in cancer mortality since a significant reduction was observed in the vitamin D_3 group upon excluding deaths occurring during the first 2 years of follow-up (HR 0.75; 95% CI, 0.59 to 0.96). With additional restriction of the analysis to cancer deaths, HR was even more reduced to 0.63 (95% CI, 0.43 to 0.92) [289]. Moreover, an updated analysis from the VITAL Research Group confirmed a significant effect on cancer mortality in vitamin D_3-supplemented individuals (HR 0.87; 95% CI, 0.79 to 0.96; P=0.005) [290]. Finally, Song and colleagues have recently shown that vitamin D_3 supplementation did not reduce the risk of colorectal adenomas and serrated polyps in a VITAL ancillary study during a follow-up period of 5.3 years. However, a stratified analysis indicated an interaction with baseline serum 25(OH)D levels, suggesting an inverse association of vitamin D_3 supplementation with the risk of conventional (OR 0.82;

95% CI, 0.6 to 1.13; P=0.07) or advanced (OR 0.60; 95% CI, 0.30 to 1.20; P=0.04) adenomas among individuals with 25(OH)D levels below 30 ng/ml, and thus a potential benefit that requires further investigation [291].

A similar RCT performed in Europe (DO-HEALTH) also showed potential benefits of vitamin D_3 supplementation in reducing cancer risk. The study evaluated the effects of daily 2,000 IU vitamin D_3 (and/or 1 g omega-3 fatty acids, and/or a simple home exercise programme, compared to placebo) in the emergence of any invasive cancer in 2,157 healthy adults aged 70 or older for a duration of 3 years. The authors observed a cumulative benefit in cancer risk reduction when combining two treatments, vitamin D_3 plus omega-3 (HR 0.53; 95% CI, 0.28 to 1.00; P=0.051), as well as for all three treatments combined (HR 0.39; 95% CI, 0.18 to 0.85; P=0.017) [292]. In contrast, no effect of vitamin D_3 supplementation on invasive cancer incidence or all-cause mortality was seen in the 5-year Finnish Vitamin D Trial, where daily 1,600 or 3,200 IU vitamin D_3 were tested against placebo in 2,495 participants aged older than 60 years. The authors postulate that the results might be related to the sufficient vitamin D status in most participants at baseline (mean baseline serum 25(OH)D concentration was 30 ng/ml) [293].

Although RCTs provide high-level evidence to establish causality, systematic reviews and meta-analysis are of great value in evaluating and synthesizing the data to reach broad generalizations across a large number of study outcomes and to give a more comprehensive picture [294]. Thus, we would like to bring up a few studies intended to resolve contradictory research outcomes regarding vitamin D_3 supplementation in cancer. A Cochrane systematic review evaluated 18 RCTs including 50,623 participants that received either vitamin D_3 or placebo/no treatment. Vitamin D_3 supplementation significantly reduced all-cause (RR 0.93; 95% CI, 0.88 to 0.98; P=0.009; 15 trials; 49,866 participants) and cancer-related (RR 0.88; 95%, CI 0.78 to 0.98; P=0.02; 4 trials; 44,492 participants; low quality evidence) mortality. However, no differences were seen in cancer incidence between vitamin D_3 and control interventions (RR 1.0; 95% CI, 0.94 to 1.06; P=0.88). Importantly, the authors remarked that all trials came from high-income countries, most trials had a high risk of bias, and the majority of the included participants did not have vitamin D deficiency [295]. Accordingly, Keum and Giovannucci conducted a brief meta-analysis including RCTs describing the effects of vitamin D_3 supplementation on cancer incidence and mortality and suggested that the benefit of vitamin D_3 was limited to cancer mortality [296]. This outcome was further confirmed in

an updated meta-analysis that incorporated new RCTs published in the following years to make a total of 10 trials (6,537 cases). The study showed that vitamin D_3 supplementation was associated with reduced total cancer mortality (13%) over a 3–10 years period of follow-up (RR 0.87; 95% CI, 0.79 to 0.96; P=0.005) [297]. In line with this, another remarkable systematic review and meta-analysis of 52 RCTs with a total of 75,454 participants found that, despite vitamin D_3 supplementation did not change all-cause mortality (RR 0.98; 95% CI, 0.95 to 1.02), it did reduce cancer specific mortality by 16% (RR 0.85; 95% CI, 0.74 to 0.97) [298]. Other meta-analysis of 30 RCTs suggested an inverse but non-significant association with cancer mortality (RR 0.88; 95% CI, 0.70 to 1.09; P=0.493) [299].

Vitamin D_3 supplementation in colorectal adenoma high-risk population

RCTs were also performed to study the effect of the VDES on individuals that had at least one colorectal adenoma removed and, thus, had a high risk of recurrence. Baron and colleagues found that supplementation with 1,000 IU/day vitamin D_3 for 3 to 5 years among 2,259 participants aged 45 to 75 years did not significantly reduce the risk of recurrent colorectal adenomas [300]. Additional work performed within the same trial suggested that the effect of vitamin D_3 supplementation on advanced adenomas, but not on overall adenoma risk, significantly varied according to the individual *VDR* genotype (SNPs rs7968585 and rs731236) rather than with the magnitude of the change in circulating 25(OH)D levels [301]. In addition, a secondary analysis of the trial explored the effect of vitamin D_3 supplementation several years after treatment (mean 4.6 years) and found that it did not modify adenoma risk [302].

Pommergaard and collaborators conducted a RCT to determine whether a combination of 0.5 μg $1,25(OH)_2D_3$, 75 mg acetylsalicylic acid and 1,250 mg calcium carbonate could interfere in colorectal adenoma recurrence in individuals aged 40 to 75 years that had at least one adenoma removed recently (<3 months). There were no differences in the recurrence rate in the treatment vs. placebo groups (OR 0.95; 95% CI, 0.61 to 1.48) after 3 years and the study was terminated precociously [303].

In a study performed by Holt and colleagues, colorectal polyps were only partially removed, and patients were daily supplemented with 400 IU vitamin D_3 and 4,500 mg calcium carbonate or placebo for a 6-month period, after which the polyps were completely removed and histologically analyzed. They found that supplementation strongly reduced proliferative indices both in the normal-appearing mucosa and in the

polyps [304]. Bostick's group studied samples from two RCTs in which individuals that had at least one adenoma removed were supplemented with vitamin D_3 to characterize its effects on cancer-related markers. Vitamin D_3 modified the expression of several markers in directions hypothesized to inhibit colorectal tumorigenesis: it increased E-cadherin, APC, p21 and BAX in the normal-appearing rectal mucosa and reduced plasma concentration of tumor-promoting pro-inflammatory markers [305-307].

Vitamin D_3 supplementation in colorectal cancer patients

Observational studies support a positive association between higher plasma 25(OH)D levels and better outcomes in CRC patients, but the potential of vitamin D compounds as an add-on treatment in the active disease is still to be established. A few studies approached vitamin D_3 supplementation intervention in CRC patients.

The SUNSHINE trial was conducted in 139 metastatic CRC patients to examine whether addition of high- vs. standard-dose vitamin D_3 to standard chemotherapy improves patient outcomes. The primary end point was PFS, and secondary outcomes included OS and changes in plasma 25(OH)D level. The high-dose group received a loading dose of 8,000 IU/day vitamin D_3 for cycle 1 followed by 4,000 IU/day for subsequent cycles while the standard-dose group received 400 IU/day vitamin D_3 during all cycles. Interestingly, median plasma 25(OH)D levels increased into the sufficient range with high-dose but remained unchanged with standard-dose. Results also showed that patients receiving high-dose vitamin D_3 had improved median PFS compared with those receiving standard-dose (13 vs. 11 months, P=0.07). A supporting analysis for PFS or death resulted in a multivariable HR of 0.64 (95% CI, 0 to 0.90; P=0.02). Median OS remained unaffected between groups. Importantly, high-dose vitamin D_3 supplementation did not result in any added toxicity [308].

The AMATERASU trial was conducted to determine whether postoperative supplementation with 2,000 IU/day vitamin D_3 improves survival of 251 patients (vs. 166 placebo) aged 30 to 90 years with non-metastatic digestive tract cancers (CRC, 48%). The primary outcome was relapse-free survival (RFS; time to cancer relapse or to death due to any cause) and the secondary was OS (time to death due to any cause). Subgroup analyses were also done based on baseline serum 25(OH)D levels as well as on the presence of relevant SNPs. Principal results were: (a) vitamin D_3 supplementation did not significantly reduce RFS at 5 years compared with placebo (HR 0.76; 95% CI, 0.50 to 1.14; P =0.18); (b) 5-year OS in the vitamin D_3 vs. placebo group was 82% vs. 81% (HR

0.95; 95% CI, 0.57 to 1.57; P =0.83); and (c) significant associations were not observed between subgroups of SNPs. However, in an adjusted analysis by age, the cumulative hazard of relapse or death was significantly lower in the vitamin D_3 group (HR 0.66; 95% CI, 0.43 to 0.99; P =0.048). Additionally, vitamin D_3 was effective in a subgroup of patients with middle (20-40 ng/ml) serum 25(OH)D levels at baseline, as the cumulative incidence of relapse was significantly lower when compared to placebo (HR 0.44; 95% CI, 0.21 to 0.89; P=0.02) [309]. Post hoc analyses of the AMATERASU study were published in a series of articles pinpointing several hypotheses on vitamin D_3 supplementation. Briefly, it improved RFS and OS in a subgroup of patients with poorly differentiated adenocarcinoma but not in any other subgroup based on histopathological characteristics [310]. It also improved RFS among patients with low bioavailable 25(OH)D levels (i.e., not bound to DBP) [311] and in p53 positive (missense mutated *TP53*) tumors [312]. Moreover, vitamin D_3 supplementation seems to influence cancer immunological mechanisms as it downregulated serum levels of the immune checkpoint protein programmed cell death ligand 1 (PD-L1) and reduced the risk of relapse/death to approximately one-third exclusively in patients with high baseline serum PD-L1 levels [313]. Finally, vitamin D_3 effectively reduced relapse in patients who had an adequate infiltration of CD56+ natural killer cells in the tumor stroma, suggesting that these cells may be involved in the antitumor action of vitamin D_3 [314].

Vaughan-Shaw and colleagues have recently performed a systematic review with meta-analysis of several RCTs (including those commented above) to examine the impact of vitamin D_3 supplementation on survival outcomes in CRC patients. In summary, the authors have found several beneficial effects of vitamin D_3 supplementation: (i) a 30% reduction in overall adverse survival outcomes (HR 0.70; 95% CI, 0.48 to 0.93); (ii) a CRC-specific survival improvement by 24% (HR 0.76; 95% CI, 0.39 to 1.13); and (iii) a 35% decrease in disease progression or death (HR 0.65; 95% CI, 0.36 to 0.94). The authors finally state that "a consistent reduction in adverse survival outcomes irrespective of the trial inclusion criteria, supplementation dose or survival outcome measure is supportive of a true causal effect, which supports observational data linking 25(OH)D level and cancer outcomes" [315].

Finally, other clinical trials: (i) evaluated the impact of supplementation with 50,000 IU vitamin D_3 weekly in non-advanced CRC patients who were candidates to receive adjuvant chemotherapy, suggesting a beneficial impact on inflammation and nutritional status [316, 317]; (ii) failed to establish an association between 2,000 IU/day vitamin D_3

together with standard chemotherapy and survival of metastatic CRC patients [318]; (iii) aimed to test whether a personalized vitamin D_3 dosing regimen (an initial individually tailored loading dose followed by a maintenance daily dose of 2,000 IU for 12 weeks) reduces or prevents fatigue and enhances quality of life among vitamin D deficient non-metastatic CRC patients (ongoing trial), as an interim analysis showed to be safe and effective in treating vitamin D insufficiency [319]. Nevertheless, the small number of patients enrolled in these trials and/or the short time of vitamin D_3 supplementation and follow-up thwart their statistical robustness and clinical significance.

In summary, nowadays we have got confounding data in which some RCTs suggest a protective or antitumoral effect of vitamin D_3 supplementation on total cancer or CRC incidence or mortality, while other studies show no effects at all. Therefore, we need new well-designed trials that clarify whether vitamin D_3 supplementation is an option to prevent or treat CRC. To accomplish this, future RCTs must be careful in the selection of participants and include a detailed characterization of parameters such as race/ethnicity, geographical location/UVB exposure, socioeconomic status, genetic heterogeneity/polymorphisms, lifestyle, dietary intake/food habits, self-supplementation, body weight/composition, physical activity, etc. Moreover, participants should bear a relevant risk of cancer and a vitamin D-depleted status at baseline. New RCTs must also consider the latency of the disease to define trial duration and exclude the premature cases unrelated to the intervention. Also, vitamin D_3 doses need to be adequate to demonstrate a protective effect. In this regard, individual doses could be adjusted to maintain serum 25(OH)D above an appropriate previously defined threshold level. Trial size must consider the expected incidence of the particular cancer type and follow-up must be exhaustive to avoid underreporting of new cancer cases, update information on lifestyle, and adjust vitamin D_3 doses based on serum 25(OH)D levels. Finally, a proper analysis using information on confounders and maximizing the statistical power, especially in subgroup analyses, is required [320, 321]. Wishfully, results from these new RCTs will align with epidemiological data and mechanistic studies and confirm the protective action of the VDES on CRC.

Mendelian randomization studies

Mendelian randomization (MR) is a research analytical method that uses measured variation in genes to determine whether an observational association between a potential modifiable risk factor (e.g., 25(OH)D plasma concentration) and a health outcome (e.g., CRC risk) is consistent with a causal effect. This method relies on the natural random assortment of genetic variants during meiosis yielding a random distribution of genetic variants in a specific population. Because these genetic variants are typically not associated with confounders, differences in the outcome between those who carry the variant and those who do not can be attributed to the difference in the risk factor. Therefore, MR studies can provide reliable evidence on the effect of modifiable risk factors for disease and can overcome some limitations of traditional observational epidemiology as they reduce both reverse causation and confounding, which often substantially impede or mislead the interpretation of results from conventional epidemiological studies [322, 323].

In the VDES field, MR studies have been conducted to test whether genetically predicted 25(OH)D levels are associated with risk of disease by using certain SNPs that have been related with 25(OH)D levels in genome-wide association studies (GWAS). In this regard, MR studies have reported null associations for the incidence of total cancer and most cancer types, including CRC [324]. Only a protective association has been observed for ovarian cancer in the Ovarian Cancer Association Consortium [325], but not in the UK Biobank [326]. Accordingly, a recent systematic review by Lawler and colleagues found similar results [327]. Regarding genetically predicted 25(OH)D levels and cancer mortality, there are currently sparse data. One study has reported a significantly reduced risk of cancer-specific mortality for individuals with higher 25(OH)D levels, while other reports have not replicated this finding [327].

In addition, it is worth mentioning that despite their advantages, MR studies have also important limitations: SNPs detected in GWAS studies only explain a small percentage of the variation in 25(OH)D plasma levels, difficulty to detect non-linear effects, most studies have been conducted in samples of European ancestry, etc. [324, 327]. Thus, further MR studies with higher statistical power are required to confirm these results.

Concluding remarks

Since the discovery of vitamin D 100 years ago, a number of milestones have, in our opinion, contributed important conceptual and/or methodological advances to the study of its role in CRC (Fig. 3). Among those, epidemiological studies strongly suggest a protective role of the VDES in CRC. This is supported by abundant experimental laboratory work that has unveiled multiple mechanisms of antitumor action of $1,25(OH)_2D_3$ in colon carcinoma cells and also in other cell types of the tumor microenvironment (Fig. 2). These mechanisms involve the regulation by $1,25(OH)_2D_3$ of genes that play important roles in tumor progression, but also the antagonism of signaling pathways commonly activated in CRC. The Wnt/β-catenin and the NF-κB pathways are possibly the most relevant targets of $1,25(OH)_2D_3$ activity in this neoplasia. CRC patient-derived organoids and primary stromal cultures provide additional and valuable new tools to complement and extend current mechanistical knowledge on $1,25(OH)_2D_3$ action in CRC and are also useful for personalized therapeutics.

Unfortunately, results from RCTs are non-conclusive, and we are still in need for new well-designed trials to definitively establish whether vitamin D_3 supplementation in healthy and high-risk population reduces CRC incidence and mortality as well as its effect on cancer-specific and all-cause mortality in CRC patients. Discrepancies between current RCT data and observational studies suggest that high 25(OH)D levels could be confounded by healthy lifestyles including outdoor physical activity and balanced diet and stress the importance of research on lifestyle factors and its critical role in cancer pathogenesis and treatment. Hopefully, future MR studies may help to overcome these confounders.

Vitamin D_3 is inexpensive, safe, and easily accessible, so robust funding and support is mandatory to conduct new RCTs and further studies on the mechanisms underlying the activity of $1,25(OH)_2D_3$ in CRC that would eventually facilitate its incorporation into standard patient care.

Author contribution

All authors wrote the manuscript. A.F.-B. created the artwork.

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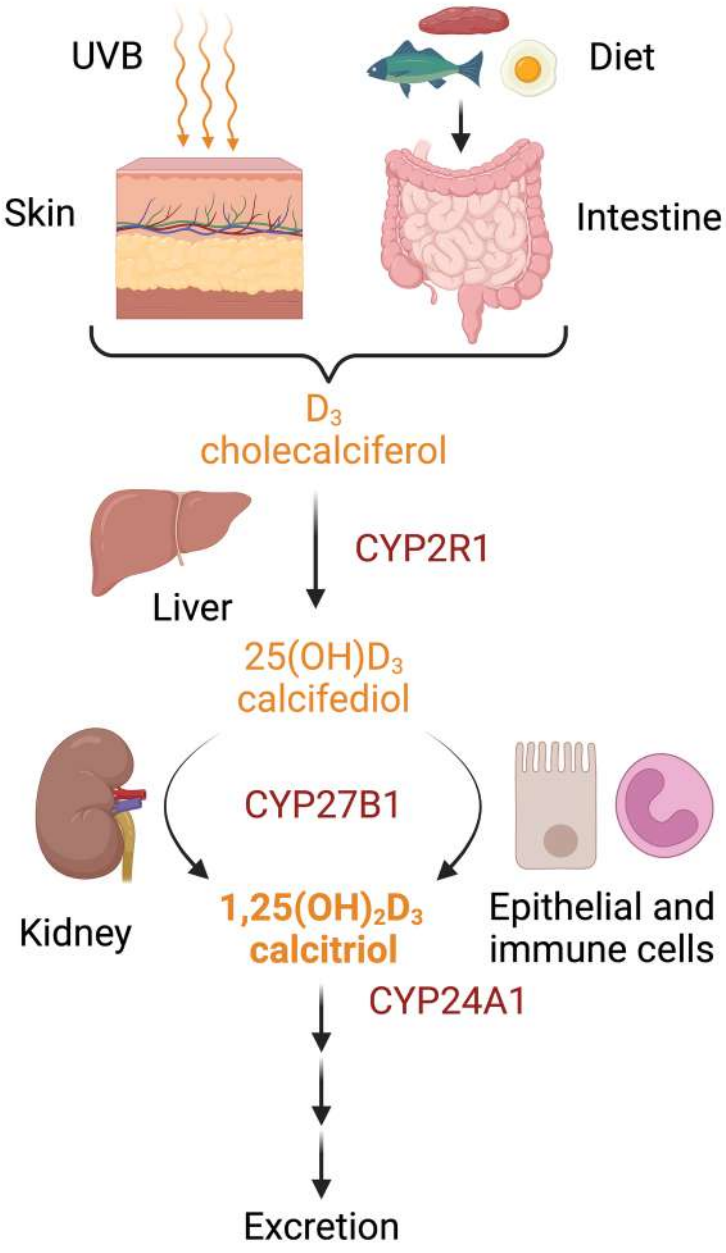
Figure Legends

Fig. 1. Schematic illustration of 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) synthesis and inactivation. Vitamin D_3 (D_3) or cholecalciferol is synthesized in the skin in response to sun ultraviolet B (UVB) light exposure, but can also be obtained from the diet, by consuming foods rich in vitamin D_3 such as fatty fish, liver, and egg yolk, and absorbed at the intestine. Once in the bloodstream, vitamin D_3 reaches the liver where it is hydroxylated by CYP2R1 to produce 25-hydroxyvitamin D_3 ($25(OH)D_3$) or calcifediol. Then, it is further hydroxylated by CYP27B1 in the kidney as well as in several epithelial and immune cells to generate $1,25(OH)_2D_3$ or calcitriol, a pleiotropic hormone which is the most active metabolite of vitamin D_3 and a major regulator of gene expression in multiple tissues. The first step in the inactivation of $1,25(OH)_2D_3$ is catalyzed by the ubiquitously expressed CYP24A1, a transcriptional target of $1,25(OH)_2D_3$ that therefore promotes its own inactivation. Created with Biorender.com.

Fig. 2. Mechanisms of 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) action in colorectal cancer (CRC). This illustration encompasses ten mechanisms by which $1,25(OH)_2D_3$ exerts its antitumoral activity on CRC cells and on the tumor microenvironment. For each mechanism, a schematic description of the most relevant effects of $1,25(OH)_2D_3$ is indicated. Red arrows pointing upwards indicate upregulation of gene/protein expression or activation of processes, whereas blue arrows pointing downwards indicate downregulation of gene/protein expression or inhibition of processes. Created with Biorender.com. COX-2, cyclooxygenase-2; DKK, Dickkopf; EMT, epithelial-to-mesenchymal transition; HIF-1α, hypoxia inducible factor 1α; IL, interleukin; MRP, multi-drug resistant-associated protein; NF-κB, nuclear factor κB; TCF, T-cell factor; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor α; VDR, vitamin D receptor; ZO, zonula occludens.

Fig. 3. Timeline of key milestones for vitamin D endocrine system research on colorectal cancer (CRC). This schematic timeline depicts some events that, in our opinion, helped to achieve conceptual or methodological advances that led to a better understanding of 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) actions in CRC. References for milestones are indicated. Created with Biorender.com. 25(OH)D, 25-hydroxyvitamin D; CAFs, cancer-

associated fibroblasts; COX-2, cyclooxygenase-2; NF-κB, nuclear factor κB; PFS, progression-free survival; RCTs, randomized controlled trials; VDR, vitamin D receptor.



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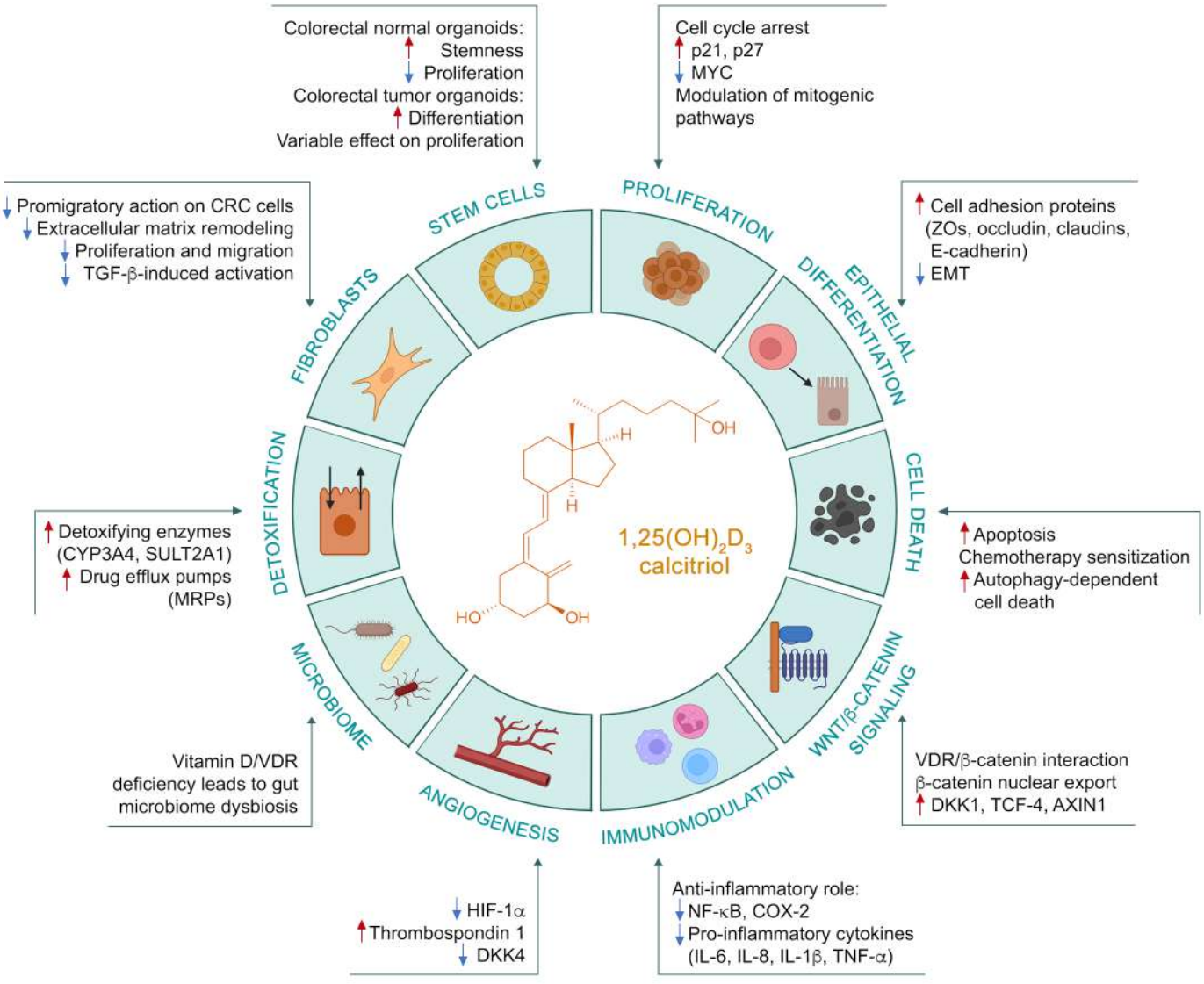

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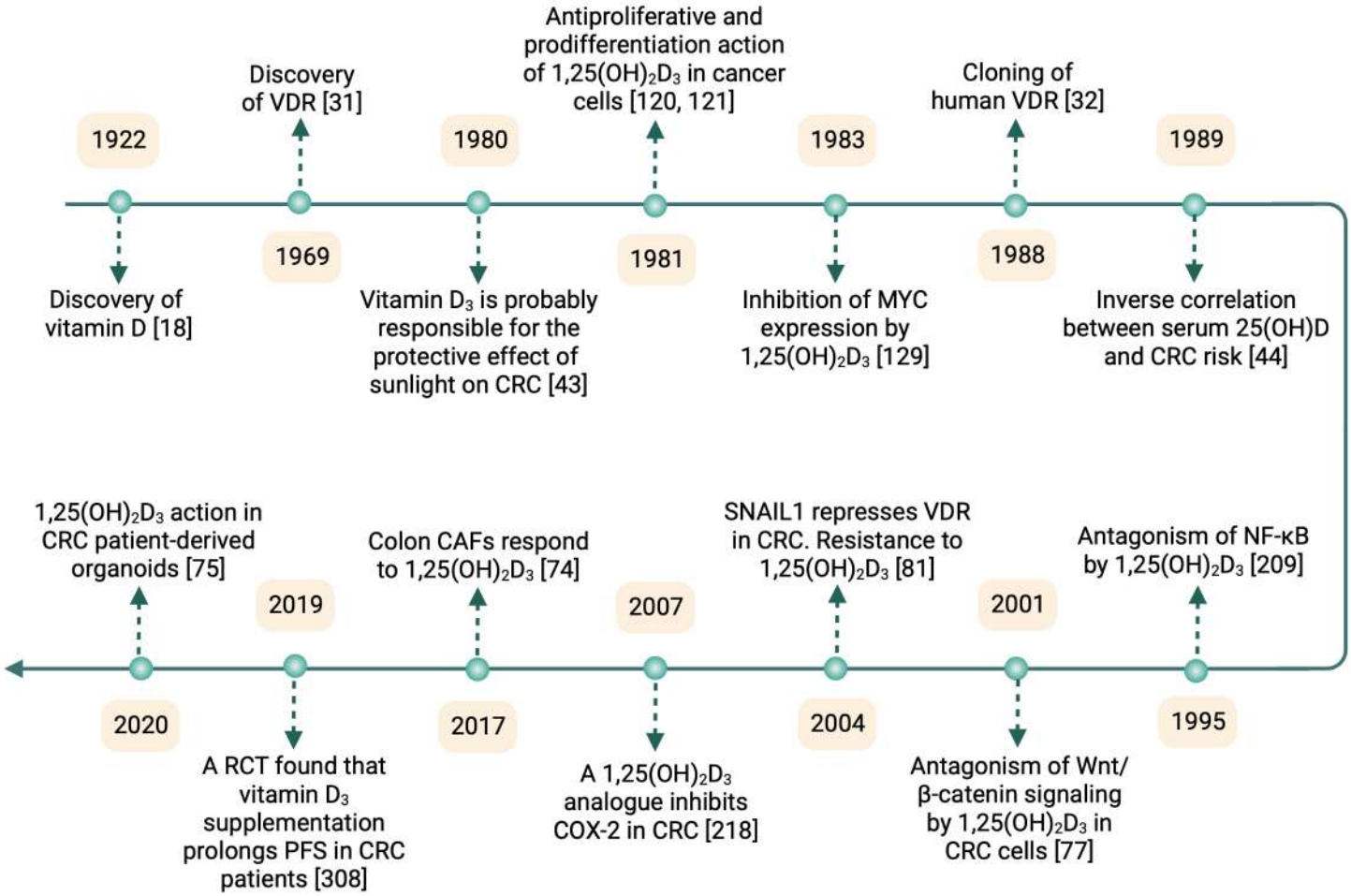

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