Vitamin D and colon cancer

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Abstract

The most active vitamin D metabolite, $1\alpha,25$ -dihydroxyvitamin D₃ ($1,25(\text{OH})_2\text{D}_3$), is a pleiotropic hormone with wide regulatory actions. Classically, vitamin D deficiency was known to alter calcium and phosphate metabolism and bone biology. In addition, recent epidemiological and experimental studies support the association of vitamin D deficiency with a large variety of human diseases, and particularly with the high risk of colorectal cancer. By regulating the expression of many genes via several mechanisms, $1,25(\text{OH})_2\text{D}_3$ induces differentiation, controls the detoxification metabolism and cell phenotype, sensitises cells to apoptosis and inhibits the proliferation of cultured human colon carcinoma cells. Consistently, $1,25(\text{OH})_2\text{D}_3$ and several of its analogues decrease intestinal tumourigenesis in animal models. Molecular, genetic and clinical data in humans are scarce but they suggest that vitamin D is protective against colon cancer. Clearly, the available evidence warrants new, well-designed, large-scale trials to clarify the role of vitamin D in the prevention and/or therapy of this important neoplasia.

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The vitamin D system

Vitamin D belongs to a group of steroids with a broken ring known as secosteroids. The most important forms of vitamin D are vitamin D₃ (cholecalciferol), produced in the human skin; and vitamin D₂ (ergocalciferol), derived from plants (Horst *et al.* 2005). Nowadays, vitamin D₃ is not categorised as a vitamin, but rather as a prohormone, given its synthesis in the skin and the multiple systemic actions of its metabolites.

The most active metabolite of vitamin D₃, $1\alpha,25$ -dihydroxyvitamin D₃ ($1,25(\text{OH})_2\text{D}_3$, calcitriol), is synthesised in a highly regulated multi-step process. When human skin is exposed to sunlight, u.v.-B irradiation causes the photolysis of provitamin D₃ (7-dehydrocholesterol) to pre-vitamin D₃, a thermodynamically unstable isomer that is rapidly converted into vitamin D₃. This passes into the bloodstream and is transported to the liver bound to the vitamin D-binding protein (DBP, encoded by the *GC* gene) and, to a lesser extent, to albumin. Alternatively, vitamin D₃ can be absorbed in the intestine from dietary sources (Holick 2005, Norman 2008). Vitamin D₃ is first hydroxylated in the liver by several

mitochondrial and microsomal vitamin D₃-25-hydroxylases. The resultant 25-hydroxyvitamin D₃ ($25(\text{OH})\text{D}_3$, calcidiol) is the main circulating form of vitamin D₃, and it is further hydroxylated in the kidney by mitochondrial $25(\text{OH})\text{D}_3$ - 1α -hydroxylase (encoded by the gene *CYP27B1*), yielding the hormonally active form $1,25(\text{OH})_2\text{D}_3$ (Lamprecht & Lipkin 2003, Deeb *et al.* 2007). Importantly, *CYP27B1* is also expressed in other human tissues and cell types, such as colon, brain, prostate, endothelial and immune cells, suggesting an extrarenal production of $1,25(\text{OH})_2\text{D}_3$ with paracrine/autocrine action (Townsend *et al.* 2005, Norman & Bouillon 2010). The rate-limiting step in vitamin D₃ metabolism is the transformation of $25(\text{OH})\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$ to the less active compounds $24,25(\text{OH})_2\text{D}_3$ and $1,24,25(\text{OH})_3\text{D}_3$ respectively through 24-hydroxylation by the ubiquitous *CYP24A1* enzyme (Deeb *et al.* 2007).

Mechanism of action of $1,25(\text{OH})_2\text{D}_3$

$1,25(\text{OH})_2\text{D}_3$ exerts its biological effects by binding to the vitamin D receptor (VDR), which is a member of the nuclear receptor super-family of transcription

factors, and regulates gene expression in a ligand-dependent manner (Deeb *et al.* 2007, Thorne & Campbell 2008). VDR dimerises with another member of the same family, the retinoid X receptor (RXR), whose ligand is 9-*cis*-retinoic acid or, at least in the mouse brain, docosahexaenoic acid (Heyman *et al.* 1992, Levin *et al.* 1992, de Urquiza *et al.* 2000). The VDR–RXR heterodimer binds to specific sequences of its target genes termed vitamin D response elements (VDREs). Curiously, studies on a few genes indicate a variable effect (additive, synergistic or none) of 9-*cis*-retinoic acid on the action of 1,25(OH)₂D₃. Results from gel-shift and transactivation assays using DNA fragments close to transcription start sites (TSS) suggested that VDREs usually comprise two hexanucleotide direct repeats separated by three or four nucleotides (AGTTCAnnnAGTTCA, being n any nucleotide; DR3 or DR4) or two everted repeats inter-spaced by six to nine nucleotides (TGAACnnnnnnAGTTCA; ER6–ER9) (Carlberg & Dunlop 2006, Carlberg & Seuter 2009).

However, this setting appears to be more complex in the light of the three chromatin immunoprecipitation sequencing (ChIP-Seq) studies published so far using human Epstein–Barr virus-transformed lymphoblastoid, THP-1 monocytic leukaemia and LS180 colon cancer cells (Ramagopalan *et al.* 2010, Heikkinen *et al.* 2011, Meyer *et al.* 2012). Although performed in different cell types and using different durations and doses of 1,25(OH)₂D₃ treatment, these genome-wide studies coincide in concluding that there are hundreds (623, 520 or 262, depending on the study) of genomic VDR-binding sites in the absence of 1,25(OH)₂D₃, which increases to thousands (2776, 1820 or 2209) after 1,25(OH)₂D₃ treatment. As expected due to the different conditions analysed, only a few of the identified VDR-binding sites overlap in the three studies (see also Carlberg *et al.* (2012)). Common findings were also the presence of most VDR-binding sites far from the TSS, the preference for the DR3-type in those located close to the TSS, and the shift from non-DR3 type to DR3-type upon ligand activation (Carlberg *et al.* 2012, Meyer *et al.* 2012). These studies revealed that though the DR3-type is the most common VDR-binding site, it represents <30% of all peaks, and that a high proportion of the peaks do not contain any classical VDRE. Very few genes have only one VDR-binding site: more complex scenarios are common, such as genes with multiple VDR-binding sites, two closely located target genes sharing one or more VDR-binding sites or even a cluster of target genes containing each of these multiple VDR-binding sites (Carlberg *et al.* 2012). In line with previous data,

in LS180 cells RXR co-occupied a high proportion of VDR-binding sites, which were most commonly of the DR3-type (Meyer *et al.* 2012). Curiously, in this system, 1073 (out of 1674) 1,25(OH)₂D₃-induced VDR–RXR-binding sites are located within repetitive DNA (long interdispersed nuclear elements) (Meyer *et al.* 2012). The characterisation of the functional relevance of data from ChIP-Seq studies will require the combination with transcriptomic results and subsequent validation analyses.

The prevailing model for the mechanism of action of 1,25(OH)₂D₃/VDR predicts that in the absence of 1,25(OH)₂D₃ the VDR–RXR heterodimer is bound to VDREs and to transcriptional corepressors, such as nuclear corepressors (NCoRs) and the silencing mediator of retinoid and thyroid hormone receptors (SMRT), that recruit complexes with histone deacetylase (HDAC) activity and maintain the chromatin in a transcriptionally repressed state. In this model, 1,25(OH)₂D₃ binding to VDR induces conformational changes in VDR that causes the release of corepressors and binding of co-activators, such as steroid receptor co-activators and the CBP-p300 coregulator, and chromatin remodelers such as switch-sucrose non-fermenting (SWI-SNF), that acetylate nucleosome histones to untie DNA for transcription. Once the chromatin is derepressed, binding of the VDR-interacting protein 205 (DRIP205) to the VDR attracts other components of the DRIP–TRIP complex, which acts as a bridge with RNA polymerase II and the transcriptional machinery, thus permitting the initiation of transcription (Haussler *et al.* 1998, Deeb *et al.* 2007, Carlberg & Seuter 2009, Pike & Meyer 2010).

The mechanism by which 1,25(OH)₂D₃ represses gene expression is less understood, although this process is probably equally important for its action, as around one-third of the target genes are inhibited (Pálmer *et al.* 2003, Heikkinen *et al.* 2011). Several possibilities have been proposed, such as direct VDRE-dependent repression, competition for DNA binding between VDR and other transcription factors or indirect repression via intermediate proteins (Kato *et al.* 2007, Kouzmenko *et al.* 2011). The best characterised transcriptional repression mechanism was elucidated for the *CYP27B1* gene. In this case, the VDR–RXR heterodimer represses gene transcription in a 1,25(OH)₂D₃-dependent manner through E-box-type negative VDREs (nVDREs: CANNTG). When bound to nVDREs, the VDR-interacting repressor (VDIR, also known as TCF3 or E47) induces the transcriptional activation of the *CYP27B1* gene. However, upon ligand binding VDR interacts with

VDIR, promoting the dissociation of co-activators and the recruitment of corepressors and complexes with HDAC activity, which results in gene repression (Murayama *et al.* 2004, Kouzmenko *et al.* 2011). In addition, the possibility that 1,25(OH)₂D₃ represses gene expression via the up-regulation of microRNAs (miRNAs) has recently emerged (Gocek *et al.* 2011, Thorne *et al.* 2011, Álvarez-Díaz *et al.* 2012). miRNAs are short non-coding RNAs that bind to the 3'-untranslated region of target mRNAs causing mRNA degradation and/or translational inhibition (Fabian *et al.* 2010). Thus, the induction of miRNAs constitutes a novel mechanism of gene repression by 1,25(OH)₂D₃.

Non-genomic actions of 1,25(OH)₂D₃ have also been described. They are rapid and transcription-independent effects that include the opening of ion channels and the activation or inhibition of kinases, phosphatases and phospholipases, and are probably relevant for the biological effects of 1,25(OH)₂D₃ (Norman *et al.* 2004, Haussler *et al.* 2011). Thus, in SW480-ADH colon cancer cells and in other cell types, 1,25(OH)₂D₃ causes a rapid increase in the cytosolic calcium concentration followed by the transient activation of the RhoA small GTPase, its effector ROCK and the p38MAPK and MSK-1 kinases that is required for the induction of its target genes (Ordóñez-Morán *et al.* 2008, 2010). This shows the convergence of non-genomic and genomic 1,25(OH)₂D₃ effects, at least in this system (Ordóñez-Morán & Muñoz 2009).

1,25(OH)₂D₃ is a pleiotropic hormone

The most widely and classically accepted physiological role for vitamin D, mediated mostly by 1,25(OH)₂D₃, is the regulation of calcium and phosphate homeostasis and bone mineralisation (Holick 2007, Verstuyf *et al.* 2010). 1,25(OH)₂D₃ enhances intestinal calcium absorption in the small intestine and affects the absorption of dietary phosphate possibly by increasing its influx through the enterocyte brush border membrane (Ajibade *et al.* 2010). In concert with these effects, 1,25(OH)₂D₃ induces the maturation of pre-osteoclasts into osteoclasts, which remove calcium and phosphate from bone (Holick 2007, Verstuyf *et al.* 2010).

In the past decades, evidence has emerged that the importance of 1,25(OH)₂D₃ for health extends far beyond bone. The discovery in 1981 that 1,25(OH)₂D₃ induces the differentiation of myeloid leukaemia cells and inhibits the growth of melanoma cells triggered the interest of cancer researchers (Abe *et al.* 1981,

Colston *et al.* 1981). Since then, numerous observations have indicated a much broader range of action for 1,25(OH)₂D₃, including the regulation of cell differentiation, proliferation, apoptosis, invasion and angiogenesis in several types of tumour cells and animal models of cancer (reviewed in Ordóñez-Morán *et al.* (2005), Deeb *et al.* (2007) and Krishnan & Feldman (2011)). These 1,25(OH)₂D₃ actions suggest a potential therapeutic role against hyper-proliferative disorders such as cancer. Nevertheless, the use of 1,25(OH)₂D₃ is restricted by its hypercalcaemic effect at therapeutic doses. This can be putatively overcome by the use of analogues that retain the anti-tumoural action but have less calcaemic effect. Currently, there are numerous clinical trials ongoing using 1,25(OH)₂D₃ or its analogues, alone or in combination with other drugs, against several neoplasias (www.clinicaltrials.gov) (Deeb *et al.* 2007).

Vitamin D and colon cancer: observational and epidemiological studies

According to the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO), about 12.7 million cancer cases and 7.6 million cancer deaths worldwide occurred in 2008. Colorectal cancer (CRC) is the third most frequent malignancy and the fourth leading cause of death from cancer worldwide, with 1 233 000 cases diagnosed and 608 000 deaths in 2008 (Ferlay *et al.* 2010).

The role of vitamin D in CRC prevention was first hypothesised by Frank and Cedric Garland based on ecological studies (Garland & Garland 1980). These authors proposed that the inverse relation between solar radiation (latitude) and CRC mortality and incidence in USA might be due to vitamin D synthesis. Later, the same authors reported an inverse association between vitamin D status and CRC in the USA (Garland *et al.* 1989). Since then, numerous studies have addressed the relationship between vitamin D status and colorectal adenoma and carcinoma incidence or mortality (Platz *et al.* 2000, Wu *et al.* 2007, Gandini *et al.* 2011). However, the integration and the overall analysis of all epidemiological studies is complex and thwarted by the fact that many of these studies do not take into account endogenous vitamin D production from the sun exposure and are limited by measurement error from the various dietary assessment methods and food composition tables used to calculate dietary intake. Such limitations can be overcome by measuring circulating 25(OH)D concentration, a biomarker that provides an overall estimate of vitamin D status (both from endogenous production

and from dietary intake). Nonetheless, the effective colonic $1,25(\text{OH})_2\text{D}_3$ concentration is determined not only by $25(\text{OH})\text{D}$ and $1,25(\text{OH})_2\text{D}_3$ serum levels but also by their uptake by the cell and by the expression and activity of CYP27B1 and CYP24A1 enzymes in the colon (Cross *et al.* 2011). In fact, it has been reported that the expression/activity of these enzymes is regulated by several factors (17β -estradiol, phytoestrogens, folate, calcium, $1,25(\text{OH})_2\text{D}_3$ itself, etc.) (Lechner *et al.* 2005, Cross *et al.* 2011) and also during colon cancer progression (discussed below). Therefore, several conditions may affect colonic $1,25(\text{OH})_2\text{D}_3$ levels and provoke that they do not correlate with $25(\text{OH})\text{D}$ serum levels in some individuals.

The substantial body of literature that examines the relationship between $25(\text{OH})\text{D}$ level and CRC risk generally supports an inverse association. A prospective study in the USA with 16 818 participants found that CRC mortality was inversely related to serum $25(\text{OH})\text{D}$ level (Freedman *et al.* 2007). A similar association was obtained in a case-control study that included an ethnically diverse population of Japanese, Latino, African-American, Caucasian and Native Hawaiian participants (Woolcott *et al.* 2010). Accordingly, a large observational nested case-control study (520 000 participants from ten Western European countries) conducted within the European Prospective Investigation into Cancer and Nutrition concluded that there is a strong inverse association between $25(\text{OH})\text{D}$ concentration and CRC (Jenab *et al.* 2010). A recent meta-analysis of 35 independent studies confirmed a consistent inverse relationship between serum $25(\text{OH})\text{D}$ levels and CRC risk (Gandini *et al.* 2011). Ma *et al.* (2011) conducted a systematic review of 18 prospective studies assessing the association of vitamin D intake or serum levels of $25(\text{OH})\text{D}$ and the risk of CRC in $\sim 1\,000\,000$ individuals and found an inverse correlation between CRC risk and both serum $25(\text{OH})\text{D}$ and vitamin D intake.

Accepting the problems of using different approaches and techniques to measure vitamin D status, the population diversity and the potential presence of confounding factors, the consistency of the association between vitamin D status and CRC risk indicates a causal association (Giovannucci 2011). Accordingly, the IARC stated that the epidemiological evidence for a relationship between serum $25(\text{OH})\text{D}$ levels and the incidence of CRC is consistent and persuasive. However, such a causal relationship awaits large, adequate prospective clinical trials (IARC 2008).

Anti-tumoural actions of $1,25(\text{OH})_2\text{D}_3$ in CRC cells

CRC arises as a consequence of the progressive accumulation of genetic and epigenetic alterations that induce the transformation and malignant progression of normal colonic epithelial cells. The initial genetic change in most colorectal adenomas, the earliest step in CRC, is the somatic mutation in the tumour suppressor gene *APC* (or, to a lesser extent, *AXIN2* or *CTNNB1*/ β -catenin genes). Each of these mutually exclusive mutations leads to aberrant activation of the canonical WNT/ β -catenin signalling pathway (Clevers 2006, Klaus & Birchmeier 2008). In addition, a large proportion of adenomas carry activating mutations in *KRAS* or *BRAF*, whereas inactivating mutations in the transforming growth factor (TGF)- β pathway (mainly in *SMAD2*, *SMAD4* or *TGFBR2*) confer additional malignant features to adenoma cells (Markowitz & Bertagnolli 2009). The inactivation of the *TP53* tumour suppressor gene matches the adenoma-carcinoma transition in around 50% of CRC tumours (Iacopetta 2003). The molecular mechanisms of carcinoma progression and the acquisition of metastatic ability remain to be fully elucidated. Recent studies indicate that the epithelial-mesenchymal transition (EMT) endows epithelial cells with the capacity to invade the surrounding tissue and then to migrate to distant organs (Thiery *et al.* 2009, Chaffer & Weinberg 2011).

Numerous studies have shown that the anti-tumoural action of $1,25(\text{OH})_2\text{D}_3$ in CRC relies on several mechanisms at the cellular level, such as inhibition of cell proliferation, sensitisation to apoptosis, induction of epithelial differentiation and cell detoxification metabolism and inhibition of angiogenesis (Fig. 1). The combined effect of these mechanisms, in a cell-type- and cell-context-dependent manner, may determine the anti-tumoural action of $1,25(\text{OH})_2\text{D}_3$ (reviewed in Lamprecht & Lipkin (2003), Deeb *et al.* (2007), Larriba *et al.* (2008) and Krishnan & Feldman (2011)). A detailed list of the most important $1,25(\text{OH})_2\text{D}_3$ target genes in human CRC cells and their role in the anti-tumoural actions of the hormone is shown in Table 1.

Proliferation

The growth-inhibitory effect of $1,25(\text{OH})_2\text{D}_3$ on CRC cells results from the accumulation of cells in the G_0/G_1 phase of the cell cycle. $1,25(\text{OH})_2\text{D}_3$ induces the expression of the cyclin-dependent kinase inhibitors $p21^{\text{CIP1}}$ and $p27^{\text{KIP1}}$, and represses that of cyclin A and cyclin F (Evans *et al.* 1999, Gaschott *et al.* 2002,

Figure 1 Summary of the anti-tumoural actions of 1,25(OH)₂D₃ in CRC cells. 1,25(OH)₂D₃ inhibits cell proliferation and angiogenesis, sensitises to apoptosis, and induces epithelial differentiation and the detoxification metabolism of cultured human CRC cells.

Pálmer *et al.* 2003, Fernández-García *et al.* 2005). A recent report by Liu *et al.* (2010) indicates that the induction of p21^{CIP1} and the inhibition of proliferation by 1,25(OH)₂D₃ in CRC cell lines are dependent on the expression of the calcium-sensing receptor, which is in turn induced by 1,25(OH)₂D₃ (Chakrabarty *et al.* 2005). 1,25(OH)₂D₃ also induces the expression of the growth-arrest and DNA damage 45α (*GADD45A*) gene (Pálmer *et al.* 2003), which is involved in cell cycle arrest after DNA damage and is required for the maintenance of genomic stability. In addition, 1,25(OH)₂D₃ regulates many other genes related to proliferation, including *MYC*, *FOS* and *JUN* (Pálmer *et al.* 2001, 2003, Meyer *et al.* 2012). The *c-MYC* oncogene is deregulated and over-expressed in most cancers, and its down-regulation by 1,25(OH)₂D₃ occurs directly, via multiple VDREs located in the promoter and intragenic regions, and indirectly, by increasing the expression and promoting the binding of intermediate proteins to regulatory regions of the gene (Pan *et al.* 1996, Pan & Simpson 1999, Toropainen

et al. 2010, Meyer *et al.* 2012). As *MYC* is a target of the WNT/β-catenin pathway, another indirect mechanism of down-regulation of this gene by 1,25(OH)₂D₃ is the antagonism of this signalling pathway (Pálmer *et al.* 2001, discussed below).

Apoptosis

Apoptosis sensitisation by 1,25(OH)₂D₃ in colorectal adenoma and carcinoma cells involves the up-regulation of the proapoptotic protein BAK1 and the down-regulation of the nuclear anti-apoptotic protein BAG1 (Díaz *et al.* 2000, Barnes *et al.* 2005). The effect of 1,25(OH)₂D₃ on the expression of other proapoptotic (BAX) or anti-apoptotic (BCL-2, BCL-X_L) proteins, which are regulated by 1,25(OH)₂D₃ in other cell systems, is not clear in CRC, and appears to be cell-specific (Díaz *et al.* 2000, Hansen *et al.* 2001). In addition, 1,25(OH)₂D₃ induces the expression of G0S2 (Pálmer *et al.* 2003), a mitochondrial protein that interacts with BCL-2 and induces apoptosis in CRC

Table 1 1,25(OH)₂D₃ anti-tumoural actions and target genes in human CRC cells

Action	Target gene	Reference
Proliferation	<i>CDKN1A</i> /p21 ^{CIP1}	Evans <i>et al.</i> (1999), Gaschott <i>et al.</i> (2002)
	<i>CDKN1B</i> /p27 ^{KIP1}	Gaschott <i>et al.</i> (2002)
	Cyclin A	Fernández-García <i>et al.</i> (2005)
	<i>CCNF</i> /Cyclin F	Pálmer <i>et al.</i> (2003)
	<i>GADD45A</i>	Pálmer <i>et al.</i> (2003)
	<i>MYC</i>	Pálmer <i>et al.</i> (2001), Meyer <i>et al.</i> (2012)
	<i>FOS</i>	Meyer <i>et al.</i> (2012)
	<i>JUN</i>	Pálmer <i>et al.</i> (2003)
	<i>TGFBR1</i>	Chen <i>et al.</i> (2002)
	<i>EGFR</i>	Tong <i>et al.</i> (1999)
	<i>IGFBP6</i>	Oh <i>et al.</i> (2001)
	<i>CST5</i>	Álvarez-Díaz <i>et al.</i> (2009)
	<i>JMJD3</i>	Pereira <i>et al.</i> (2011)
	<i>miR-22</i>	Álvarez-Díaz <i>et al.</i> (2012)
Apoptosis	<i>BAK1</i>	Díaz <i>et al.</i> (2000)
	<i>BAG1</i>	Barnes <i>et al.</i> (2005)
	<i>G0S2</i>	Pálmer <i>et al.</i> (2003)
	<i>BIRC5</i> /Survivin	Liu <i>et al.</i> (2010)
	<i>TYMS</i> /Thymidylate synthase	Liu <i>et al.</i> (2010)
Differentiation	Alkaline phosphatase	Giuliano <i>et al.</i> (1991), Halline <i>et al.</i> (1994), Díaz <i>et al.</i> (2000)
	Maltase	Brehier & Thomasset (1988)
	<i>OCN</i> /Occludin	Pálmer <i>et al.</i> (2001)
	<i>ZO-1 (TJP1)</i>	Pálmer <i>et al.</i> (2001)
	<i>ZO-2 (TJP2)</i>	Pálmer <i>et al.</i> (2001)
	<i>CLDN2</i> /Claudin-2	Fujita <i>et al.</i> (2008)
	<i>CLDN12</i> /Claudin-12	Fujita <i>et al.</i> (2008)
	<i>CDH1</i> /E-cadherin	Pálmer <i>et al.</i> (2001)
	<i>PLEC</i> /Plectin	Pálmer <i>et al.</i> (2003)
	<i>KRT13</i> /Keratin-13	Pálmer <i>et al.</i> (2003)
	<i>VCL</i> /Vinculin	Pálmer <i>et al.</i> (2001)
	<i>FLNA</i> /Filamin A	Pálmer <i>et al.</i> (2003)
	<i>CST5</i>	Álvarez-Díaz <i>et al.</i> (2009)
	<i>SPRY2</i>	Barbáchano <i>et al.</i> (2010)
	<i>JMJD3</i>	Pereira <i>et al.</i> (2011)
Angiogenesis	<i>HIF1A</i>	Ben-Shoshan <i>et al.</i> (2007)
	<i>VEGFA</i>	Fernández-García <i>et al.</i> (2005)
	<i>THBS1</i>	Fernández-García <i>et al.</i> (2005)
	<i>DKK4</i>	Pendás-Franco <i>et al.</i> (2008)
Detoxification	<i>CYP3A4</i>	Thummel <i>et al.</i> (2001), Thompson <i>et al.</i> (2002), Pfrunder <i>et al.</i> (2003)
	<i>SULT2A1</i>	Echchgadda <i>et al.</i> (2004)
	<i>MDR-1 (ABCB1)</i>	Pfrunder <i>et al.</i> (2003), Aiba <i>et al.</i> (2005), Fan <i>et al.</i> (2009)
	<i>MRP2 (ABCC2)</i>	Fan <i>et al.</i> (2009)
	<i>MRP3 (ABCC3)</i>	McCarthy <i>et al.</i> (2005)
	<i>MRP4 (ABCC4)</i>	Fan <i>et al.</i> (2009)

cells by preventing BCL-2 from forming anti-apoptotic heterodimers with BAX (Welch *et al.* 2009). Although previous reports indicated that the apoptosis potentiated by 1,25(OH)₂D₃ does not require an intact *TP53* tumour suppressor gene (Díaz *et al.* 2000, Hansen *et al.* 2001), a recent study by Stambolsky *et al.* (2010) has revealed that mutant p53 protein interacts with VDR and modulates the transcriptional activity of 1,25(OH)₂D₃ leading to the increase of the expression

of survival-promoting genes and the decrease of that of proapoptotic genes, thus converting 1,25(OH)₂D₃ into an anti-apoptotic agent. In CRC cells, 1,25(OH)₂D₃ promotes sensitivity to the chemotherapeutic agent 5-fluorouracil by down-regulating the expression of survivin, an anti-apoptotic protein, and that of thymidylate synthase, an enzyme involved in DNA *de novo* synthesis and the molecular target of 5-fluorouracil (Liu *et al.* 2010). Moreover, in an

in vitro model developed to evaluate the cross talk between tumour-associated macrophages and CRC cells, 1,25(OH)₂D₃ restores the sensitivity of CRC cells to TRAIL-induced apoptosis by interfering with the release of IL1 β by macrophages (Kaler *et al.* 2010).

Differentiation

1,25(OH)₂D₃ has specific and multiple prodifferentiation effects in CRC cells. It increases the expression and/or activity of several brush border enzymes, such as alkaline phosphatase and maltase, and enhances the formation of microvilli (Brehier & Thomasset 1988, Giuliano *et al.* 1991, Halline *et al.* 1994, Díaz *et al.* 2000). In addition, 1,25(OH)₂D₃ increases the expression of several components of cell adhesion structures that are essential for the maintenance of the epithelial phenotype: i) occludin, *zonula occludens* (ZO)-1 and -2, and claudin-2 and -12 in tight junctions (Pálmer *et al.* 2001, Fujita *et al.* 2008); ii) E-cadherin in *adherens junctions* (Pálmer *et al.* 2001); and iii) plectin in hemidesmosomes (Pálmer *et al.* 2003). Moreover, 1,25(OH)₂D₃ induces the expression of proteins associated with the actin cytoskeleton and intermediate filaments such as keratin-13, vinculin and filamin A (Pálmer *et al.* 2001, 2003). Filamin A has been implicated in cell motility, adhesion and invasion (Nakamura *et al.* 2011). However, novel functions of filamin A have recently been described within the cell nucleus, such as the regulation of nuclear shape during EMT and of DNA double-strand break repair (Yue *et al.* 2009, Gay *et al.* 2011).

Angiogenesis

The angiogenic capacity of CRC cells may be affected by 1,25(OH)₂D₃, as it represses the expression and transcriptional activity of hypoxia-inducible factor (HIF)-1 α , a key transcription factor involved in hypoxia-induced angiogenesis (Ben-Shoshan *et al.* 2007). In addition, 1,25(OH)₂D₃ regulates the expression of vascular endothelial growth factor (VEGFA) and thrombospondin-1 (THBS1), two major opposing factors that control tumour angiogenesis, leading to a balanced change in the angiogenic potential of SW480-ADH cells (Fernández-García *et al.* 2005). Further, 1,25(OH)₂D₃ strongly represses DICKKOPF-4 (DKK4), a weak WNT antagonist that promotes invasion and angiogenesis in cultured CRC cells and is up-regulated in human colon tumours (Pendás-Franco *et al.* 2008).

Detoxification

Intestinal epithelial cells express a variety of enzymes involved in the detoxification and metabolism of xenobiotics from dietary sources and of deleterious endobiotic compounds that may contribute to CRC development. Phase I enzymes, most of which belong to the cytochrome P450 (CYP) super-family, initiate the enzymatic detoxification of these compounds by oxidation, hydrolysis or reduction (Kaminsky & Zhang 2003). In CRC cells, 1,25(OH)₂D₃ induces the expression of CYP3A4, the member of the CYP super-family most expressed in the intestine (Thummel *et al.* 2001, Thompson *et al.* 2002, Pfrunder *et al.* 2003). Likewise, several members of the CYP2C sub-family contain VDREs in their promoters and are regulated by 1,25(OH)₂D₃ in hepatic tissues. As CYP2C is the second most abundant CYP sub-family in the human intestine, its putative regulation by 1,25(OH)₂D₃ in this tissue could be highly relevant (Drocourt *et al.* 2002, Chen & Goldstein 2009).

Phase II enzymes conjugate different charged groups to the compounds previously modified by phase I enzymes, which increases their solubility in water and facilitate their excretion (Kaminsky & Zhang 2003). In CaCo-2 cells, 1,25(OH)₂D₃ activates the transcription of SULT2A1, a phase II sulphotransferase that acts preferentially on hydroxysteroids and cholesterol-derived sterol bile acids, but may also modify several drugs and other xenobiotics (Echchgadda *et al.* 2004).

In addition, 1,25(OH)₂D₃ regulates the expression of several transporters responsible for the efflux of phase II conjugates to the extracellular medium. One of them is the drug efflux pump multi-drug resistance protein (MDR)-1, which is located in the enterocyte apical membrane (Pfrunder *et al.* 2003, Aiba *et al.* 2005, Fan *et al.* 2009). 1,25(OH)₂D₃ also modulates the expression of members of the multi-drug resistance-associated protein (MRP) family, which are involved in the transport of conjugated and unconjugated bile acids, and also in that of glutathione and sulphate conjugates (McCarthy *et al.* 2005, Fan *et al.* 2009).

Thus, 1,25(OH)₂D₃ has a wide-ranging effect on the expression of several proteins involved in intestinal detoxification, which is consistent with a preventive and therapeutic action of this hormone against CRC.

Interference with the WNT/ β -catenin pathway and inter-play with other signalling pathways

1,25(OH)₂D₃ interferes with several signalling pathways, which may partially mediate its anti-tumoural effects. Results from our group and others have demonstrated that 1,25(OH)₂D₃ inhibits the

WNT/ β -catenin pathway and the activation of its target genes in CRC cells, which contributes to the inhibition of cell proliferation and to the maintenance of the differentiated phenotype (Pálmer *et al.* (2001) and reviewed in González-Sancho *et al.* (2011)). 1,25(OH) $_2$ D $_3$ inhibits the WNT/ β -catenin pathway through several mechanisms. First, it rapidly increases the amount of VDR bound to β -catenin, thus reducing the interaction between β -catenin and the transcription factors of the TCF/LEF family and leading to the repression of its target genes (Pálmer *et al.* 2001). Shah *et al.* (2006) confirmed the VDR/ β -catenin interaction and characterised the protein domains involved, while Egan *et al.* (2010) reported that wild-type APC enhances the inhibition of β -catenin/TCF transcriptional activity by 1,25(OH) $_2$ D $_3$. Second, 1,25(OH) $_2$ D $_3$ induces β -catenin nuclear export linked to E-cadherin accumulation at the plasma membrane *adherens junctions* (Pálmer *et al.* 2001). Third, it induces the expression of DKK1, an extracellular WNT inhibitor (Aguilera *et al.* 2007). In addition, 1,25(OH) $_2$ D $_3$ impedes the production of IL1 β by THP-1 monocytic leukaemia cells and, if extrapolated to tumour-associated macrophages, this would represent another mechanism of inhibition of β -catenin/TCF-dependent gene activation in CRC cells, as IL1 β inhibits β -catenin phosphorylation and labelling for degradation by GSK-3 β (Kaler *et al.* 2009). Thus, 1,25(OH) $_2$ D $_3$ exerts a complex set of regulatory actions leading to the inhibition of the WNT/ β -catenin pathway. As this pathway is aberrantly activated in most adenomas and colorectal tumours and is considered the main driving force in this neoplasia, its inhibition is probably crucial for the anti-tumoural action of 1,25(OH) $_2$ D $_3$ in CRC.

Additionally, 1,25(OH) $_2$ D $_3$ sensitises CRC cells to the growth inhibitory action of TGF- β by up-regulating the expression of its type I receptor (TGFBR1) (Chen *et al.* 2002). Moreover, 1,25(OH) $_2$ D $_3$ inhibits the stimulation of cell proliferation by EGF via the reduction of EGF receptor (EGFR) expression and the induction of its internalisation upon ligand binding (Tong *et al.* 1999). 1,25(OH) $_2$ D $_3$ may also inhibit EGFR activity by inducing the expression of E-cadherin (Pálmer *et al.* 2001) and by repressing that of SPROUTY-2 (Barbáchano *et al.* 2010, see below), which are respectively negative and positive regulators of EGFR activity (Andl & Rustgi 2005, Cabrita & Christofori 2008). Likewise, 1,25(OH) $_2$ D $_3$ interferes with the growth-stimulatory effect of insulin growth factor 2 (IGF2) through the inhibition of IGF2 secretion and the modulation of the expression of

several genes encoding IGF-binding proteins (IGFBP; Oh *et al.* 2001, Pálmer *et al.* 2003).

Immunomodulation

1,25(OH) $_2$ D $_3$ has the potential to be an important regulator of the immune system in the colon as epithelial and immune cells of the gut mucosa express VDR and CYP27B1 (Verstuyf *et al.* 2010). Several studies have shown that 1,25(OH) $_2$ D $_3$ and VDR play a role in the innate immunity against bacterial infections in the colon. It has been observed that bacterial infiltration of the colon is higher in vitamin D-deficient animals than in controls (Lagishetty *et al.* 2010). Accordingly, in CRC cells 1,25(OH) $_2$ D $_3$ induces the expression of cathelicidin anti-microbial peptide in a VDRE-dependent mechanism (Gombart *et al.* 2005) and VDR mediates the up-regulation of the anti-microbial peptide β -defensin-2 by dietary sulphoraphane (Schwab *et al.* 2008). Besides their anti-microbial activity, these peptides exert other functions such as chemoattraction of immune cells, degranulation of mast cells, stimulation of wound vascularisation and induction of cancer cells apoptosis and cytotoxicity (Kamysz *et al.* 2003, Oppenheim *et al.* 2003, Okumura *et al.* 2004, De Smet & Contreras 2005).

Several evidences suggest that 1,25(OH) $_2$ D $_3$ could play a role in the prevention and therapy of inflammatory bowel disease (IBD), and thus may also reduce the risk of colitis-associated CRC. *Cyp27b1* knockout mice are more susceptible to colitis, which is associated with increased levels of IL1 and 17 in the colon (Liu *et al.* 2008). In addition, vitamin D deficiency accelerates the development of the IBD symptoms that spontaneously occur in *Il10* knockout mice (Cantorna *et al.* 2000, 2004). Accordingly, administration of 1,25(OH) $_2$ D $_3$ to these mice inhibits the TNF- α pathway and significantly prevents disease progression and alleviates the symptoms of established IBD (Cantorna *et al.* 2000, 2004, Zhu *et al.* 2005). Additionally, work by Froicu *et al.* (2006) showed that VDR is required to control gastrointestinal inflammation in *Il10* knockout mice, as *Il10/Vdr* double knockout mice develop exacerbated IBD symptoms associated with high local expression of IL2, IFN- γ , IL1 β , TNF- α and IL12. In line with this, 1,25(OH) $_2$ D $_3$ was shown to antagonize the NF- κ B pathway and the production of IL8 in CRC cells treated with IL1 β (Sun *et al.* 2008). These results suggest that 1,25(OH) $_2$ D $_3$ /VDR signalling has a potent anti-inflammatory action *in vitro* and *in vivo* that may influence the colonic inflammatory scenario that pre-dispose to the development of CRC.

1,25(OH)₂D₃ target genes in CRC cells

The development of high-throughput techniques for gene expression profiling, such as microarrays, has allowed a more detailed study of 1,25(OH)₂D₃ effects in the transcriptome of cancer cells (reviewed in Kriebitzsch *et al.* (2009)). We and others performed oligonucleotide microarrays in CRC cells treated with 1,25(OH)₂D₃ (Pálmer *et al.* 2003, Wood *et al.* 2004). Our results showed that around two-thirds of 1,25(OH)₂D₃ target genes are up-regulated, while one-third of these genes are down-regulated after 1,25(OH)₂D₃ treatment. A high proportion of the regulated genes are related to transcription, cell adhesion and metabolism, although genes related to many different cell functions were also found (Pálmer *et al.* 2003). In the past decade, our laboratory has dedicated much effort to characterizing the role of several of these genes in the anti-tumoural action of 1,25(OH)₂D₃ in CRC.

One of the genes regulated by 1,25(OH)₂D₃ in our transcriptomic analysis was *CST5*, encoding cystatin D, an inhibitor of cysteine proteases of the cathepsin family. We validated the strong induction of cystatin D by 1,25(OH)₂D₃ and found that it mediates the anti-proliferative and prodifferentiation effects of 1,25(OH)₂D₃ in CRC cells (Álvarez-Díaz *et al.* 2009). Cystatin D regulation by 1,25(OH)₂D₃ also occurs in xenografted mice treated with the 1,25(OH)₂D₃ analogue EB1089/Seocalcitol, and a direct correlation between cystatin D and VDR protein levels was observed in human CRC biopsies, suggesting that 1,25(OH)₂D₃ also regulates cystatin D *in vivo* (Álvarez-Díaz *et al.* 2009). In addition, ectopic cystatin D inhibits proliferation, migration and anchorage-independent growth of cultured CRC cells and growth of xenografted tumours in mice. Moreover, cystatin D expression is down-regulated during colorectal tumourigenesis and is associated with tumour dedifferentiation. All together, these data led us to propose *CST5* as a candidate tumour suppressor gene in CRC (Álvarez-Díaz *et al.* 2009). The mechanism by which this protease inhibitor mediates its actions is not fully understood. Experiments using mutant cystatin D proteins with reduced anti-proteolytic activity indicate that at least some of its anti-tumour effects may be independent of cathepsin inhibition (Álvarez-Díaz *et al.* 2009). In line with this, 1,25(OH)₂D₃ regulates a number of genes related to the protein degradation machinery, such as proteases, other protease inhibitors, and components of the ubiquitin–proteasome system (Álvarez-Díaz *et al.* 2010).

SPRY2 is another gene shown by transcriptomic assays to be regulated by 1,25(OH)₂D₃ (5.6-fold repression at 4 h of treatment) (Pálmer *et al.* 2003). *SPRY2* encodes SPROUTY-2, an intracellular modulator of EGFR and other growth factor tyrosine kinase receptors involved in the regulation of cell growth, migration and angiogenesis (Cabrita & Christofori 2008). SPROUTY-2 strongly inhibits the inter-cellular adhesion induced by 1,25(OH)₂D₃, and gain- and loss-of-function experiments showed that SPROUTY-2 and E-cadherin repress reciprocally in CRC cell lines and have opposite actions on cell differentiation (Barbáchano *et al.* 2010). Accordingly, the expressions of SPROUTY-2 and E-cadherin are mutually exclusive in xenografted tumours and correlate inversely in human CRC biopsies, where SPROUTY-2 is up-regulated in undifferentiated high-grade tumours and at the invasive front of low-grade carcinomas (Barbáchano *et al.* 2010). Therefore, SPROUTY-2 is inhibited by 1,25(OH)₂D₃ and is a potential novel marker of malignancy and a therapeutic target in CRC.

Several candidate 1,25(OH)₂D₃ target genes encode for proteins implicated in epigenetic regulation of gene expression. We have recently reported that 1,25(OH)₂D₃ induces the expression of the *JMJD3* (*KDM6B*) gene (Pereira *et al.* 2011), which codes for a histone demethylase of the trimethyl repressive mark on lysine 27 of histone H3, and which may have an important tumour suppressor function (De Santa *et al.* 2007, Agger *et al.* 2009). *JMJD3* partially mediates the prodifferentiation, anti-proliferative and gene regulatory actions of 1,25(OH)₂D₃, and also its antagonism of the WNT/β-catenin pathway (Pereira *et al.* 2011). Moreover, *JMJD3* depletion up-regulates the expression of EMT inducers and mesenchymal markers, while it down-regulates that of epithelial proteins. Importantly, and in line with data in cultured cells, the expression of *JMJD3* RNA was lower in tumour tissue than in normal tissue in 56% of human colorectal tumours and directly correlates with that of *VDR* (Pereira *et al.* 2011). Recent data indicate that 1,25(OH)₂D₃ modulates the expression of several genes coding for histone demethylases of the Jumonji C domain and lysine-specific demethylase families (Pereira *et al.* 2012). These findings link chromatin epigenetic regulation with the transcriptional activity of 1,25(OH)₂D₃.

In addition, our group has recently performed an miRNA-specific microarray study to identify miRNAs under the control of 1,25(OH)₂D₃ in SW480-ADH cells. We have found that several miRNA species are up-regulated or down-regulated by 1,25(OH)₂D₃ treatment (Álvarez-Díaz *et al.* 2012).

miR-22 (*MIR22*) is induced by $1,25(\text{OH})_2\text{D}_3$ in a time-, dose- and VDR-dependent manner and is required for the anti-proliferative and anti-migratory effects of $1,25(\text{OH})_2\text{D}_3$ in colon cancer cells (Álvarez-Díaz *et al.* 2012). Consistently, *miR-22* expression is lower in tumour than in the matched normal tissue in 78% CRC patients and correlates directly with VDR RNA expression, suggesting that *miR-22* is also a $1,25(\text{OH})_2\text{D}_3$ target *in vivo* and may contribute to the anti-tumoural action of $1,25(\text{OH})_2\text{D}_3$ in human colon cancer (Álvarez-Díaz *et al.* 2012).

The new generation of ChIP-Seq studies mentioned above are expected to contribute to our understanding of $1,25(\text{OH})_2\text{D}_3$ actions on a genome-wide scale, and to the identification of its target genes. In their work, Meyer *et al.* (2012) report new genes activated by $1,25(\text{OH})_2\text{D}_3$ in LS180 colon cancer cells, such as several members of the peptidylarginine deiminase family, a class of enzymes that convert histone methylated residues of arginine to citrulline (Klose & Zhang 2007), thus expanding the number of epigenetic modifiers that are regulated by $1,25(\text{OH})_2\text{D}_3$ in CRC cells.

Proteomic approaches have also identified $1,25(\text{OH})_2\text{D}_3$ -regulated proteins in CRC cells. Our laboratory has used 2D-DIGE electrophoresis combined with MALDI-TOF-TOF mass spectrometry to identify nuclear proteins regulated by $1,25(\text{OH})_2\text{D}_3$ in SW480-ADH cells (Cristobo *et al.* 2011). Interestingly, several of these proteins (SFPQ, SMARCE, KHSRP, TARDBP and PARP1) are involved in the regulation of the spliceosome machinery, suggesting a role of $1,25(\text{OH})_2\text{D}_3$ in alternative splicing, which is frequently altered in cancer (David & Manley 2011, Miura *et al.* 2011). In addition, $1,25(\text{OH})_2\text{D}_3$ also regulates the expression of a few cytoskeletal and actin-binding proteins (EZR, RDX and CORO1C), some of which perform transcription-related functions in the cell nucleus in addition to modulating cell morphology and adhesion (Cristobo *et al.* 2011).

Mechanisms of resistance to $1,25(\text{OH})_2\text{D}_3$ action in CRC

CRC cell responsiveness to $1,25(\text{OH})_2\text{D}_3$ depends mainly on the expression of VDR and on the bioavailability of $1,25(\text{OH})_2\text{D}_3$ within the cell. The intracellular level of $1,25(\text{OH})_2\text{D}_3$ is determined by the circulating levels of $25(\text{OH})\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$, and by the activity of CYP27B1 and CYP24A1 within the cell. CYP27B1 expression and activity are up-regulated at early stages of colorectal tumorigenesis and drastically decreased in advanced CRC,

while those of CYP24A1 are elevated in colorectal carcinomas. These changes lead to attenuated $1,25(\text{OH})_2\text{D}_3$ synthesis and accelerated $1,25(\text{OH})_2\text{D}_3$ catabolism in advanced CRC, causing resistance to $1,25(\text{OH})_2\text{D}_3$ (Bareis *et al.* 2001, Cross *et al.* 2001, Bises *et al.* 2004). As discussed above, several compounds (17β -estradiol, phytoestrogens, folate, calcium, etc.) are known to up-regulate the colonic expression and activity of CYP27B1 and/or to inhibit those of CYP24A1 (Lechner *et al.* 2005, Cross *et al.* 2011). In addition, several CYP24A1 inhibitors have been developed to prolong $1,25(\text{OH})_2\text{D}_3$ biological lifetime (Schuster *et al.* 2003). The use of these compounds in combination with $1,25(\text{OH})_2\text{D}_3$ or its analogues could circumvent the $1,25(\text{OH})_2\text{D}_3$ resistance caused by the aberrant expression and/or activity of these enzymes.

Several studies have shown that VDR is expressed by normal and certain tumour colon epithelial cells (Sheinin *et al.* 2000, González-Sancho *et al.* 2006, Modica *et al.* 2010) and is associated with a high degree of cell differentiation (Shabahang *et al.* 1993, Zhao & Feldman 1993). VDR expression is enhanced in early stages of colorectal tumorigenesis (adenomas, polyps), whereas it decreases in advanced stages (Sheinin *et al.* 2000, Cross *et al.* 2001, Larriba & Muñoz 2005, Matusiak *et al.* 2005, Anderson *et al.* 2006). Accordingly, elevated VDR expression is associated with high tumour differentiation, absence of node involvement and good prognosis in CRC (Cross *et al.* 1996, Evans *et al.* 1998).

VDR down-regulation causes unresponsiveness to $1,25(\text{OH})_2\text{D}_3$ and failure of the therapy with $1,25(\text{OH})_2\text{D}_3$ or its analogues. For this reason, our group has searched for mechanisms to explain the loss of VDR expression in advanced CRC. We found that SNAIL1, a repressor of E-cadherin and a strong EMT inducer, represses the expression of VDR and blocks $1,25(\text{OH})_2\text{D}_3$ anti-tumoural actions in CRC cells in culture and such cells xenografted in mice (Pálmer *et al.* 2004, Larriba *et al.* 2007). Moreover, SNAIL1 is up-regulated in ~60% of colorectal tumours and is associated with VDR down-regulation (Pálmer *et al.* 2004, Peña *et al.* 2005, 2006). In addition to SNAIL1, its family member SNAIL2 (but not other EMT inducers such as ZEB1, ZEB2, TWIST1 or E47) also represses VDR expression and blocks $1,25(\text{OH})_2\text{D}_3$ action in CRC cells (Larriba *et al.* 2009). Moreover, SNAIL1 and SNAIL2 show an additive repressive effect on VDR expression in cultured cells and in colorectal tumours (Larriba *et al.* 2009, 2010). Thus, it is probable that efficient VDR repression during CRC progression requires the expression of both SNAIL1

and SNAIL2, and that their expression in colorectal tumours could be used to identify patients who are likely to be resistant to the therapy with $1,25(\text{OH})_2\text{D}_3$ or its analogues.

Post-transcriptional down-regulation of VDR by miRNAs may be another mechanism responsible for the low VDR levels found in advanced CRC. Thus, it has been reported that *miR-27b* (MIR27B) and *miR-298* (MIR298) reduce VDR levels in LS-180 colon cancer cells (Pan *et al.* 2009). In addition, Mohri *et al.* (2009) have shown that *miR-125b* (MIR125B) down-regulates VDR expression and reduces $1,25(\text{OH})_2\text{D}_3$ action in breast cancer cells. Interestingly, *miR-125b* has been found to be over-expressed in colon cancer metastases (Baffa *et al.* 2009). Therefore, the over-expression of miRNAs that target VDR may constitute a novel strategy used by colon cancer cells to escape from the anti-tumoural action of $1,25(\text{OH})_2\text{D}_3$.

Extensive work by Campbell's group has shown that lack of $1,25(\text{OH})_2\text{D}_3$ sensitivity in prostate and breast cancer cells is associated with elevated expression of the VDR corepressors SMRT and NCoR respectively (Khanim *et al.* 2004, Banwell *et al.* 2006). Accordingly, cotreatment with inhibitors of HDACs (trichostatin A, sodium butyrate) or DNA methyltransferases (5-aza-2'-deoxycytidine) reverses the resistance to the anti-proliferative effects of $1,25(\text{OH})_2\text{D}_3$ in different types of cancer cells, including CRC cells (Abedin *et al.* 2006). These results suggest that altered expression patterns of corepressors inappropriately sustain epigenetic modification at $1,25(\text{OH})_2\text{D}_3$ target genes and generate resistance to $1,25(\text{OH})_2\text{D}_3$ action. However, the effect of sodium butyrate on the sensitivity of CRC cells to $1,25(\text{OH})_2\text{D}_3$ seems to be mainly mediated by butyrate-induced VDR over-expression (Gaschott *et al.* 2001a,b, Gaschott & Stein 2003). Notably, increased levels of VDR corepressors have not been reported in colon cancer, instead, NCoR and SMRT have been found aberrantly located in the cytoplasm of cells from human CRC biopsies (Fernández-Majada *et al.* 2007a,b, Tzelepi *et al.* 2009). Thus, the possible contribution of this epigenetic mechanism to the development of $1,25(\text{OH})_2\text{D}_3$ resistance in CRC is unclear.

Anti-tumoural action of vitamin D in animal models of CRC

Results obtained in several types of studies using experimental animals support a protective and therapeutic action of vitamin D against CRC. Administration of a western-style diet (high in fat and low in vitamin D and calcium) generates colonic crypt

hyperplasia and colon dysplasia in wild-type mice, and increases the incidence of pre-neoplastic lesions in various pre-clinical mouse models of intestinal tumourigenesis. These effects are suppressed upon diet supplementation with calcium and vitamin D (reviewed in Lamprecht & Lipkin (2003) and Ordóñez-Morán *et al.* (2005)).

Human tumour cells implanted s.c. into immunosuppressed mice (xenografts) are commonly used as an *in vivo* approach in pre-clinical anti-cancer drug development. Numerous studies have shown that $1,25(\text{OH})_2\text{D}_3$ and several analogues clearly reduce the growth of colorectal xenografts (reviewed in Ordóñez-Morán *et al.* (2005), Deeb *et al.* (2007) and Kang *et al.* (2011)). Similar results were obtained using different chemical carcinogens (*N*-methyl-*N*-nitrosourea, azoxymethane and others) to generate colorectal tumours in mice or rats. The number of tumours generated after chronic treatment with the carcinogens was reduced by the administration of $1,25(\text{OH})_2\text{D}_3$ or several of its analogues (reviewed in Ordóñez-Morán *et al.* (2005)).

Both $1,25(\text{OH})_2\text{D}_3$ and its less calcaemic derivative $1,25(\text{OH})_2\text{-16-ene-19-nor-24-oxo-D}_3$ reduce tumour load (the sum of all polyp areas) in *Apc*^{min/+} mice (Huerta *et al.* 2002), a mouse model of intestinal carcinogenesis that carries a mutated allele of the tumour suppressor gene *Apc* and spontaneously develops multiple neoplasias throughout the intestinal tract (Su *et al.* 1992). Xu *et al.* (2010) confirmed these results and also found that treatment with $1,25(\text{OH})_2\text{D}_3$ or two of its analogues increases the expression of E-cadherin, inhibits that of Myc and reduces β -catenin nuclear levels in the small intestine and colon of *Apc*^{min/+} mice. However, a recent study by Irving *et al.* (2011) has shown that treatment with $25(\text{OH})\text{D}_3$ or two $1,25(\text{OH})_2\text{D}_3$ analogues did not affect intestinal tumour development in the *Apc*^{Pirc/+} rat or in the *Apc*^{min/+} mouse models in the absence or presence of the colonic tumour inducer dextran sodium sulphate. As the authors comment, it is however possible that the length of treatment with the vitamin D compounds or the putative loss of *Vdr* expression in the tumours might have influenced the results of the study (Irving *et al.* 2011).

Genetically modified mice have also been used to study the effects of the disruption of VDR expression on colorectal tumourigenesis. *Vdr*-deficient mice do not show a spontaneous increase in cancer but are more prone to oncogene- or carcinogen-induced tumours (reviewed in Bouillon *et al.* (2008)). These animals display colonic crypt hyper-proliferation, and increased levels of DNA damage and oxidative stress in the

intestine (Kállay *et al.* 2001, 2002). Recently, two teams have bred $Vdr^{-/+}$ and $Apc^{min/+}$ mice in order to evaluate the consequences of VDR deficiency on the initiation and development of intestinal cancer (Larriba *et al.* 2011, Zheng *et al.* 2012). These studies described increased tumour burden in $Vdr^{-/-}Apc^{min/+}$ mice as compared to $Vdr^{+/+}Apc^{min/+}$. Importantly, $Vdr^{-/-}Apc^{min/+}$ mice also show a higher activation of the WNT/ β -catenin pathway in the lesions and an increase in the number of colonic aberrant crypt foci (Larriba *et al.* 2011).

Human studies: clinical and molecular data

Few large-scale cancer intervention trials on vitamin D treatment in human CRC patients have been reported. The first of these was a randomised, double-blind, placebo-controlled trial of 2686 individuals (65–85 years of age) receiving 100 000 IU vitamin D₃ or placebo every 4 months ($\sim$ 833 IU/day). This study did not find any significant effect of vitamin D₃ supplementation on CRC incidence or mortality after 5 years (Trivedi *et al.* 2003). Accordingly, another randomised, double-blinded, placebo-controlled trial performed by the US Women's Health Initiative, including 36 282 post-menopausal women (50–79 years of age) who were treated with 400 IU/day vitamin D₃ plus 1 g/day calcium vs placebo, failed to find any effect of the treatment on CRC incidence (Wactawski-Wende *et al.* 2006). However, the study had serious drawbacks that challenge its validity: the low amount of vitamin D₃ used did not increase the circulating 25(OH)D₃ levels, the degree of patient adherence to the treatment was low and the follow-up period was short (7 years) (Wactawski-Wende *et al.* 2006). Still, re-analysis of the data showed that concurrent estrogenic therapy increased CRC risk and that the treatment with vitamin D₃ plus calcium was beneficial in its absence (relative risk (RR) 0.71; 95% confidence interval (CI) 0.46–1.09) (Ding *et al.* 2008). The third study with published results was a smaller trial performed in Nebraska (USA) with 1179 post-menopausal women (mean age 67 years) who were treated with 1100 IU/day vitamin D₃ plus 1.4–1.5 g/day calcium vs calcium alone for 4 years (Lappe *et al.* 2007). Treatment with vitamin D₃ plus calcium reduced total cancer incidence (RR 0.40; 95% CI 0.20–0.82), including CRC, particularly if the first-year cases were not considered. However, the low number of CRC cases reduces the validity of the study (Lappe *et al.* 2007).

At the molecular level, Holt *et al.* (2006) showed that combined daily supplementation of 11 patients with vitamin D₃ (400 IU) and calcium (1.5 g) for 6 months reduced the proliferation of colonic flat mucosa and polyps, and increased the expression of BAK1 and diminished that of the CRC-associated mucin MUC5AC in the polyps when compared with the placebo group (8 patients). In a series of recent papers, Fedirko *et al.* reported that 800 IU/day vitamin D₃ and/or 2 g/day calcium (in 92 patients with at least one adenoma, 6 months treatment) increased BAX and p21^{CIP1} expression in colorectal crypts (Fedirko *et al.* 2009a,b). These authors also observed a decreased labelling of the DNA oxidation marker 8-OH-dG (only by vitamin D₃ or calcium alone, but not by the combination) and a reduced expression of the marker of long-term proliferation hTERT in the upper part of the crypt (only in the vitamin D₃ plus calcium group) (Fedirko *et al.* 2009b, 2010). In addition, the group treated with vitamin D₃ alone showed a reduction in the expression of the proinflammatory cytokines TNF- α , IL6, IL1 β and IL8, and the proinflammatory marker C-reactive protein (Hopkins *et al.* 2011).

Clearly, there is a need for large and well-designed randomised, double-blind, placebo-controlled clinical studies. Up to now, clinical trials have included mostly elderly people, frequently post-menopausal women and so on, they may not fully reveal the potential action of vitamin D. Additional problems are the lack of an optimal vitamin D₃ dose to be used, its possible combination with other agents (calcium), and the long follow-up period that seems recommendable in the case of a disease such as CRC, which probably develops over one or two decades. One promising ongoing trial is the VITamin D and Omega-3 Trial (VITAL), a study designed to investigate the role of high doses of vitamin D₃ (2000 IU/day), omega-3 fatty acids (1 g/day) and their combination on cancer and cardiovascular diseases in 20 000 initially healthy men and women aged 60 and 65 years and older respectively (Nicholas 2011, Okereke & Manson 2011). In addition, a recent look at the US National Institutes of Health clinical trials website (<http://clinicaltrials.gov>) showed that more than 1000 trials are currently underway to assess the effects of vitamin D compounds on cancer and other diseases.

Conclusions and perspectives

In summary, the vast majority of molecular and genetic data suggests a protective role of vitamin D against CRC, and epidemiological studies also indicate that vitamin D deficiency is linked to high risk of this

neoplasia. Globally, the results available on the loss of VDR expression and on the alteration of CYP27B1 and CYP24A1 levels during CRC progression support a role for vitamin D in the prevention and/or in the therapy of early stages rather than in the treatment of advanced cases of this neoplasia.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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