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METHYLATION STATUS OF VITAMIN D RECEPTOR GENE PROMOTER IN ADRENOCORTICAL CARCINOMA

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TABLE OF CONTENTS

SUMMARY

Context: Previous data of my research group showed a decreased expression of vitamin D receptor (VDR) mRNA/protein in a small group of adrenocortical carcinoma (ACC) tissues, suggesting the loss of a protective role of VDR against malignant cell growth in this type of cancer. Downregulation of VDR gene expression may result from epigenetics events, that is, methylation of cytosine nucleotide of CpG islands in VDR gene promoter.

Objective: To analyse methylation of CpG sites in the VDR gene promoter in a series of ACC tissue specimens, comparing malignant adrenocortical tumour samples with those from various benign forms and normal adrenals.

Methods: Methylation of CpG-rich 5′ regions was assessed by bisulfite sequencing PCR using bisulfite-treated DNA from archival microdissected paraffin-embedded adrenocortical tissues. Three normal adrenals and twenty-three various adrenocortical tumour samples including eight carcinomas and fifteen adenomas were studied.

Results: Methylation in the promoter region of VDR gene was found in three of eight ACCs, while no VDR gene methylation was observed in normal adrenals and adrenocortical adenomas. VDR mRNA and protein levels were lower in ACCs than in benign tumours. VDR immunostaining was weak or negative in ACCs, including all three methylated tissue samples.

Conclusion: The association between VDR gene promoter methylation and reduced VDR gene expression is not a rare event in ACC, suggesting that VDR epigenetic inactivation may have a role in adrenocortical carcinogenesis. Other epigenetic mechanisms in the upstream signalling pathway involved in silencing VDR gene expression in ACC should be investigated.

INTRODUCTION

PART 1: ADRENOCORTICAL CARCINOMA

1.1 EPIDEMIOLOGY

Adrenal tumours are common, affecting 3% to 10% of the human population, and the majority are small benign non-functional adrenocortical adenomas (ACA). Conversely, adrenocortical carcinoma (ACC) is a very rare disease with an estimated incidence of 0.7-2 cases per million population per year [1,2]. The US database Surveillance, Epidemiology, and End Results (SEER) reports an estimation of incidence of approximately 0.72 per million cases per year leading to 0.2% of all cancer deaths in the United States. The same database provides a mean age of 55 years [2]. The German ACC Registry reports a slightly younger median age at diagnosis of 46 years, and a large single centre series in France reported the same age [3]. Epidemiological data on ACC from larger cancer registries is sparse, as they are often grouped with other endocrine malignancies.

A second peak of increased incidence affect childhood; approximately 1.3% of all childhood cancers are ACCs as opposed to 0.02% to 0.2% of adult cancers, confirming a higher relative incidence early in life [4-7]. In Southern Brazil the incidence during childhood ranges from 2.9 - 4.2 per million per year, and this is mainly dependent on the high prevalence of regional predisposing factors, particularly the p.R337H low penetrance germline mutation of *TP53* [8,9].

In the adult as well as in the paediatric population, there is a predilection for the female gender (ratio of female to male ranges from 1.5–2.5:1) [7,10]. A relative increase of diagnosis of ACC occurs during pregnancy [10,11].

1.2 GENETIC PREDISPOSITION

Most ACCs occur sporadically, but ACCs can also be associated with various genetic syndromes (Table 1), e.g. Li Fraumeni syndrome (LFS) [12], Beckwith–Wiedemann syndrome (BWS) [13], multiple endocrine neoplasia type 1 (MEN1) [14] and Lynch syndrome [15]. To a lesser extent, ACC can be associated with familial adenomatous polyposis (FAP) [16], neurofibromatosis type 1 [17], and Werner syndrome [18].

Hereditary syndromes in patients with ACCs are summarised in Table 1. Aside from genetic predisposition, no risk factor have been firmly established.

Table 1. Hereditary syndrome in patients with ACC [3].

Syndrome	Prevalence in ACC patients	Prevalence in general population	Gene mutation	Phenotype
LFS	3-7% of adults, 50-80% of children	1:20 000 to 1:1 000 000	*TP53*	Sarcoma, choroid plexus tumour, brain cancer, early brest cancer, leukemia, lymphoma
MEN1	1-2% of adults	1:30 000	*MENIN*	Foregut neuroendocrine tumours, pituitary tumours, parathyroid hyperplasia, collagenoma, angiofibroma, adrenal adenoma/hyperplasia
Lynch syndrome	3% of adults	1:440	*MSH2, MSH6, MLH1, PMS2*	Colorectal cancer, endometrial cancer, sebaceous neoplasms, ovarian cancer, brain cancer
BWS	Very rare, only children	1:13 000	*IGF2, CDKN1C, H19* locus changes on 11p15	Wilm's tumour, hepatoblastoma, macrosomia, adrenocortical cytomegaly, adreanle adenoma, adrenal cyst, hemihypertrophy, macroglossia, omphalocele, ear pits
FAP	Very rare (<1%)	1: 30 000	*APC*	Intestinal polyps, colon cancer, duodenal carcinoma, thyroid cancer, desmoid tumour, adrenal adenoma, supernumerary teeth, congenital hypertrophy of the retina, osteoma, epidermoid cysts
NF1	Very rare (<1%)	1: 3 000	*NF1*	Malignant peripheral nerve sheet tumour, pheocromocytoma, cafè au lait spots, neurofibroma, optic glioma, Lisch nodule, skeletal abnormalities
Carney complex	Very rare (case reports)	~700 cases worldwide	*PRKAR1A*	Primary pigmented nodular adrenal disease, large cell calcifying Sertoli cell tumours, thyroid adenoma, myxoma, somatotroph pituitary adenoma, lentigines

The relative high incidence of ACC in childhood is mainly explained by germline *TP53* mutations, which are the underlying genetic cause of ACC in ~50% to 80% of children with ACC [19]. Childhood ACC is a core malignancy of LFS syndrome. Approximately 3% to 10% of LFS-associated cancers are ACCs, suggesting that germline *TP53* mutations infer a significant contribution to increase risk of ACC development [20]. Prevalence *TP53* mutations in ACC adult patients ranges between 3% and 7% [21]. *TP53* germline testing is recommended for any patient with a diagnosis of ACC [22], even when family history is lacking. Up to 25% of *TP53* mutations occur de novo. Most of *TP53* mutation affect the DNA binding and tetramerization domains. One particular hot spot

mutation has been reported, the low penetrance tetramerization domain p.R337H in Southern Brazil [23], related to a funder effect in most cases.

A hallmark of BWS is alteration of DNA methylation of the 11p15 locus, which harbours the coding regions for *IGF2*, the cell cycle regulator *CDKN1C*, and the non translated RNA *H19*. The result is an upregulation of *IGF2* expression and a downregulation of other two transcripts. ACC comprises 5-15% of malignancies in BWS. Cancer risk of children with BWS decreases through adolescence and then remains at the level of the general population [13].

A small fraction of patients with MEN1 will develop ACC, but in this setting adrenal lesions are very frequent and require special attention in order to recognise signs suggestive of ACC [14].

Recently, a systematic analysis reported the prevalence of Lynch syndrome in patients with ACC to be near to 3%. All tested ACCs resulted microsatellite stable [15].

Carney complex is a familial lentiginosis syndrome caused by *PRKAR1A* mutations and perturbations of the cyclic AMP-dependent protein kinase (PKA) signalling pathway. In addition to the cutaneous findings, the main tumours associated with Carney complex are endocrine. ACC has been reported in 2 cases of patients with Carney complex [24,25].

The association between ACC and hereditary cancer syndromes led to the discovery of the role of several genes and signalling pathways involved in adrenocortical tumorigenesis, such as β-catenin signalling (FAP) and IGF-1 (BWS).

1.3 CLINICAL PRESENTATION

Clinical presentation of patients with ACC can be heterogeneous. Symptoms and signs of hormone excess are the major complaints in up to 60% of ACC patients. About a third of patients present with nonspecific symptoms, due to local tumour growth, such as abdominal or flank pain, abdominal fullness, or early satiety [10]. In the remaining cases (20% to 30%), ACCs are incidentally diagnosed by imaging procedures for unrelated medical issues. Paraneoplastic syndromes are uncommon, but hypoglycaemia could occur and is attributed to IGF-2; other rare paraneoplastic syndromes are hyperreninemic hyperaldosteronism, erythropoietin-associated polycythemia, and leucocytosis [26-28].

Generally, signs/symptoms of hormone excess are often not readily recognized by physicians, leading to delay in diagnosis and subsequent surgical and/or medical therapy.

Hypercortisolism is the most common presentation of patients presenting with hormone excess (50%–80% of hormone-secreting ACCs). Frequently, very high cortisol levels in ACC saturate the renal 11-Beta-Hydroxysteroid Dehydrogenase Type 2 (HSD11B2) system, resulting in glucocorticoid-mediated mineralocorticoid receptor activation. Therefore, hypokalemia and hypertension are commonly observed in ACC patients with hypercortisolism. The second most commonly produced hormones in patients with ACC are adrenal androgens (40%–60% of hormone-secreting ACCs), leading to hirsutism, virilisation, and menstrual irregularities in women. Concurrent androgen and cortisol production is not rare. Oestrogen production occurs in 1% to 3% of male ACC patients, causing gynecomastia and testicular atrophy. Androgen or oestrogen overproduction should always raise the suspicion of a malignant tumour. Steroid precursors such as 11-deoxycorticosterone could mimicry mineralocorticoid effects [29,30].

At the time of presentation, ACCs are generally large tumours, measuring on average 10 to 13 cm [31,32]. A minority of tumours are < 6 cm (9%–14%), with only 3% presenting as lesions < 4 cm [31,33]. Contralateral metachronous or synchronous adrenal tumours can be found in ~5% of patients. The most common metastatic sites are lung (40%–80%), liver (40%–90%), and bone (5%–20%) [3].

1.4 DIAGNOSTIC WORK-UP

The initial evaluation should include patient history, family history to identify possible hereditary contribution, and physical examination with particular respect to symptoms and signs of hormone excess. Patients should undergo biochemical assessment with particular respect to hormonal workup. Staging should at the minimum include a computed tomography (CT) scan or magnetic resonance imaging (MRI) of the abdomen/pelvis and a CT of the chest. Other imaging should be guided by clinical suspicion.

1.4.1 Biochemistry

Measurement of steroid hormones produced by the tumour is the hallmark of biochemical evaluation in ACC. To note, biochemical exclusion of a pheochromocytoma by measuring levels of metanephrine and normetanephrine in plasma or 24-hour urine is mandatory, especially when no steroid hormone production is evident, in order to prevent unexpected complications during surgery or treatment.

Suppressed ACTH (≤10 pg/mL) and increased cortisol on a blood sample collected at 8:00 AM are common features in patients with cortisol-secreting tumours. Diagnosis of hypercortisolism is usually established by a 1-mg dexamethasone suppression test (DST), midnight salivary cortisol, or elevated 24-hour urine free cortisol. Screening for aldosterone production includes measurement of plasma renin activity and serum aldosterone levels. Isolated suppression of renin without elevated levels of aldosterone is related to volume repletion, reflecting the mineralocorticoid activity of cortisol and/or steroid precursors. Dehydroepiandrosterone sulfate (DHEAS) and total or bioavailable testosterone should be measured in every patient. Measurement of other steroid metabolites, such as 17-hydroxyprogesterone (17-OH-progesterone), androstenedione, and oestrogen is generally recommended, allowing specific treatment with hormonal antagonists to alleviate symptoms [3].

ENSAT suggested preoperative laboratory workup comprises assessment of basal cortisol, ACTH, DHEAS, 17-OH-progesterone, androstenedione, testosterone, and oestradiol as well as a dexamethasone suppression test and urinary free cortisol excretion. Aldosterone/renin ratio is measured in patients with hypertension or hypokalemia. Although the cost effectiveness of this approach is unproven, this extensive panel appears useful for several reasons: it may prove the adrenocortical origin of the lesion, suggest malignancy, and document autonomous glucocorticoid excess that, if missed, regularly entails postoperative adrenal failure [1].

Despite the presence of a large tumour, signs or symptoms of steroid hormone excess and blood levels of hormones in ACC can be absent or minimal. In fact, in comparison with the normal adrenal cortex, steroid hormone synthesis in ACC is relatively inefficient, resulting in elevated levels of a variety of steroid hormone precursors but only modestly elevated hormone levels, even in the presence of a large lesion. Although most of these metabolites are not routinely measured clinically, they can be detected by gas chromatography/mass spectrometry analysis [3]. Arl et coll. identified 11-deoxycortisol metabolite tetrahydro-11-deoxycortisol as the most discriminative marker, although integrated profile of several metabolites provided more information. Routine use of the recently introduced urine steroid metabolomic analysis might further increase this number and may serve as a fingerprint of the tumour, facilitating early detection of recurrence [34].

1.4.2 Imaging

Together with a careful endocrine workup, modern cross-sectional imaging is able to correctly diagnose an adrenal mass as ACC before surgery in most cases. ACCs are generally large tumours, often measuring more than 6 cm in diameter and frequently combining the presence of internal haemorrhage, necrosis, and calcification. Contrast-enhanced computed tomography (CT) or magnetic resonance imaging (MRI) is the diagnostic imaging modality of choice for initial imaging and staging as well as for detecting local recurrence and metastatic disease [35]. Functional imaging by positron emission tomography (PET) with [^{18}F]fluorodeoxyglucose (FDG) and [^{11}C]Metomidate (MTO) or [^{123}I]MTO (where available) may be used to confirm diagnosis of a malignant lesion or establish the adrenocortical origin of a tumour. [131I]-iodocholesterol scans are no longer available.

CT and MRI

On CT imaging, ACCs are large and heterogeneous masses and can be distinguished from lipid-rich ACAs, which tend to be small, homogeneous masses that measure ≤ 10 Hounsfield Units (HU) on unenhanced CT. On state-of-the-art MRI, ACCs appear isointense to hypointense relative to liver parenchyma on T1-weighted images and hyperintense relative to liver parenchyma on T2-weighted images, and demonstrate loss of signal on chemical-shift MRI. In ACC, contrast-enhanced imaging often demonstrates heterogeneous, predominantly irregular peripheral enhancement with central non-enhancing areas due to haemorrhage or necrosis. Internal hemorrhage is seen as ill-defined areas of increased attenuation on non–contrast-enhanced CT and as areas of high signal intensity on T1-weighted images. Areas of necrosis have low attenuation on non–contrast-enhanced CT, high signal intensity on T2-weighted images and do not enhance after administration of intravenous contrast [36]. Calcifications can be present in approximately 30% of cases and are best detected on CT imaging; calcification is not a distinguishing feature as it is also present in other adrenal pathologies such as myelolipoma and approximately 10% of pheochromocytomas. Due to the multiplanar capability of MRI, direct invasion of adjacent organs may be better depicted [3].

[^{18}F]FDG PET/CT imaging

ACCs typically present intense FDG uptake greater than liver background. In a study of 77 patients with surgically proven diagnosis of ACA or ACC, [^{18}F]FDG PET/CT had a sensitivity of 100% and specificity of 88% in distinguishing benign from malignant

lesions by using cut-off value above 1.45 for adrenal to liver maximum standardized uptake value (SUV) [37]. In a meta-analysis of published data aimed at determining the diagnostic utility of [^{18}F]FDG PET/CT for distinguishing benign from malignant adrenal tumours, [^{18}F]FDG PET/CT had sensitivity of 97% and specificity of 91%. [^{18}F]FDG PET/CT, however, is not a tumour-specific tracer and cannot distinguish ACC from other pathologies like metastases, lymphoma, or pheochromocytoma, which also have high metabolic activity [38]. [^{18}F]FDG PET/CT can be an imaging modality complementary to CT for evaluating local recurrence or diagnosis of metastasis in selected cases, but sensitivity decreases for lesions less than 1 cm in diameter. A maximum SUV of >10 was found to be related to survival, indicating poor prognosis [39].

Experimental imaging modalities

Proton MR spectroscopy may be helpful in differentiating ACAs and pheochromocytomas from ACC and metastases using choline to creatine ratios of greater than 1.2 (92% sensitivity and 96% specificity) and choline to lipid ratios greater than 0.38 (92% sensitivity and 90% specificity) [35]. However, more research data and prospective clinical evaluation are needed to substantiate this approach. Metomidate is an inhibitor of 11β-Hydroxylase (CYP11B1) and aldosterone synthetase (CYP11B2), [^{11}C]Metomidate and [123I]Iodometomidate are highly specific tracers for PET-imaging of adrenocortical tissue but they cannot distinguish benign from malignant lesions [40].

1.5 STAGING

Consensus has been obtained during the last years that the tumour staging classification suggested by the European Network for the Study of Adrenal Tumors (ENSAT), reliably predicts the outcome of patients (Table 2) [41,42]. In this staging system, which is a modification of the Lee classification from 1995, stage I and stage II are defined as strictly localized tumours with a size of ≤ 5cm or > 5 cm, respectively. Stage III tumours are characterized by infiltration in surrounding tissue, positive regional lymph nodes, or a tumour thrombus in the vena cava/renal vein. Stage IV is restricted to patients with distant metastasis. Although this staging system can differentiate patient cohorts with different prognosis and a 5-year stage-dependent survival of 81, 61, 50, and 13% [42] (Figure 1), there is a need for further improvements; e.g., by adding a grading system [43]. Recently,

Asare et al. reported that predicting 5-year overall survival rates in patients with stage I/II ACC would improve if patient age is added to the ENSAT staging [44].

ENSAT Staging System for ACC	
STAGE I	T1, N0, M0
STAGE II	T2, N0, M0
STAGE III	T1-2, N1, M0 T3-4, N0, M0
STAGE IV	T1-4, N0-1, M1

Table 2. ENSAT staging system for ACC. Tumours are classified as follows: T1, tumour ≤5 cm; T2, tumour >5 cm; T3, tumour infiltration into surrounding (fat) tissue; T4, tumour invasion into adjacent organs or venous thrombus in vena cava or renal vein; N0, no spread into nearby lymph nodes; N1, positive lymph node(s); M0, no distant metastasis; M1, presence of distant metastasis [3].

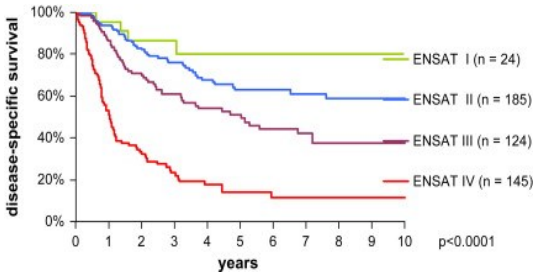


Figure 1. Disease-specific survival stratified according to the European Network for the Study of Adrenal Tumors (ENSAT) staging classification for adrenocortical carcinoma (ACC) using Kaplan-Meier analysis. Disease-specific survival was defined as the time elapsed from primary diagnosis to death from ACC. Patients who were alive or who had died of other causes were censored. Four hundred sixteen patients were analysed (stage I: n=23 patients, 4 deaths, 19 censored; stage II: n=164 patients, 48 deaths, 116 censored; stage III: n=107 patients, 52 deaths, 55 censored; stage IV: n=122 patients, 89 deaths, 33 censored). HR indicates estimated hazard ratio (with 95% confidence intervals are indicated in parentheses); P indicates log-rank P value assessing differences [31].

1.6 PATHOLOGY

The Weiss score is currently the most widely employed classification system for the pathological assessment of adrenocortical tumours [45]. It is based on the recognition at light microscopy of at least three among nine morphological parameters [46] that are focused on invasion by tumour into capsule and adjacent vessels, changes in growth patterns, presence of tumour necrosis, increased mitotic rates, and the presence of atypical mitotic figures. Tumours with an abundance of these features (score 3 or more according to the Weiss system) most often behave in a malignant fashion and can be classified as ACC, whereas tumours without these features (score 0–2 in the Weiss system) do not metastasize and can be classified as ACA. The Weiss scoring system is able to diagnose metastatic ACC with 100% sensitivity and 99.4% specificity.

More simplified algorithms have been proposed because of the lack of reliability for some Weiss criteria (Table 3). This modified system, based on the most reliable criteria (2 x mitotic rate + 2 x cytoplasm + abnormal mitoses + necrosis + capsular invasion) has a significant correlation with the Weiss system (r = 0.98) [47]. The Helsinki score, developed by Pennanen et al., consists of the sum of 3 x mitotic rate + 5 x presence of necrosis + maximum proliferation index and it has been recently validated [48,49].

Table 3. Original Weiss criteria and modified Weiss criteria

Original Weiss criteria for malignancy [50] (requires 3+ of these factors):

- ✓ Nuclear grade III or IV based on Fuhrman criteria
- ✓ > 5 mitotic figures/50 HPF (40x objective), counting 10 random fields in area of greatest number of mitotic figures on 5 slides with greatest number of mitoses
- ✓ Presence of atypical mitotic figures (abnormal distribution of chromosomes or excessive number of mitotic spindles)
- ✓ Clear or vacuolated cells comprising 25% or less of tumour
- ✓ Diffuse architecture (more than 1/3 of tumour forms patternless sheets of cells; trabecular, cord, columnar, alveolar or nesting pattern is not considered to be diffuse)
- ✓ Microscopic necrosis
- ✓ Venous invasion (veins must have smooth muscle in wall; tumour cell clusters or sheets forming polypoid projections into vessel lumen or polypoid tumour thrombi covered by endothelial layer)
- ✓ Sinusoidal invasion (sinusoid is endothelial lined vessel in adrenal gland with little supportive tissue; consider only sinusoids within tumour)
- ✓ Capsular invasion (nests or cords of tumour extending into or through capsule with a stromal reaction); either incomplete or complete

Each criterion is scored 0 when absent and 1 when present in the tumour

Modified Weiss criteria [47] (score of 3 or more suggests malignancy):

- ✓ Mitotic rate > 5 per 50 high power fields
- ✓ Cytoplasm (clear cells comprising 25% or less of the tumour)
- ✓ Abnormal mitoses
- ✓ Necrosis
- ✓ Capsular invasion
- ✓ Calculate: 2 x mitotic rate criterion + 2 x clear cytoplasm criterion + abnormal mitoses + necrosis + capsular invasion

Each criterion is scored 0 when absent and 1 when present in the tumour

The reliability of the Weiss score is challenged in borderline cases, where a Weiss score of 2 can be suggestive for ACC [51,52]. The Weiss score lacks reproducibility and is difficult to apply in paediatric ACCs and in ACC variants. The most common is the oncocytic variant because the predominant cell type in this variant is an oncocyte, a cell with abundant, granular cytoplasm related to accumulation of mitochondria and endoplasmic reticulum. To prevent overdiagnosis in oncocytic variants with the classic Weiss score, an alternative diagnostic system was proposed [53] and also validated to correctly predict malignancy in this ACC subtype [54]. Rare ACC variants are the myxoid

variety, due to the production of abundant extracellular myxoid substances, and the sarcomatoid variety (carcinosarcoma), both generally portending aggressive tumour behaviour [55].

Because of these diagnostically challenging cases, many pathologists have tried to develop ancillary techniques to refine the approach to these tumours. Volante et al. demonstrated that disruption of reticular networks, defined as the loss of continuity of reticular fibres or basal membrane network as highlighted by histochemical staining was present in all 92 ACCs included in their study. This observation is related to the altered growth pattern observed in ACCs and reflects one of the Weiss criteria (diffuse growth pattern greater than 25%). By adding at least one of the following three parameters – necrosis, high mitotic rate or vascular invasion – this reticulin algorithm identified malignancy with a sensitivity and specificity of 100% [56]. After a specific training, the interobserver reproducibility was 86% in a study aiming to validate the presence of reticulin fibre disruptive changes in a series of 178 adrenocortical tumours [57]. This simple approach is intriguing and awaits further validation, concerning cortical tumour variants like oncocytic and myxoid subtypes [55].

In addition to histochemical approaches, adrenal pathologists developed immunohistochemical methods in order to separate benign from malignant tumours. Most of these studies focus on tumour cell proliferation [3]. Using accepted proliferation immunomarkers, such as Ki67, a general consensus has emerged that ACCs have a Ki67 labelling index > 5%. Conversely, ACAs generally show a much lower index, although there is some overlap observed depending on the particular study. Ki67 evaluation seems to be reproducible, with intra- and inter-observer differences of 3.7 and 4.2% respectively [58]. Proliferation markers generally correlate with mitotic accounts and do have a role to play in the evaluation of these tumours. In a large study (n=319, validation cohort n=250; all patients after complete resection of the tumour) evaluating the prognostic value of histopathological, clinical and immunohistochemical markers, Ki67 alone most powerfully predicted recurrence-free and overall survival [59]. In addition, the Authors recommend that based on their results Ki67 should be introduced in the routine pathology for adrenocortical tumours.

ACCs can be graded into low- and high-grade based on their mitotic rates (≤ 20 mitoses per 50 high-power fields [HPFs] vs >20 mitoses per 50 HPFs). ACCs exhibit a large degree of intra-tumour heterogeneity, an unsurprising finding given their large size and

the evolutionary nature of cancer progression. Tumours consisting of numerous areas with different histological phenotypes are not uncommon. Similarly, it is possible to find tumour nodules within a given mass with different immunohistochemical phenotypes. These observations provide support for a clonal model in which ACC can exhibit step-wise progression from low to high-grade carcinoma [60].

Most adrenocortical tumours are readily apparent on routine hematoxylin and eosin stains and do not require supplemental immunostains to document adrenocortical differentiation. In difficult cases, a battery of immunostains could provide evidence of adrenocortical differentiation, including but not limited to the following proteins that are expressed in most ACCs: α-inhibin, calretinin, synaptophysin, melanA (Mart1), and steroidogenic factor 1 (SF1). In general, ACC does not express the common cytokeratins most often used in practice. Chromogranin A expression is universally not present, and if it is present, an adrenomedullary tumour should be strongly considered [3].
Primary or metastatic tumours of unknown origin would involve a larger panel of the adrenocortical and adrenomedullary markers as well as other non-adrenal markers. The most common tumours metastasizing to the adrenal gland are lung carcinoma, melanoma, renal cell carcinoma, and breast carcinoma. With the exception of renal cell carcinoma, these tumours generally possess a distinct morphology that will immediately suggest metastatic disease. Bilateral adrenal masses strongly suggest metastatic carcinoma or lymphoma.

Much work is proceeding on how the molecular pathobiology of adrenocortical tumours can be translated into practical tools that will enhance the routine pathological evaluation of these tumours beyond standard histopathology, immunohistopathology, mitotic grading, and tumour staging.

1.7 MOLECULAR PATHOLOGY

Classical genetic tools (i.e., DNA content assessment, metaphase spreads, and comparative genomic hybridization [CGH]) and the advent of high resolution analytic methods (i.e., tiled arrays and whole genome sequencing) have revealed several genomic aberrations and molecular markers that are predicted to contribute to neoplastic transformation of adrenocortical cells.

1.7.1 DNA content

Aneuploidy is a genomic aberration related to chromosomal instability consistently reported in most cancers. One of the first genetic study on ACC based on flow cytometry showed aneuploidy in 4 of 4 ACCs, yet only diploidy or tetraploidy in normal adrenal cortices and benign adrenal tumours [61]. The results were validated in other two studies, reporting aneuploidy in 5 of 6 ACCs [62] and in 6 of 8 ACC samples [63] respectively, the latter one revealing a high correlation with Weiss score > 3. No significant difference in overall survival was observed in patients with ACC exhibiting aneuploidy vs patients with ACC exhibiting diploid neoplasms. Further investigation is required in order to establish the role of aneuploidy and hyperploidy as etiological factors that drive tumorigenesis or as an epiphenomenon.

1.7.2 Chromosomal aberrations

Comparative genomic hybridization (CGH) can identify structural chromosomal abnormalities within ACCs at a higher resolution. A complex pattern of chromosomal alterations occurs in ACCs, while ACAs present few regions of chromosomal gains and losses [64], suggesting that genes critical for carcinogenesis, i.e. oncogenes and tumour suppressor genes, rely on regions of gains and losses respectively.

In ACCs, chromosomal gains were frequently observed in regions 4q, 4p16, 5p15, 5q12–13, 5q32-qter, 9q34, 12q13, 12q24, and 19p, and chromosomal losses were reported at 1p, 2q, 11q 17p, 22p, and 22q. Interestingly, 9q34 contain the steroidogenic factor 1 (*SF1*) gene, supporting the hypothesis of its direct involvement in adrenocortical cell proliferation [65]. Microsatellite studies identified frequent allelic losses in regions 17p13, 11q15, and 2p16 (85%, 92%, and 90% of samples, respectively) [66]. Gains in chromosome 5 and 12 with additional gains in chromosome 7 and 16 were identified in a series of adrenocortical tumours including both ACCs and ACAs. The same study reported multiple loci of high-level, multiple amplifications specifically at 19p13.3 and 19q13.4 and revealed a positive correlation between the number of aberrations and the size of tumours [67].

A recent study with higher-resolution CGH arrays in a large series encompassing 86 ACAs and 52 ACCs confirmed increased alterations in ACCs (44%) compared with ACAs (10%). In ACCs, the frequently observed chromosomal gains at 5, 7, 12, 16, 19, and 20 and losses at 13 and 22. The group identified genes within these regions with potential tumorigenic potential including fibroblast growth factor 4 (*FGF4*), cyclin-

dependent kinase 4 (*CDK4*), and cyclin E1 (*CCNE1*). Moreover, Barreau et al. also developed a diagnostic tool to identify malignancy of adrenal tumours with a sensitivity of 100% and a specificity of 83% by combining DNA copy number estimates at six loci (5q, 7p, 11p, 13q, 16q, and 22q). Cluster analysis based on gains and losses in DNA could also identify two groups of ACC with different survival rates [64].

A separate CGH study identified a similar increase in copy number in chromosomes 5, 6q, 7, 8q, 12, 16q, and 20 and allelic losses in 1, 2q, 3, 6p, 7p, 8p, 9, 10, 11, 13q, 14q, 15q, 16, 17, 19q, and 22q. A subgroup of these alterations (gains in 6q, 7q, and 12q and losses in chromosomes 3, 8 10p, 16q, 17q, and 19q) resulted associated with decreased overall survival [3].

Partly in concordance with the previous reports, chromosomes 1, 5, 7, and 12 were selected to separate ACCs (n=22) from ACAs (n=24), which appeared more evident when considering only chromosome 5 [68]. More recently, frequent recurrent copy number variations were identified at 5p15 and deletions at 22q12.1 [69]. Regions contain *TERT*, encoding telomerase reverse transcriptase, and the *ZNRF3* gene, which is recently reported to act as a tumour suppressor gene respectively.

All together, these studies indicate genetic diversity and heterogeneity of chromosomal gains and losses in ACC. The utility of chromosomal aberrations in diagnosing malignancy of adrenocortical tumours remains to be fully elucidated.

1.7.3. Differential gene expression

Global gene expression studies aim to identify biomarkers that could provide diagnostic and prognostic utility in addition to the classic histological analyses. Furthermore, this approach hold the promise of new potential targets for ACC therapy.

ACAs and ACCs show distinct gene expression profiles [70-72]. *IGF2* is the most widely known overexpressed gene in ACCs, nevertheless *IGF2* alone is not able to sufficiently distinguish ACCs from ACAs. Using microarray analysis, De Frapoint et al. identified two clusters of genes whose combined levels of expression could correctly discriminate ACCs from ACAs: 75% of ACCs expressed high levels of eight genes of the *IGF2* cluster, whereas 93% of ACAs highly expressed fourteen genes representing the steroidogenic cluster [70]. Soon et al. reported a high diagnostic accuracy (96% sensitivity, 100% specificity) combining *IGF2* and Ki67. *MAD2L1*, *CCNB1*, *ABLIM1*, *NAV3*, *SEPT4*, and *RPRM* were identified differentially expressed in ACCs compared to ACAs [73]. Among

a group of 614 genes, *TOP2A* and *IGF2*, *CCNB2*, *CDC2*, *CDC25C* and *CDKN1C* were the most differentially expressed genes in the series analysed by Tombol et al. [74].
Recently, several Authors have correlated expression profiles in ACC with clinical outcome. Specifically, Giordano et al. [72] determined that ACCs with high histological grade exhibited marked overexpression of cell cycle and functional aneuploidy genes, which correlated with decreased overall survival. After reporting *ALDH1A1*, *IGF2*, *USP4* and *UFD1L* as the four most upregulated genes in ACCs compared with ACAs, Laurell et al. employed hierarchical clustering and identified two subclusters of patients with short survival (<9 months) and long survival (>67 months) [75].
Expression levels of *BUB1B* and *PINK1* alone identified subgroups of paediatric ACCs with different overall survival, regardless of tumour stage. Similarly, the expression levels of *DLG7* and *PINK1* identified subgroups of ACCs with distinct disease-free survival, regardless of tumour grade [71]. These findings were later validated in a separate cohort of adult patients [76].

1.7.4 DNA methylation

DNA methylation involves the addition of a methyl group to the cytosine pyrimidine ring or adenine purine ring, occurring typically at CpG dinucleotides. It acts as a regulatory mechanism for proper gene expression in normal cells. Aberrant methylation is a mechanism of altered gene expression often occurring in tumorigenesis [77]. To date, research has focused on candidate gene approaches as well as genome-wide methylation level analysis.

Insights into the possible role of gene methylation in ACC tumorigenesis come from the observation of the association of ACC with the Beckwith-Wiedemann syndrome. Many of these subjects show abnormal DNA methylation in different areas of 11p15 chromosomal region – containing *IGF2*, *H19*, and *CDKN1C* – meaning that normal epigenetic marks that regulate imprinted genes in this region are altered. As a result, overactivity of the *IGF2* gene and/or no active copy of the antiproliferative gene *CDKN1C* occur. In sporadic ACC, DNA methylation of the *H19* promoter has been shown to be correlated with *H19* and *IGF2* expression [78]. Very recently, Creemers et al. analysed methylation of regulatory regions of *IGF2* using pyrosequencing, and they found that specific methylation patterns of these regions can discriminate ACCs from ACAs with high diagnostic accuracy [79]. In contrast to some other cancer types, *TP53*

methylation is not reported as a mechanism of tumour suppressor gene inactivation in ACC [80].

A genome-wide approach to study methylation status was first performed by Rechache et al. Global hypomethylation was found in primary (n=8) and metastatic (n=12) ACC samples compared to normal adrenals (n=19) and ACAs (n=48). Fifty-two genes were down-regulated and hypermethylated in primary adrenocortical tumour samples, suggesting methylation as a potential regulator of expression in ACC [81].

Fonseca et al. analysed 27578 CpG sites in 6 normal adrenals, 27 ACAs and 15 ACCs. 212 CpG islands in promoter regions of genes involved in cell cycle regulation, apoptosis, and transcriptional regulation, were significantly hypermethylated in ACCs compared to ACAs and normal adrenal tissues. Of six selected genes, mRNA expression levels were concordantly significantly reduced in ACCs compared to ACAs and normal adrenal tissue [82].

Along with this finding, Barreau et al. also confirmed ACC-specific hypermethylation in promoter regions in a series of 51 ACCs and 84 ACAs, identifying *H19*, *PLAGL1*, *G0S2*, and *NDRG2* as silenced genes. In addition, the same Authors also correlated the methylation levels with prognostic features in patients with ACC [83] (for details see the section 'Prognostic factors and predictive markers').

1.7.5 MicroRNAs

MicroRNAs (miRNAs) are evolutionarily conserved, non-coding, 18- to 25-nucleotide RNAs that are involved in post-transcriptional regulation of gene expression [84]. Mature miRNAs in association with the RNA induced silencing complex are loaded onto the 3′-untranslated region of the targeted mRNA to inhibit translation or to cause degradation. Dysregulation of miRNAs, such as overexpression or deletion, plays an important role in various diseases, including cancer [85].

Examination of 36 adrenocortical samples (10 normal tissues, 10 non-functional ACAs, 9 cortisol-secreting adenomas, and 7 ACCs) revealed differential expression of 22 miRNAs, with 14 miRNAs upregulated in ACCs. Preferentially expressed miRNAs in ACCs included miR-184, miR-210, and miR-503. Downregulated miRNAs included miR-214, miR-375, and miR-511. Levels of miR-184, miR-503, and miR-511 alone were able to distinguish benign from malignant adrenal tumours (specificity, 80%–97%; sensitivity, 100%) [74].

Similarly, in a series of 55 adrenal samples (6 normal tissues, 22 ACAs, and 27 ACCs) Soon et al identified 14 upregulated miRNAs and 9 downregulated miRNAs unique to ACC [86]. MiR-483-5p and miR-483-3p are the most overexpressed miRNAs in ACCs compared to ACAs, whereas miR-195 is often found underexpressed, comparably with other reports [87-89]. MiR-483, which is located in an intron of IGF2, was found to be significantly upregulated in paediatric ACCs, although a majority of the differentially expressed miRNAs were downregulated in ACCs, particularly miR-99a and miR-100, both involved in IGF1 signalling pathway [90].

Combinations of several miRNAs (miR-483-5p, miR-195, miR-503, miR-511, miR-335, miR-675, miR-139-3p) have been proposed as a tool for identifying malignancy of adrenal tumours [74,87]. Moreover, overexpression of miRNA-processing enzymes, particularly TARBP2, strongly discriminated carcinomas from adenomas [91] but data still need a validation for clinical use.

1.7.6 Gene mutations

Targeted genetic analyses, such as sequencing and single-strand confirmation analyses have identified somatic genetic changes in *TP53*, *MEN1*, *IGF2*, *IGF2R*, and *CDKN2A* (*p16/INK4A*).

The association of TP53 gene mutations with ACC has been discovered in the setting of the Li–Fraumeni syndrome. *TP53* located on 17p13 is the most commonly mutated gene in ACC, present in at least one third of ACCs [92], but other more recent studies reported frequencies of *TP53* mutations ranging from 15 to 19.5% in ACC [69,93,94]. Prevalence of *TP53* mutations is higher in paediatric ACC [55].

The second most frequently mutated driver gene in ACC is *CTNNB1* (*β-catenin*). Mutations in *CTNNB1* lead to activation of the WNT signalling pathway and these mutations have been shown to be a common event in both ACCs and ACAs, varying from 20 to 30% of samples [95]. Upregulation of β-catenin in adrenocortical tumours was also confirmed with immunohistochemistry [96]. More recently, the high frequency of *CTNNB1* mutations in ACC was confirmed by several studies, which reported somatic mutation frequencies of 10–16% [69,93,94]. Notably, *TP53* and *CTNNB1* mutations are mutually exclusive.

Recently, Assié et al. identified *ZNRF3* as a new tumour suppressor gene driving ACC pathogenesis, with inactivation of *ZNRF* in 21% of ACCs. The frequency of *ZNRF3*

mutations was even higher than *TP53* mutations (16%). Inactivation was caused by a homozygous deletion in 75% of the mutated cases, whereas the other 25% were caused by missense and nonsense mutations [94]. In addition, mutations in *ZNRF3* and *CTNNB1* appeared to be mutually exclusive. A second recent study confirmed this mutually exclusive behavior, although the frequency of *ZNRF3* mutations was lower (10%) compared to the former study [69].

Other genes frequently mutated in ACC include *ATM* (~13%), *CDKN2A* (~11%), *RB1* (~4 to 7%), *MEN1* (~7%), *KREMEN1* (~7%), *DAXX* (~6%), *TERT* (~6%), *MED12* (~5%) *and JAK3* (~4%), which almost always co-occurs with mutations in *TP53, CTNNB1*, or *ZNRF3* [69,93,94,97]. Three additional studies screened for *EGFR* mutations in ACC and reported different frequencies, i.e. 0, 11 and 0% [55]. Four studies have screened ACCs simultaneously for mutations and copy number alterations using (targeted) next generation sequencing and CGH. In the first study, in which a large number of structural DNA changes in ACC was analysed, *TP53* was found to be mutated in 15% of cases, *ATM* in 12.5% of cases and *CTNNB1* in 10% [93]. Most frequent copy number alterations were amplification of the *CDK4* gene, and deletion of the *CDKN2A and CDKN2B* genes. Interestingly, these genes are known actors of the RB/E2F pathway. Overall, 19/40 ACCs (47.5%) had at least one molecular abnormality. In a second study, Ross et al. performed a comprehensive genomic profiling of 29 ACC samples and found at least one alteration (a mutation, amplification, deletion, or truncation) in 22 cases (76%). Genomic alterations in *NF1* (14%), *CDKN2A* (14%), *ATM* (10%), *CCND2* (7%), *CDK4* (7%) and *DNMT3A* (7%) were considered as the most common and potentially clinically relevant at the same time [98]. The third study showed, considering the different omics classifications, a strong correlation between clustering of patients with different prognosis based on transcriptome clusters, DNA methylation and miRNA expression [94]. The fourth study investigated recurrent copy number variations using the coverage of paired exome sequencing results (patient's tumour vs normal), and reported somatic amplification of the *TERT* gene and deletion of *ZNRF3* and *KREMEN1* genes [69]. Based on two recent studies that used different genomic approaches, it is possible to conclude that the WNT signalling pathway is most frequently altered in ACCs [69,94]. Because of the lack of a discriminative value and the relative rarity of genetic abnormalities in ACCs, mutation studies are not primarily used to diagnose ACCs, but specifically to identify potential novel targets for therapy.

1.8 PATHOPHYSIOLOGY OF CELLULAR SIGNALLING PATHWAYS

At least three cellular signalling pathways appear to be relevant for adrenocortical carcinogenesis and for identification of novel potential therapeutic targets.

1.8.1 IGF-mTOR pathway

The IGF signalling pathway consists of ligands (IGF1 and IGF2), receptors (IGF1 receptor [IGF1-R], IGF2-R, and insulin receptor), IGF binding proteins 1–6, and IGF binding protein proteases. The binding of the mitogenic polypeptides to their receptors activates the downstream AKT/PI3K and MAPK pathways to regulate cellular processes of metabolism, differentiation, proliferation, and apoptosis. The IGF pathway regulates adrenal growth and maintenance, and steroidogenesis [3,99] (Figure 2).

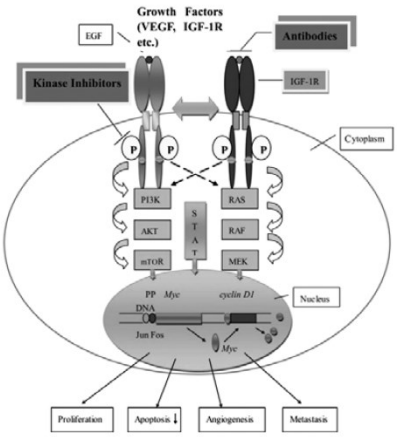


Figure 2. Scheme of growth factor pathways and potential therapeutic targets for ACC [100].

The findings of high *IGF2* expression levels and the knowledge of an increased incidence of ACC in BWS led to the investigation of IGF-1R as a therapeutic target. In an NCI-H295 xenograft mouse model, IGF pathway inhibition by the small-molecule inhibitor NVP-AEW541 and the monoclonal IGF-1R antibody IMCA12 showed an antitumor effect. Furthermore, the combined treatment of NCI-H295 cells with IGF-1R antagonists and mitotane resulted in a synergistic antiproliferative effect in vitro and in vivo in tumour xenografts [101,102].

Linsitinib (OSI-906) was the first IGF1 blocker that reached a phase III trial, but unfortunately did not show an increased overall survival compared to placebo [103]. Various clinical studies targeting IGF signalling showed disappointing results. A potential explanation can be found in compensatory activation of other growth promoting pathways. Combination therapy with other targeting drugs could be considered.

Interestingly, Sirianni et al. highlighted a critical role for estrogen receptor (ER)-α in 17β-estradiol and IGF2-dependent ACC proliferation, providing a rationale for targeting ERα to control ACC growth [104]. In addition, the same research group investigated estrogen-related receptor (ERR)-α, a downstream nuclear effector of multiple pathways, as possible target for innovative treatment modalities in ACC [105].

The role of the mammalian target of rapamycin (mTOR), a downstream effector of IGF2, has been investigated in adrenal tumours by several studies, and mTOR appeared to be a potential therapeutic target in a subset of patients with ACC [106]. Targeting mTOR signalling by everolimus caused tumour cell growth reduction in vitro and in mouse xenografts [107]. Preclinical studies support the idea that mTOR inhibitors can upregulate AKT phosphorylation in tumour tissue. To address and circumvent the problem of induction of upstream receptor tyrosine kinase signalling, Doghman & Lalli showed that a PI3K/mTOR dual inhibitor (NVP-BEZ235) significantly inhibited ACC cell proliferation. Phosphatidylinositol 3-kinase (PI3K) is a downstream signalling pathway. NVPBEZ235 antagonized rebound AKT activation, but induced ERK phosphorylation. In this light, the ERK inhibitor FR180204 in combination with NVP-BEZ235, synergistically inhibited ACC cell proliferation [108]. On the other hand, IGFs can activate escape mechanisms from mTOR inhibitors by stimulation of AKT or ERK activation [106]. This finding demonstrates the potential benefit and rationale for combination of an IGF1R antagonist with an mTOR inhibitor. De Martino et al. showed the effect of the mTOR inhibitor sirolimus on basal and IGF2 stimulated ACC cells in vitro. Sirolimus inhibited basal, as well as IGF2-induced, colony formation and colony size of ACC cells [109]. In a phase II study, the combination of cixutumumab, a fully human IGF1 monoclonal antibody directed at IGF1R, with temsirolimus, an mTOR inhibitor, was well tolerated and resulted in prolonged (6–21 months) stable disease in 42% of the 26 patients with metastatic ACC [110].

1.8.2 WNT signalling pathway

In the normal adrenal gland, the WNT/β-catenin signalling pathway plays a crucial role in both embryonic development and maintenance of the adrenal cortex. Recent examinations of adrenocortical tumours suggest that the WNT/β-catenin signalling pathway plays an important role in sporadic adrenocortical tumorigenesis.

The pathway is differentiated into 3 diverging signalling cascades dependent on signal conduction through β-catenin (canonical pathway), ras homolog gene family small

GTPase (planar cell polarity pathway), or phospholipase C (WNT/calcium pathway). β-catenin is normally sequestered in a destruction complex with adenomatous polyposis coli (APC), glycogen synthase kinase 3, and axin. In the canonical pathway, binding of the WNT ligand to its respective frizzled receptors results in release of β-catenin from the complex and translocation to the nucleus where it serves as a transcriptional cofactor [3]. Immunohistochemical analysis of 39 adrenal tumours revealed accumulation of β-catenin in 10 of 26 ACAs and in 11 of 13 ACCs, consistent with stabilized and hence activated β-catenin. Mutational analysis of the β-catenin gene *CTNNB1* identified activating point mutations in both ACAs and ACCs [96,111]. The fact that both nuclear β-catenin accumulation and activating *CTNNB1* mutations are present in ACAs as well as in ACCs suggests that WNT activation may be an early step in adrenocortical tumorigenesis, which precedes malignant transformation.

The most widely investigated WNT inhibitor is CWP232291, which is in a Phase I trial for refractory acute myeloid leukemia (AML) (NCT01398462). CWP232291 can promote β-catenin degradation. The first results of effectiveness of targeting the WNT signalling pathway in ACC comes from in vitro inhibition of ACC cell proliferation by the small-molecule inhibitor PKF115-584 [112]. Gaujoux et al. showed that *β-catenin* silencing caused decreased cell proliferation, alterations in the cell cycle and increased apoptosis in adrenocortical cancer cells in vitro [113]. Clinical trials with WNT inhibitors in ACC have not yet been performed.

1.8.3 Vascular endothelial growth factor

Sustained angiogenesis is a hallmark of virtually all types of cancer and the vascular endothelial growth factor (VEGF) is a chief regulator of cancer angiogenesis. Elevated VEGF levels were identified in blood samples from ACC patients, and overexpression of VEGF receptor has been shown in ACC samples [55].

An earlier clinical trial using bevacizumab, an anti-VEGF monoclonal antibody, proved to be ineffective [114]. Several studies have been undertaken with VEGF receptor inhibitors in patients with ACC. Three phase II studies evaluated sorafenib in combination with paclitaxel, sunitinib or axitinib respectively [115-117]. Sorafenib did not show an anti-tumour effect in patients, whereas sunitinib and axitinib showed a partial response in 14 and 62% of the patients respectively.

The lack of efficacy of tyrosine kinase inhibitors monotherapy might depend on compensatory hyperactivation of other signalling pathways. In two ACC cell lines, Lin et

al. confirmed the activation of multiple tyrosine kinases during treatment with sunitinib, with ERK as the most activated tyrosine kinase [118]. An additive antiproliferative effect was observed when sunitinib was given in combination with an ERK inhibitor. Furthermore, induction of CYP3A4 by mitotane treatment may enhance drug metabolism, limiting the therapeutic efficacy of tyrosine kinase inhibitors [119].

1.9 THERAPY

Briefly, complete surgical resection ('R0') is currently the gold standard treatment for non-metastatic ACC or following local recurrence. Mitotane is the only FDA-approved drug for locally advanced inoperable and metastatic disease displaying single-agent activity of 10–30% tumour response rates based on its adrenolytic action, although with a high toxicity profile [3]. The only prospective phase III randomised clinical trial, FIRM-ACT, showed that in advanced ACCs mitotane combined with etoposide, doxorubicin and cisplatin (EDP-M) provided some additional clinical benefit compared to mitotane plus streptozocin alone, but was associated with more serious adverse events [120]. A further prospective study evaluating mitotane vs. placebo in high-risk for recurrence patients, ADIUVO (https://clinicaltrials.gov/ct2/show/study/NCT00777244), is currently under way [121].

1.9.1 Surgery

Surgery is the treatment of choice for non-metastatic ACC. Complete R0 resection of ACC is currently the keystone and the only curative treatment option for patients with ACC. An operative planning by a surgeon experienced in the resection of malignant adrenal tumours is highly recommended in order to assure long-term local control of malignancy. Requiring ACC specialised knowledge of surgical technique and management strategies, adrenalectomies for suspected ACC should be performed only in specialised centres performing at least 20 adrenalectomies per year [122]. Poor initial surgical treatment can rarely be corrected, whether by reoperation, radiotherapy, or chemotherapy. When imaging characteristics could not exclude malignancy clearly, surgeons are obligated to approach the resection as a cancer operation. Failure to do so often leads to dismal outcomes. Despite accurate preoperative diagnostics, approximately 25% of stage III cases are initially suspected to be stage II ACC but ultimately found to

have microscopic extension through the adrenal capsule. These cases highlight the importance of careful surgical technique including resection of all surrounding soft tissue and adjacent organs if necessary [3].

Preoperative considerations include management and optimization of those patients with hormone excess, especially those with Cushing's syndrome because of the numerous deleterious effects due to elevated cortisol, such as poor wound healing, infection, and metabolic derangements. Debulking for control of hormone excess in the setting of known metastatic disease is also performed in some situations. The long-term durability of hormone control is usually limited by the metastatic disease progression. The benefits of debulking must outweigh the risks of surgery, so that preoperative evaluation should include estimation of recovery periods and postoperative quality of life with respect to life expectancy.

Resection of the primary tumour in stage IV disease needs to be individually addressed. In general, those with widespread distant metastatic disease in multiple organs or those with multiple metastatic deposits in one organ system unable to be completely resected should not undergo adrenalectomy. However, surgical treatment could be considered in selected cases, i.e. tumour burden remains stable or decreases after chemotherapy and/or mitotane and/or palliative radiation [120].

Careful attention should be paid to the adrenal and renal veins, the inferior vena cava, and the aorta, including the take-off of the celiac and superior mesenteric arteries. Adrenalectomy in the setting of tumour thrombus within the vena cava (if the tumour is otherwise technically resectable) is reasonable. Obstruction or occlusion of the vena cava by tumour thrombus can lead to significant lower body and gastrointestinal tract oedema, which leads to significant patient suffering. Lack of resection in the setting of vena cava thrombus can quickly lead to death. If tumour resection is not technically feasible for other reasons, vena cava stents can be placed, leading to temporary prevention of occlusion [3].

Lymph node dissection

The role of lymph node sampling or formal regional lymph node dissection in the treatment of ACC remains unknown, and consensus within the field is needed . There is also no formal agreement on the extent of lymph node dissection. The main lymphatic

areas are the renal hilum and the origin of the celiac and mesenteric artery. Because lymph nodes ideally should be removed as part of the 'en bloc' resection, surgeons need to individually balance the increased risk due to extended surgery (e.g., bleeding) with the presumed benefit of radical lymph node dissection. The impact of regional lymph node metastasis upon overall survival provides impetus for earlier or more aggressive use of additional therapies when disease is present in the lymphatic system [123].

Open vs laparoscopic surgery

Controversy surrounds the appropriateness of laparoscopic adrenalectomy (LA) for patients with ACC, since a complete margin-negative tumour resection at the initial operation is critical. Published data comparing the efficacy of LA vs open adrenalectomy (OA) for ACC are limited. All large series are retrospective, include fewer than 200 patients (with most reports including fewer than 10 patients), provide limited or no follow-up, are hampered by referral bias, and include patients who did not undergo their initial surgical resection at the referral centre. Recent recommendations by the American Association of Clinical Endocrinologists and the American Association of Endocrine Surgeons advocate OA by an experienced surgeon as the procedure of choice [124]. Conversely, the European Society of Endocrine Surgeons and European Society for Medical Oncology suggest LA could be performed for stage 1 and 2 ACC tumours less than 8 or 10 cm if an R0 resection is performed and surrounding peri-adrenal tissue removed [125]. However, it remains undetermined how to differentiate stage I and II ACCs from microscopic or unappreciated stage III ACC definitely before surgery. To date, existing data are inconclusive and more studies are needed to better judge the equivalence of LA to OA.

Surgery for recurrent disease

In the setting of recurrence, surgery is indicated in those patients with disease confined to one site or organ. Beyond that, decisions regarding resection must be carefully individualised. Disease recurrence in the peritoneum outside the tumour bed has the worst survival. Tumour grade influences the decision for reoperation because it correlates with survival. In patients with low-grade tumours, time of disease progression can be slower and lead to longer survival with resection of sites of recurrence or metastasis. In contrast, those patients with high-grade tumours benefit less from reresection, because other sites of disease often appear quickly [3].

1.9.2 Adjuvant therapy

Despite complete surgical tumour excision, patients with ACC remain at high risk for recurrence, typically ranging from at least 19% to 34% [3]. There is no doubt that an effective adjuvant treatment would be of great benefit [1]. Treatment modalities currently considered include mitotane, irradiation of the tumour bed, cytotoxic agents, or combinations of them.

1.9.2.1 Mitotane

Mitotane is a synthetic derivative of the insecticide dichlorodiphenyltrichloroethane whose adrenolytic activity was first described in dogs in 1948. In 1960 Bergenstal et al. reported responses to therapy with the isolated 1-(*o*chlorophenyl)-1-(*p*-chlorophenyl)-2,2-dichloroethane isomer (o,*p*′DDD, mitotane) that harbors the adrenolytic activity [3]. Since then, further modifications and isolations of enantiomers have aimed to improve the adrenolytic activity, improve pharmacokinetics and reduce side effects, but unfortunately only marginal improvement occurred. To date, mitotane remains the only drug approved by the U.S. Food and Drug Administration and European Medicine Executive Agency for treatment of ACC [5].

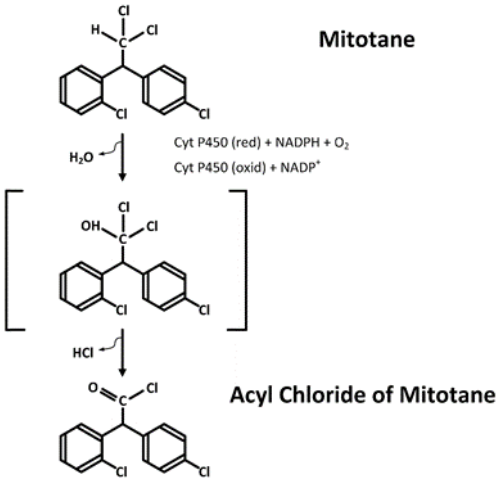


Figure 3. Mitotane chemical structure (up) and transformation to the active metabolite (down) [126].

The pharmacological mechanism by which mitotane exerts its adrenolytic effect is still not completely understood. Mitotane leads with relative specificity to a destruction of the inner zones of the adrenal cortex, the zona fasciculata, and zona reticularis. The specificity of mitotane towards adrenal cortex may derive from metabolic transformation of the drug to an active product *via* an enzyme system that is unique to this tissue (Figure

3). Active metabolites produced by adrenal mitochondria, in turn, covalently bind to mitochondrial proteins hypothesized to inhibit mitochondrial respiration [126]. Furthermore, mitotane metabolites inhibit several enzymes in the adrenocortical steroidogenesis pathway, mainly at the level of the cholesterol side-chain cleavage enzymes CYP11A1 (which appears to be one of the covalently bound mitotane targets) and CYP11B1.

Approximately 40% of mitotane is absorbed from the gastrointestinal tract, and a significant amount is distributed to fatty tissues. After a usual daily dose of 5 to 15 g/d, plasma levels range between 0 and 90 mg/L. Doses greater than 20 g regularly result in neurological side effects, which are reversible with normalization of plasma levels [3].

Mitotane for adjuvant therapy

Adjuvant treatment is routinely started as early as possible after surgery, generally within 3 months. Previous studies showed a large variation in responses, however all were retrospective, with older studies lacking the advantage of cross-sectional imaging.

In a recent large retrospective study, adjuvant mitotane therapy showed significant improvement in median tumour-free survival in patients with completely resected ACCs (42 vs 10 and 25 months in 2 control groups). Median overall survival was significant only in comparison with one of the control groups (110 vs 52 and 67 months) [121]. It seems that only a subgroup of patients may benefit, and primarily those with cortisol-producing tumours, but results are inconclusive. These limitations have led to the currently only prospective randomized multicentre study for mitotane as an adjuvant therapy for low to moderate risk for recurrence ACC (ADIUVO).

Although often usual practice, no study has formally evaluated the combination of mitotane and radiation therapy. This approach is supported by in vitro findings of mitotane acting as a radiation sensitizer [3].

Mitotane for recurrent and advanced disease

The efficacy of mitotane therapy in the setting of not completely resectable, metastasized, or recurrent ACC is well established. Overall, 30% of patients show stable disease or partial remission after treatment with mitotane, but possibly confounding interpretation

of these results comes from a subgroup of patients that shows a very slow disease progression [3].

The most important prognostic factor is the mitotane plasma level. Most studies, including a large retrospective analysis, have defined the therapeutic mitotane level to be 14 to 20 mg/L [127,128]. Only very few studies have analyzed patient-, tumor-, or drug-related factors that may influence patient outcome and predict patients who may respond to mitotane therapy. On the molecular level, *RRM1* expression has been found to be inversely correlated with mitotane response. Low *RRM1* expression was a predictor of response to mitotane therapy with prolonged tumour-free survival [129].

Mitotane management.

Managing mitotane therapy is an intensive process and requires experience. The dose is initiated at 1 g twice daily and increased every 4 to 7 days by 0.5 to 1 g/d until a daily dose of 5 to 7 g is reached. A low-dose loading protocol has also been described, probably leading to fewer side effects, but the same efficacy, and increased patient compliance. Regardless of the initial protocol, appropriate monitoring of blood levels is key and readily available in most countries. After the initial loading phase, the mitotane dose is titrated to a blood level of 14 to 20 mg/L. Side effects are mainly gastrointestinal, neurological, and metabolic/endocrinological.

Nausea and diarrhoea are most commonly dependent on the actual dose in contact with lumen of the gastrointestinal tract. These effects are rarely dose limiting and can be attenuated by distributing the mitotane amount into 3 or 4 daily doses. Gastrointestinal side effects are also often ameliorated by taking mitotane with food, specifically lipid-rich foods. Mild to moderate side effects can also be treated with antiemetic and antidiarrheal medications. Patients should be carefully evaluated whether gastrointestinal symptoms could be due to adrenal insufficiency, in which case a hydrocortisone increase may ameliorate symptoms.

Neurological side effects have a wide range from minor mental slowing, ataxia, and dysphasia to severe somnolence and lethargy. Neurological side effects are dependent on plasma mitotane levels and usually do not occur until blood levels rise higher than 20 mg/L. Neurological side effects are the main limiting side effect.

Mitotane therapy almost invariably leads to an increase in liver enzymes and hypercholesterolemia. Alkaline phosphatase and γ-glutamyl transferase (GGT) can

increase significantly, usually without clinical significance, but aspartate aminotransferase (AST) and alanine aminotransferase (ALT) show only mild elevation. When rapidly rising levels of AST and ALT or levels greater than 3-fold the normal range, mitotane therapy should be withheld and evaluation for mitotane-induced hepatotoxicity or other liver pathologies initiated. Hypercholesterolemia is best treated with a statin, preferably using a compound not metabolized by CYP3A4 [3].

Major endocrine abnormalities result from the effect of mitotane on steroid hormone biosynthesis. Three main mechanisms lead to adrenal insufficiency and decreased bioavailability of cortisol: 1) inhibition of steroid hormone biosynthesis at the level of CYP11B1 and CYP11A1; 2) induction of CYP3A4 and increased 6β-hydroxylation of cortisol; and 3) induction of cortisol binding globulin (CBG). Adrenal insufficiency occurs invariably and is treated pre-emptively. All patients are started on a minimum of 30- to 40-mg daily dose of hydrocortisone. Supraphysiological hydrocortisone doses up to 50 to 100 mg daily may be necessary because of the increased cortisol catabolism [3]. Hydrocortisone therapy needs to be continued after cessation of mitotane until the patient does not show any clinical or biochemical evidence of adrenal insufficiency. Even after discontinuation of mitotane therapy, CYP3A4 induction and mitotane levels persist up to several months. Occasionally, mitotane may affect mineralocorticoid synthesis and replacement therapy with fludrocortisone therapy may become necessary.
Other common endocrine side effects during mitotane treatment include hypogonadism in male patients, which often requires replacement therapy, and hypothyroidism [1].

Several drugs regularly used in combination with mitotane, such as platinum-based cytotoxic drugs, doxorubicin, and etoposide are also metabolized by CYP3A4, potentially reducing their antineoplastic effect. This is especially important when evaluating new drugs and targeted agents. A study using sunitinib, which is metabolized by CYP3A4, raised concerns that several of the study subjects did not reach therapeutic levels of this drug [116].

1.9.2.2 Cytotoxic chemotherapy

Cytotoxic chemotherapy is currently a mainstay of treatment for advanced and metastasized ACC. The overall response to chemotherapeutic regimens is 30% and 50%,

when counting stable disease as a response. However, the response is invariably transient and short-lived (6–18 months).

To establish a gold standard of cytotoxic chemotherapy for ACC, a recent phase 3 trial (FIRM-ACT, First International Randomized Trial in Locally Advanced and Metastatic Adrenocortical Carcinoma Treatment) compared the most promising regimens (etoposide, doxorubicin, cisplatin, mitotane [EDPM] vs streptozotocin, mitotane). This study confirmed the efficacy of chemotherapy and proved the superiority of EDPM [120]. The response rate was 20% and 50%, when stable disease was included. However, the median progression free survival, was short with a median of 5 months. Because of CYP3A4 induction potentially affecting cisplatin metabolism, there is criticism regarding whether chemotherapy without mitotane may be more successful .

1.9.2.3 Targeted therapy

The unfavourable prognosis of ACC using traditional therapies has led to the exploration of targeted agents, compounds with defined molecular targets, such as receptors or intracellular enzymes.

The most data for targeted therapy exist for the IGF-1R antagonists. Several studies investigated drugs targeting IGF-1R in patients with stage 4 disease. The first studies investigated figtilimumab and IMCA12 (cixutumab) but results have been disappointing [55]. The phase 3 trial GALACCTIC investigated OSI906 (Linsitinib), a small-molecule inhibitor of IGF-1R and insulin receptor. Despite failing to show an effect on OS and PFS in the overall population, the promising responses seen in individual patients suggest the therapeutic potential of inhibiting IGF-1R in selected ACC cases [130].

A study using the multikinase inhibitor sunitinib showed stable disease in 5 of 35 patients. Concomitant mitotane treatment negatively affected patient response [116].

Trials with new targeted drugs are under way, and altered regimens and combination therapies may hold some promise.

Therapy for hormone excess

In 40–60% of patients with ACC, the main complaints are due to hormone overproduction [31]. Treatment of these elevated hormone levels is mandatory. By different mechanisms, mitotane treatment can already result in control of hormone levels to some extent. Adrenal steroidogenesis inhibitors like ketoconazole or metyrapone (alone or in combination) can also be used, or more rarely aminoglutethimide or etomidate [55]. Mifepristone, a

glucocorticoid receptor antagonist, is another treatment modality against cortisol excess. To control androgen effects in women with androgen-secreting tumours and mineralocorticoid effects in patients with mineralocorticoid-secreting tumours, spironolactone can be administered [3]. Monitoring of the patient parameters is important in all cases, considering the risk on adrenal insufficiency.

Radiation therapy

Although traditionally considered ineffective for ACC, radiotherapy has been shown in several recent series to offer a significant improvement in disease control in both the adjuvant and palliative settings, although such an improvement has not been universally demonstrated [3]. Apart from the adjuvant setting, radiotherapy can be indicated: i) when microscopic tumour residues are visible after surgery; ii) when patients are not suitable for surgery (in this case radiotherapy is often in combination with mitotane); and iii) for palliative care. Several studies have shown efficacy of radiotherapy for adequate palliation, but with divergent results and mainly based on case series [55].

Other local therapies

In case of inoperable metastatic disease, palliation is possible with local treatment modalities, such as radiofrequency ablation (RFA) or transarterial chemoembolization (TACE). None of these methods have been explored in clinical trials. However, both methods are an alternative to surgery, when surgery is not desired or contraindicated [3]. Adrenal tumours, including ACC, have a tendency to undergo hemorrhage and might lead to bleeding complications.

1.10 PROGNOSTIC FACTORS AND PREDICTIVE MARKERS

Despite the globally poor prognosis of ACC, there is a marked individual variation in disease progression, recurrence, and overall survival.

Unfavourable prognostic indicators are tumour extent (e.g., stage), specifically the presence of metastatic disease and number of organs involved, high Weiss score, high tumour grade (>20 mitoses per HPF), and high Ki67 [59]. Although older studies did not show any differences in prognoses for patients harboring different hormone secretion subtypes of ACC, recent studies including a total of 274 patients identified cortisol production as an adverse prognostic factor [4,125].

Novel potential factors have been described in order to improve outcome stratification and to identify subpopulations of subjects in which therapies could be effective.

Two subgroups of ACCs have been reported based on transcriptome characteristics: cluster C1A and cluster C1B, the latter one with a remarkable better 5-years survival rate (20 vs 91%) [71,72,75,131]. Genes involved in cell cycle regulation predominated in the poor outcome group (C1A). Moreover, all *TP53* and *CTNNB1* mutations were exclusively observed in the C1A ACC group. The C1A group was further divided into three subgroups, with inactivated *p53* (C1A-p53), activated *β-catenin* (C1A-β-catenin) and one with a still unidentified molecular alteration (C1A-x) [132], but validation of these microarray based prognostic factors is needed. C1A and C1B groups differed also in microRNAs and DNA methylation [94].

Barreau et al. made the first correlation between DNA methylation levels and patient outcome in ACC [83]. After analysis of DNA methylation profiles of a series of ACC samples, two groups of ACCs were identified, one exhibiting a higher methylation compared to ACAs, which was termed the CpG island methylation phenotype (CIMP) group, similarly to what previously reported in other types of cancer (e.g. colorectal cancer) [133]. Of note, the two ACC subgroups with poor prognosis previously identified as C1A-p53 and C1A-x showed a CIMP pattern. In contrast, the poor prognosis subgroup named C1A-β-catenin and the good prognosis C1B group belonged to the non-CIMP cluster [83]. The fact that not all poor prognosis groups show a CIMP could potentially mean that the prognostic value of methylation patterns is less effective compared to gene expression. The CIMP group was further divided into two subgroups, with different levels of methylation (CIMP-high and CIMP-low). Hypermethylation was associated with a poor survival [83,94].

Dismal prognosis of ACC is associated with overexpression of the pituitary tumour transforming gene 1 (PTTG1), low expression of the transforming growth factor β signalling mediator SMAD and diminished expression of GATA-6, cyclin E overproduction, and overexpression of SF1 [55]. Combined assessment of Ki67 and VAV 2, a factor overexpressed by increased SF1 and essential for tumour spreading, improved prognostic prediction in ACC [134].

Recently, several studies have identified potential factors associated with response to mitotane. CYP2W1 immunoreactivity was associated with a longer overall survival and time to progression in mitotane monotherapy treated patients, even when adjusted for ENSAT stage [135].

Ribonucleotide reductase large subunit 1 (RRM1) gene expression was associated with a shorter disease-free survival and overall survival [129]. Subjects with low RRM1 expression who received adjuvant mitotane had a significantly longer disease-free survival compared to patients who only received follow-up, whereas this was not the case in patients with high RRM1 expression. It has been suggested that the *RRM1* gene might interfere with mitotane metabolism in ACC cells.

Ronchi et al. investigated protein expression of excision repair cross complementing group 1 (ERCC1) as a predictor for response to platinum-based chemotherapy in patients with ACC. High ERCC1 expression was correlated with a worse overall survival in patients treated with platinum-based chemotherapy [136].

PART 2: VITAMIN D

2.1 VITAMIN D AND ITS BIOACTIVATION

Vitamin D is member of the secosteroid family widely known for its role in phosphate and calcium homoeostasis and its preventive action in rickets and osteomalacia by increasing bone mineralisation and osteogenesis. The most widely accepted physiological effect of vitamin D is mediated primarily by $1\alpha,25(OH)_2D_3$, also known as calcitriol, which represents the most active product of vitamin D synthesis. $1\alpha,25(OH)_2D_3$ is synthesized in a highly regulated multistep process (Figure 4).

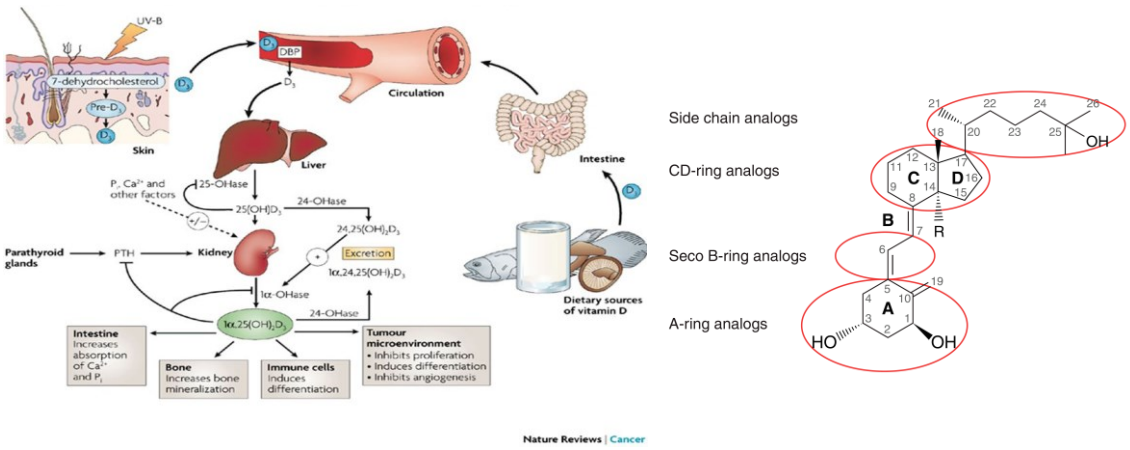


Figure 4. On the left, principal steps of vitamin D_3 metabolism in humans [137]**. On the right, chemical structure of $1\alpha,25(OH)_2D_3$** [138]**.**

The first step in vitamin D synthesis is the formation of vitamin D_3 in the skin through the action of ultraviolet irradiation with spectrum 280-320 nm (UVB), which transforms pro-vitamin D in pre-vitamin D_3. Temperature-dependent isomerisation of pre-vitamin D_3 in the basal layers of the epidermis leads to vitamin D_3 (cholecalciferol). Reduced sun exposure as well melanin and sunscreen limit vitamin D synthesis. Vitamin D_3 can also be taken in the diet but in North America and Europe dietary vitamin D_3 intake represents a minor component of vitamin D_3 acquisition as it is present in only a few foods (dairy products, eggs, fish and fortified products) that contain only small amounts of vitamin D. Already formed in the skin or ingested by the diet, vitamin D_3 travels through the bloodstream joined to DBP (vitamin D Binding Protein) and albumin, to a lesser extent. Both DBP-bound vitamin D and free vitamin D stay in balance to maintain adequate levels of the active hormone [138].

Vitamin D_3 itself is not biologically active. Vitamin D_3 is hydroxylated at C-25 by liver mitochondrial and microsomal 25-hydroxylases (25-OHase) to produce 25-hydroxyvitamin D_3 [25(OH)D_3]. The synthesis of 25(OH)D_3 has not been reported to be highly regulated [139]. Many cytochrome P-450 enzymes including CYP2R1, CYP27A1, and CYP2D25 (among other 25 hydroxilases) have been considered as candidates for the enzyme responsible for the conversion of vitamin D to 25(OH)D_3 [140]. The 25(OH)D_3 is the major circulating form of vitamin D and its serum concentration has served as one of the most reliable biomarkers of vitamin D status [138,141].

25(OH)D_3 is transported by DBP to the kidney and is filtered by the glomerulus. In the kidney megalin, a 600-kDa transmembrane protein, and a member of the low-density lipopoprotein receptor superfamily, acts as a cell surface receptor for DBP resulting in uptake of 25(OH)D in the tubular epithelial cells by endocytic internalization [141]. In the proximal renal tubule, 25(OH)D_3 is hydroxylated at the position of carbon 1 of the A ring, resulting in the formation of 1,25$(OH)_2D_3$ (calcitriol), which is the functional, hormonally active form of vitamin D, responsible for most of the biologic actions of vitamin D.

The renal 25(OH)D_3 1α-hydroxylase (mitochondrial CYP27B1), which metabolizes 25(OH)D_3 to 1,25$(OH)_2D_3$, comprises a cytochrome P-450, a ferredoxin, and a ferredoxin reductase and is present predominantly in the kidney (proximal straight tubules) and contributes to the circulating concentrations of 1,25$(OH)_2D_3$ [142].

In the kidney, besides conversion to 1,25$(OH)_2D_3$ by CYP27B1, 25(OH)D_3 can also be converted to 24,25$(OH)_2D_3$ by hydroxylation at C-24 by CYP24A1 (24-hydroxylase, 24-OHase), a mitochondrial inner membrane cytochrome *P*-450 enzyme [142]. This enzyme can hydroxylate not only 25(OH)D_3 but also 1,25$(OH)_2D_3$. 1,25$(OH)_2D_3$ has been suggested to be the preferred substrate for CYP24A1. CYP24A1 limits the amount of 1,25$(OH)_2D_3$ when circulating 1,25$(OH)_2D_3$ is elevated by catalysing the conversion of 1,25$(OH)_2D_3$ into 24-hydroxylated products targeted for excretion or by producing 24,25$(OH)_2D_3$ thus decreasing the pool of 25(OH)D_3 available for 1-hydroxylation [138]. Additionally, 1α,25$(OH)_2D_3$ concentrations are feedback regulated: an increase in 24,25$(OH)_2D_3$ induces the synthesis of 1α,25$(OH)_2D_3$; whereas Ca^{2+}, Pi and 1α,25$(OH)_2D_3$ itself suppress 1α,25$(OH)_2D_3$ synthesis. *CYP27B1* (which encodes 1α-OHase) expression is induced by parathyroid hormone (PTH) and repressed by 1α,25$(OH)_2D_3$ [137]. Furthermore, *CYP24A1* is strongly induced by 1α,25$(OH)_2D_3$ to produce the less active vitamin D metabolites 1α,24,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ [138].

It has been suggested that in healthy animals and humans CYP27B1 is only expressed in kidney and, during pregnancy, in placenta. However, in addition to the kidney, it has been reported that CYP27B1 is present in a number of extrarenal sites. Extrarenal production of CYP27B1 has been convincingly demonstrated in patients with sarcoidosis [143]. Macrophages were identified as the source of extrarenal production of $1,25(OH)_2D_3$ resulting in hypercalcemia and hypercalciuria in these patients. In addition to sarcoidosis, hypercalcemia has also been identified in patients with Crohn's disease [144]. It was suggested that activated macrophages of Crohn's granuloma are responsible for the hypercalcemia in Crohn's disease. CYP27B1 produced by macrophages, unlike renal CYP27B1, is not suppressed by elevated $1,25(OH)_2D_3$ but is upregulated by immune stimuli [interferon-γ and lipopolysaccharide (LPS)]. Regulation by immune stimuli has been reported to involve multiple pathways (including JAK/STAT and NF-κB) [137]. Cancer cells have also been shown to express CYP27B1. In addition, CYP27B1 expression has been noted in parathyroid gland and in many other tissues. However, whether there is a functional impact of CYP27B1 activity in vivo at sites other than the kidney and placenta under normal physiological conditions remains to be determined [138].

2.2 THE VITAMIN D RECEPTOR

The biological actions of $1,25(OH)_2D_3$ are mediated by the VDR. VDR belongs to the steroid receptor family which includes receptors for retinoic acid, thyroid hormone, sex hormones, and adrenal steroids [145]. The *VDR* gene is evolutionarily conserved among fishes, birds, and mammals. The human and mouse *VDR* genes are localized on chromosomes 12 and 15, respectively and both are comprised of eight coding exons [138]. Human VDR protein consists of 427 aminoacids. It functions as an obligate heterodimer with RXR for activation of vitamin D target genes [137,145]. The two core functional domains of the VDR are the highly conserved NH2-terminal DNA binding domain (DBD), a cysteine-rich zinc finger region, and the more variable COOH-terminal ligand binding domain (LBD). LBD contains sequences critical for interactions with co-regulators (Figure 5).

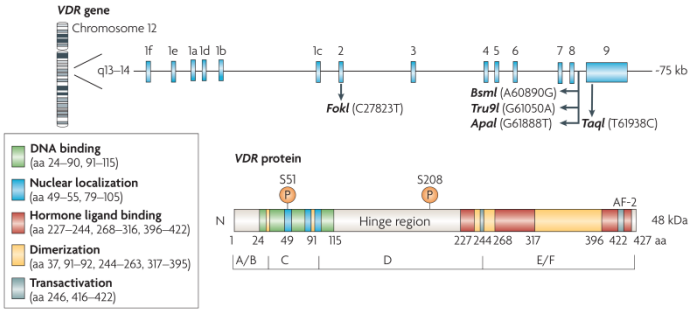


Figure 5. The vitamin D receptor and its critical regions [137].

$1,25(OH)_2D_3$ binding induces a conformational change that facilitates interaction with RXR and coregulatory complexes required for the transcription of target genes. X-ray crystallographic data of the VDR/RXR complex is currently not available. Recently, the structure of the liganded VDR/ RXR DNA complex was characterized using cryoelectron microscopy [138]. Recent studies using small angle X-ray scattering and hydrogen-deuterium exchange technology also enabled characterization of the VDR/RXR DNA complex and indicated cooperative effects between the VDR DBD and VDR LBD, suggesting mechanisms by which ligands and DNA can act together to fine-tune regulation of gene expression [138].

2.3 GENOMIC MECHANISM OF $1,25(OH)_2D_3$-VDR COMPLEX

The classical genomic mechanism of $1,25(OH)_2D_3$ action involves the direct binding of $1,25(OH)_2D_3$ activated VDR/RXR to specific DNA sequences known as vitamin D response elements (VDREs) in and around target genes (Fig. 2.3). The heterodimerization of $1,25(OH)_2D_3$-VDR with RXR leads to high-affinity binding to VDREs. Following the binding of VDR-RXR heterodimer to the VDRE, changes in gene expression are mediated through the ability of the liganded receptor to recruit transcriptional coactivators (Figure 6). The p160 coactivators, steroid receptor activator 1, 2, and 3 (SRC-1, SRC-2, and SRC-3), that exhibit histone acetylase (HAT) activity, are primary coactivators which bind to the liganded VDR. Members of the p160 family recruit proteins as secondary coactivators, such as CBP/p300 (which also have HAT activity), resulting in a multisubunit complex that modifies chromatin and destabilizes histone/DNA interaction [137,146]. In addition to acetylation, methylation also occurs on core histones. Recent studies have shown that methyltransferases may also play a fundamental role in VDR-mediated transcription [147,148]. VDR-mediated transcription is facilitated by Mediator,

a multi-protein complex (the 205 subunit binds to VDR) which functions through recruitment of RNA polymerase II and promotes formation of the preinitiation complex. A number of other transcription factors have been reported to affect the transcriptional activity of VDR. Since VDR coregulatory proteins are master regulators of $1,25(OH)_2D_3$ action, further studies identifying VDR coactivators and corepressors as well as epigenetic regulation of VDR function will yield significant new insight into the complex mechanisms by which $1,25(OH)_2D_3$ acts to direct its multiple biological activities [147].

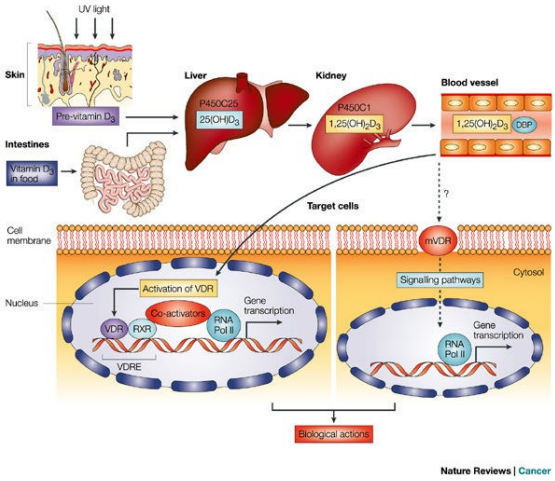


Figure 6. Illustration of genomic mechanism of $1,25(OH)_2D_3$/VDR complex action (on the left side). Schematic view of proposed non-genomic mechanism of liganded VDR (on the right side) [149].

The complexity of the molecular mechanisms involved in $1,25(OH)_2D_3$ action is not only indicated by the diversity of coregulators and their activities but also through genome-wide studies which have shown that the actions of $1,25(OH)_2D_3$, similar to other steroids, involve regulation of gene activity at a range of locations many kilobases upstream as well as downstream of the transcription start site and within introns and intergenic regions. VDR binding to these sites is largely but not exclusively dependent on activation by $1,25(OH)_2D_3$. Global networks regulated by VDR are beginning to be addressed in osteoblastic, intestinal carcinoma, immune, and hepatic stellate cells [138,148,150]. Genome-wide studies have provided a new perspective on mechanisms involved in the regulation of gene expression by $1,25(OH)_2D_3$ and suggest a chromatin looping mechanism whereby the regulatory regions can be brought into close proximity with the gene's promoter via protein-protein interaction [147].

Non-genomic actions mediated by $1,25(OH)_2D_3$ are rapid and not dependent on transcription. However, non-genomic signalling may indirectly affect transcription

through cross-talk with other signalling pathways [137]. The most well-described non-genomic effect of $1,25(OH)_2D_3$ is the rapid intestinal absorption of Ca^{2+}.

There is no agreement on how the non-genomic actions are initiated. Data suggest that these effects begin at the plasma membrane and involve a non-classical membrane receptor (mVDR) described in intestinal caveolae, and a $1,25(OH)_2D_3$-membrane-associated rapid response steroid binding protein ($1\alpha,25D_3$-MARRS) isolated from chick intestinal basal-lateral membrane, with subsequent activation of numerous signalling cascades [137,138].

2.4 CLASSICAL ROLES OF VITAMIN D

2.4.1 Intestine

The principal action of $1,25(OH)_2D_3$ and the VDR is intestinal calcium absorption. This conclusion is based on the observation from patients with hereditary vitamin D-resistant rickets (HVDRR), a rare autosomal recessive disorder characterized by hypocalcemia, hyperparathyroidism, early-onset rickets, and organ resistance to $1,25(OH)_2D_3$. Resistance to $1,25(OH)_2D_3$ is caused by heterogeneous loss of function mutations in the *VDR* gene. In the setting of HVDRR, mineral and skeletal phenotypes are reversed when treated with intravenous or high oral calcium [151].

The facilitated diffusion model is the most studied mechanism of vitamin D-regulated calcium absorption, involving stimulation of transcellular intestinal calcium transport by increasing the expression of the apical membrane calcium channel TRPV6 and of calcium binding protein calbindin-D_{9k}. However, the fine-tuned mechanisms involved in vitamin D regulation of intestinal calcium absorption have remained incompletely understood. Moreover, it has been suggested that $1,25(OH)_2D_3$ can also stimulate active phosphate absorption in the intestine [138] (Figure 7).

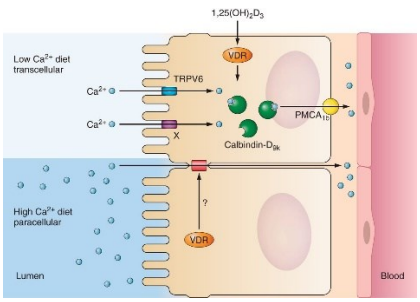


Figure 7. Effects of $1,25(OH)_2D_3$ on calcium absorption in the intestine cells [138].

Most of what is known about intestinal calcium absorption mediated by $1,25(OH)_2D_3$ comes from studies that utilize the duodenum. However, even though the high efficiency, only 8-10% of calcium absorption takes place in this tract. Although little is known about $1,25(OH)_2D_3$ action in other parts of the intestine, distal segments of the intestine are suggested to play an important role in intestinal calcium absorption and proper bone mineralization. Vitamin D and $1,25(OH)_2D_3$-regulated calcium transport has been reported in ileum, cecum, and colon [152].

In addition to transcellular transport, calcium is absorbed by the paracellular path that occurs between epithelial cells. To date, little is known about the vitamin D dependency of this non-saturable component of calcium absorption recently suggested [153].

2.4.2 Kidney

Only 1% to 2% of filtered calcium appears in the urine. Approximately 65% of the filtered calcium is passively reabsorbed at the proximal tubules in a $1,25(OH)_2D_3$-independent way. The proximal tubules of the kidney are the major site of $1,25(OH)_2D_3$ synthesis and of phosphate absorption . CYP27B1 expression is upregulated by PTH but downregulated by FGF23 and $1,25(OH)_2D_3$. Phosphate reabsorption in the proximal tubules is regulated by several factors including FGF23, PTH, and $1,25(OH)_2D_3$ [138].

In the distal tubules, calcium absorption is regulated by $1,25(OH)_2D_3$ and PTH, involving TRP channels (transient receptor potential cation channels), especially TRPV5, as active transcellular mechanism that resembles intestinal calcium absorption [154].

2.4.3 Bone

Bone formation involves the mineralization of the extracellular matrix formed by osteoblasts. In this process the role of $1,25(OH)_2D_3$ is both direct and indirect.

Indirect effects occur via control of calcium and phosphate absorption in the intestine and renal reabsorption of calcium [155]. In VDR knockout mice, a rescue diet containing high levels of calcium and phosphorus prevents rickets and osteomalacia, but did not rescue all bone defects. This suggests the importance of the direct role of $1,25(OH)_2D_3$ for bone mineralization [156].

Direct effects of $1,25(OH)_2D_3$ are targeted to bone forming cells (osteoblasts) to enhance differentiation and mineralization, as was found in *in vitro* cultures using human osteoblasts [157]. Direct effects of vitamin D on osteoblasts are exerted by binding to the nuclear VDR [158]. However, differences occur in VDR protein expression during

osteoblast development. In fact, VDR is highly expressed in immature osteoblasts, while low or no expression is detected in matured osteoblasts, including lining cells and osteocytes [159].

Direct effects of $1,25(OH)_2D_3$ on osteoblasts involve the control of the production of extracellular matrix proteins (collagen type I, osteopontin, osteocalcin, matrix Gla protein) and effects on the activity of the alkaline phosphatase enzyme to supply phosphate for the deposition of mineral [160]. Woeckel et al. showed that $1,25(OH)_2D_3$ positively interferes in the mineralization process of human osteoblasts by accelerating their production of alkaline phosphatase positive extracellular vesicles [157,160] eventually resulting in an increased formation and deposition of hydroxyapatite crystals leading to increased mineralization. This direct effect of vitamin D occurred early, in the period prior to the onset of mineralization and involved also an accelerated extra cellular matrix maturation [157]. Direct effects of $1,25(OH)_2D_3$ on human osteoblast activity depend on phase of differentiation of osteoblasts, thus different features of vitamin D action may take place during different stages of differentiation.

VDR expression was found in hypertrophic chondrocytes, and not in osteoclasts, chondroclasts and bone marrow stromal cells. The VDR absence in osteoclasts may indicate that the effects of $1,25(OH)_2D_3$ on osteoclast differentiation are indirect via increasing the expression of receptor activator of NF-κB ligand (RANKL) in osteoblasts, which is an important osteoclastogenic factor. RANKL binds to its cognate receptor RANK in osteoclast precursors and increases osteoclast formation and action [138].

Human osteoblasts express 1α-hydroxylase (CYP27B1) as well as the vitamin D binding protein receptors cubulin and megalin to metabolize $1,25(OH)_2D_3$ that is active in stimulating differentiation and mineralization. Besides the advantages of this local $1,25(OH)_2D_3$ production to the bone, also the precursor $25(OH)D_3$ as well as the 24-hydroxylated 'degradation' products $24R,25(OH)_2D_3$ and $1,24R,25(OH)_3D_3$ are stimulatory to osteoblast differentiation and mineralization in human preosteoblasts and mesenchymal stem cells [161].

Understanding the exact role of vitamin D in this process remains difficult to achieve, since vitamin D effects are part in many networks. The differences in experimental conditions used in combination with the different models used in studying vitamin D further contribute to the complexity, and require careful interpretation of the data [160].

2.5 PLEIOTROPIC EXTRA-SKELETAL ACTIONS OF VITAMIN D

In addition to its role in calcium and bone homeostasis, vitamin D potentially regulates many other cellular functions. The vitamin D receptor (VDR) is nearly universally expressed in nucleated cells. Furthermore, at least 10 tissues outside the kidney express 1-alpha-hydroxylase (*CYP27B1*), the enzyme responsible for converting vitamin D to its active form, and therefore, the active hormone can be generated in an auto- or paracrine way. In other words, now the concept is that all cells may be targets for $1,25(OH)_2D_3$, at least at some stage of their differentiation, and that many of these cells make their own $1,25(OH)_2D_3$ and are not totally dependent on the renal production of this metabolite. Thus, the spectrum of activity of the vitamin D endocrine system is much broader than calcium/bone homeostasis. In this regard, the vitamin D-VDR system resembles that of other ligands of nuclear receptors, such as thyroid hormone [162-164].

2.5.1 Muscle and falls

A body of evidence suggest a relationship between vitamin D and muscle function [165]. In humans vitamin D deficiency leads to a preferential loss of type II muscle fibers associated with fatty infiltration, fibrosis, and loss of strength, and is associated with muscle weakness and increased falls [164,165]. VDR and CYP27B1 expression decreases with differentiation of muscle precursor cells into adult fibres; however, in adult muscle VDR is still expressed albeit at very low levels [166]. The non-genomic actions of $1,25(OH)_2D_3$ have also received attention in muscle. Vitamin D supplementation improved muscle weakness and recovery of energy stores (maximal mitochondrial oxidative phosphorylation rate, as measured by in vivo magnetic resonance spectroscopy) after physical exercise in severely vitamin D-deficient but otherwise healthy adults [167]. Observational studies suggest an association between poor vitamin D status (<10 or <20 ng/mL [<25 or <50 nmol/L]) and muscle weakness in children and older individuals [162,168,169]. However, a causal relationship between vitamin D supplementation and improvement in muscle weakness has not been clearly demonstrated in randomized trials, and the optimal 25-hydroxyvitamin D_3 ($25[OH]D_3$) concentration for muscle function is still unknown [168]. Probably, benefit of vitamin D supplementation on muscle strength occurs when baseline $25(OH)D_3$ levels are below 10 or 20 ng/mL (25 or 50 nmol/L). Efforts to provide very large doses of vitamin D (or calcifediol) at infrequent intervals (e.g., 500,000 IU annually or 60,000 IU every month) have not been successful and may even increase the fall risk [170].

2.5.2 Cardiovascular system

In vitro studies show that $1,25(OH)_2D_3$ suppresses myocyte hypertrophy, $1,25(OH)_2D_3$ suppresses endothelin expression in cardiac fibroblasts, a known profibrotic/hypertrophic factor for the heart, whereas the expression of several metalloproteinases are increased and their inhibitors are decreased. VDR- and CYP27B1-null mice have increased levels of renin, increased blood pressure, increased cardiac hypertrophy, impaired systolic and diastolic function, increased arterial stiffness, and reduction in nitric oxide synthase (NOS) activity [164,171]. VDRKO mice are prone to develop accelerated atherosclerosis, whereas $1,25(OH)_2D_3$ can reduce such lesions in ApoE gene knockout mice, a mouse model of accelerated atherosclerosis, in part by suppressing the immune response in the atherosclerotic plaques. $1,25(OH)_2D_3$ showed promising results in mouse models of hypertension, such as reduction of procoagulant status [164].

Severe vitamin D deficiency is associated with cardiomyopathy and congestive heart failure in children, which are reversible with vitamin D supplementation [164]. Epidemiologic studies investigated the relationship between low $25(OH)D_3$ levels and increased risk of cardiac events, strokes, coronary artery calcification, and atherosclerosis in various settings. Not all such studies are positive but recent metanalyses of a number of these studies have demonstrated an association [172,173]. Endothelial function was found to improve in vitamin D–deficient diabetic patients when given vitamin D. However, RCTs have generally been disappointing [174].

The potential benefit of vitamin D supplementation on prevention and treatment of cardiovascular disease needs confirmation.

2.5.3 Immune system

The overall picture is that vitamin D signalling suppresses adaptive immunity but promotes innate immunity. The VDR and CYP27B1 are expressed in most if not all cells of the immune system including the epithelial cells at least when activated [164]. Moreover, several of these cells express CYP2R1 and so theoretically can produce $1,25(OH)_2D_3$ from circulating vitamin. The regulation of CYP27B1 in these cells differs substantially from that in the kidney, being insensitive to hormonal regulators such as PTH and FGF23, its product $1,25(OH)_2D_3$, and calcium and phosphate levels. In these immune cells, CYP27B1 is stimulated by cytokines such as TNF-α and interferon-γ (IFN-γ) [164]. Furthermore, transcription of the enzyme that controls $1,25(OH)_2D_3$ levels within cells, CYP24A1, is absent, defective, or blocked, essentially leaving $1,25(OH)_2D_3$

with minimum regulation. Thus, activation of these immune cells in diseases such as sarcoidosis or lymphomas can lead to hypercalcemia with elevated $1,25(OH)_2D_3$ levels [143].

The relevance of $1,25(OH)_2D_3$ on the immune system has been suggested by *in vivo* studies in mouse models of autoimmunity. $1,25(OH)_2D_3$ can protect against a number of experimental autoimmune diseases including inflammatory bowel disease and experimental autoimmune encephalomyelitis (mouse model for multiple sclerosis) [138]. Studies on animal experimental models of rheumatoid arthritis, psoriasis, type 1 diabetes mellitus, systemic lupus erythematosus, multiple sclerosis, and inflammatory bowel disease have been ameliorated with the use of $1,25(OH)_2D_3$ or one of its analogues [138]. Association studies in humans have found inverse correlations between $25(OH)D_3$ levels and/or vitamin D intake and a number of autoimmune diseases, including multiple sclerosis, type 1 diabetes, Crohn's disease, rheumatoid arthritis, lupus, and Graves thyroiditis. Although experimental findings are suggestive of a protective effect of $1,25(OH)_2D_3$ against the pathogenesis of autoimmune inflammation, whether vitamin D supplementation or treatment with analogues of $1,25(OH)_2D_3$ is beneficial clinically in the treatment of autoimmune diseases is not known [164].

2.5.4 Skin

The epidermis is the major source of vitamin D_3 in the body. Keratinocytes, having the 25-hydroxylase *CYP27A1*, possibly *CYP2R1*, the 1α-hydroxylase *CYP27B1*, and the *VDR*, can make their own 1,25(OH)2D from their own substrates and respond to the $1,25(OH)_2D_3$ they produce. The highest levels of VDR and CYP27B1 are found in the basal stratum. The VDR is also highly expressed in the stem cells of the hair follicle.

$1,25(OH)_2D_3$ ligand-dependent VDR transcriptional activity promotes differentiation of keratinocites by blocking β-catenin regulated proliferation. Coactivator complexes control different aspects of VDR action in keratinocytes, with Mediator 1 more involved in proliferation, whereas SRC2 and SRC3 more involved in maintenance of the barrier function of the skin including its innate immune function [175].

Vitamin D suppresses the immunologic aspects of psoriasis, a complex disease of autoimmunity characterised by skin hyperproliferation, and $1,25(OH)_2D_3$ have a well-validated clinical application in this setting. In fact, a number of clinical trials have demonstrated the efficacy and safety of $1,25(OH)_2D_3$ and its analogues in the treatment of psoriasis, as monotherapy or in combination with topical glucocorticoids [164].

2.5.5 Type 2 diabetes mellitus

Pancreatic β-cell expresses both VDR and CYP27B1. $1,25(OH)_2D_3$ stimulates insulin secretion *in vivo* and *in vitro*, and promotes glucose uptake by peripheral tissues. Calcium levels seem to be important in determining impaired insulin secretion in in vitamin D deficiency and in VDRKO mice [164]. The renin/angiotensin system (RAS) may also play a role by impairing β-cell function and insulin sensitivity. Several clinical studies investigated the relationship between increased risk of type 2 diabetes mellitus with low $25(OH)D_3$ levels, but not all studies showed this association [164,176]. A large multicentre placebo-controlled RCT is ongoing to study the role of vitamin D in type 2 diabetes mellitus [176].

2.5.6 Obesity

The adipocyte expresses both the VDR and CYP27B1 [138]. The role of vitamin D in adipogenesis depends on species and stage of cell differentiation by processes involving Wnt/β-catenin signalling.

Vitamin D deficiency is very common in obese patients, probably due to a sum of factors including increased storage in fat tissue, decreased sunlight exposure, and decreased efficiency of vitamin D production in the skin. Clinical trials with vitamin D and calcium have had limited success with respect to reducing obesity or increasing energy expenditure [164].

2.6 VITAMIN D AND CANCER

2.6.1 Epidemiological data

The inverse relationship between solar exposure and cancer mortality in North America was first noted by Apperly [177] in 1941. This association was related to ultraviolet B exposure as the protective element by the Garland brothers in 1980 in their epidemiologic studies with colon cancer, introducing the possible link with vitamin D status. With the exception of skin cancer, the inverse relationship between solar exposure and cancer has been described for many other cancer types in several countries [178].

There is now a very extensive medical literature on vitamin D and cancer epidemiology. Most of the studies have focused on epithelial cancers, i.e. colorectal, breast, and prostate cancer.

Colon cancer

Observational studies in humans (cross-sectional studies and especially long-term, prospective studies) revealed a link between poor vitamin D status (either evaluated by serum 25(OH)D_3 or a surrogate estimation) and the risk of nearly all cancers [178,179], but a reanalysis by a World Health Organization (WHO) working group identified colon cancer as the greatest risk associated with poor vitamin D status. This finding was supported by the results of a meta-analysis of nine case-control studies [180]. For each 4 ng/mL (10 nmol/L) increase in prediagnosis serum 25(OH)D_3 concentration, there was a 6 percent (95% CI 3-9 percent) reduction in colorectal cancer risk. In one of the largest studies in the meta-analysis, a nested case-control study in European populations including 2496 cases and controls, serum 25(OH)D_3 levels between 10 and 20 ng/mL (25 to 50 nmol/L) compared with 20 to 30 ng/mL (50 to 75 nmol/L) were associated with a higher incidence of colorectal cancer (incidence rate ratio 1.28, 95% CI 1.05-1.56) [181]. In contrast to these results, a prospective case-control study did not show a significant association between vitamin D status and colon or colorectal cancer [182]. In addition, some observational studies have shown an elevated risk of some cancers (i.e., pancreatic) at higher 25(OH)D_3 levels (relative risk [RR] 2.12, 95% CI 1.23-3.64 for levels ≥40 versus 20 to 30 ng/mL [≥100 versus 50 to 75 nmol/L]) [183].

Breast cancer

Observational studies examining the relationship between vitamin D and breast cancer report inconsistent results. A meta-analysis of prospective studies examining the relationship between serum 25(OH)D_3 concentrations and breast cancer risk showed a significant inverse association in post- but not premenopausal women [184]. The risk of postmenopausal breast cancer decreased with 25(OH)D_3 levels between 27 and <35 ng/mL (67 to 87 nmol/L), with no further reduction for levels above 35 ng/mL.

A metanalysis of 8 case control studies demonstrated an OR of 0.55 (CI 0.38–0.80) comparing the highest quintile of 25(OH)D_3 levels to the lowest. In general, these studies did not evaluate premenopausal and postmenopausal women separately [164]. Chlebowski performed a similar review of 10 case control and 10 cohort studies with respect to vitamin D intake and breast cancer and 4 case control and 6 nested case control studies with respect to 25OHD levels and breast cancer [185]. A meta-analysis of five of the case control studies examining vitamin D intake failed to show a significant effect of vitamin D overall, but when only the premenopausal/perimenopausal women were included in the analysis a significant negative association between increased intake and

breast cancer incidence was found (RR 0.83, CI 0.73–0.95). Of the six nested case control studies assessing the relationship of serum $25(OH)D_3$ and breast cancer, only one study showed a significant negative association between high $25(OH)D_3$ levels and incidence of breast cancer, although one other study was close (P = 0.06). In a separate metanalysis by Gandini et al., a RR of 0.89 (0.82–0.98) for a 10 ng/ml increase in $25(OH)D_3$ was found when all studies were included and 0.83 (0.79–0.87) when only case control studies were pooled. Epidemiologic data tend to support a protective role for vitamin D and breast cancer, but the data are not as compelling or consistent as for colorectal cancer [178].

Prostate cancer

A relationship between serum $25(OH)D_3$ levels and prostate cancer incidence has not been consistently found. In observational studies, higher (highest compared with lowest quartiles or quintiles) serum $25(OH)D_3$ levels have been associated with both an increased and reduced risk of more aggressive disease [186]. A meta-analysis of six cohort/nested case control studies (8,722 cases) examining the association of dietary vitamin D intake to prostate cancer found a relative risk of 1.14 (CI 0.99–1.31) for an increase in dietary vitamin D of 1,000 IU. Similarly, a meta-analysis of 14 cohort/nest case control studies including 4,353 cases examining the association of serum $25(OH)D_3$ and prostate cancer found a relative risk of 1.04 (CI 0.99–1.1) for a 10 ng/ml increase in $25(OH)D_3$ for all prostate cancers. Likewise, no association was found for serum $1,25(OH)_2D_3$ levels [187]. Similar negative results were observed in the meta-analysis by Gandini et al [178]. Although an initial clinical trial (ASCENT I) with high dose $1,25(OH)_2D_3$ and docetaxol seemed to show promise in the treatment of castration resistance prostate cancer, this initial success could not be repeated in a larger trial (ASCENT II) potentially flawed by a change in the docetaxol only arm of the study [164]. Thus, the clinical evidence weighs against vitamin D being beneficial in the prevention/treatment of prostate cancer.

2.6.2 Intervention trials

The majority of vitamin D intervention trials do not show a reduction in cancer risk [187]. In a meta-analysis of 18 randomized trials in predominantly older, community-dwelling women, vitamin D supplementation had no effect on the incidence of cancer [188]. In a trial published after the meta-analysis, 2303 healthy, postmenopausal women (mean baseline $25(OH)D_3$ 32.8 ng/mL [81.9 nmol/L]) were randomly assigned to 2000 international units vitamin D3 and 1500 mg of calcium daily or identical placebos]. After four years, the proportion of patients in each group with newly diagnosed cancer did not

significantly differ (3.89 and 5.58 percent, respectively, hazard ratio [HR] 0.70, 95% CI 0.47-1.02) [189]. An analysis by cancer site showed no difference in the incidence of breast cancer between the two groups; there were too few cancers at other sites to analyze. Study limitations include selection of patients with a relatively high baseline vitamin D level and permission to supplement with vitamin D (up to 800 international units daily) outside of the intervention, both of which may have contributed to the absence of an effect. Additional trials are ongoing [164,189].

To date, the relationship between cancer and vitamin D remains controversial. The expert panel of the Institute of Medicine (IOM) declared that the data were inconclusive as to whether vitamin D had a protective role in cancer [178]. Indeed, clinical data are mixed, and definitive evidence from randomized clinical trials is lacking. Given the lack of funding support for a sufficiently large trial, such evidence may be difficult in the near future.

2.6.3 Cellular mechanisms by which vitamin D is supposed to be an anticancer agent

$1,25(OH)_2D_3$ regulates the expression of hundreds of genes, both those encoding mRNAs that are translated into proteins and those encoding RNA that are non (miRNAs, lncRNAs). Different cell types exhibit different profiles of genes that are regulated by $1,25(OH)_2D_3$, so that generalisations are difficult to make. Nevertheless, there are a number of cellular pathways that contribute to vitamin D regulation of cancer growth and metastasis that are found in several cancers. Mechanisms are summarised in Table 4.

Table 4. Mechanisms of vitamin D tumour suppression (modified from [178]**).**

Antiproliferation	1. Arrest of cell cycle: G0/G1 and G1/S 2. Dephosphorilation of FOXO 3. ↓ level of myc, fos, jun 4. ↓ activity of growth factors: IGF-I, IHH, EGF 5. ↑ activity of TGFβ 6. ↓ activity Wnt/β-catenin signalling 7. Regulation of miRNA and lncRNA
Apoptosis	1. ↑ expression GOS 2 and Bax, ↓expression Bcl2 and Bcl-XL 2. ↑ expression DAP-3, CFKAR, FADD, ↓ caspases 3. ↑ expression PTEN 4. ↑ autophagy
DNA repair	1. ↑ clearance of CPDs and 6,4-PPs (in UVB irradiated skin) 2. ↓ oxidative DNA damage by ↑ expression antioxidant enzymes

	3. ↑ expression of DNA repair enzymes XPC and DDB2
Prostaglandin metabolism	1. ↓ COX2 expression 2. ↓ PG receptors 3. ↑ 15-PDGH expression
Angiogenesis	1. ↓ proliferation of endothelial cells 2. ↓ VEGF expression
Invasion and Metastasis	1. ↓ Cell migration and invasion capacity 2. ↓ expression of laminin and its receptors 3. ↑ expression of E-cadherin 4. ↓ expression of CEACAMI

Antiproliferation

$1,25(OH)_2D_3$ is antiproliferative for most cellular types. $1,25(OH)_2D_3$ typically causes arrest at the G0/G1 and/or G1/S transitions in the cell cycle. This is associated with a decrease in cyclins and an increase in the inhibitors of the cyclin-dependent kinases (CDK) such as $p21^{cip1}$ and $p27^{kip1}$ again in a cell specific fashion. The antiproliferative actions of $1,25(OH)_2D_3$ and its induction of $p21^{cip1}$ in colorectal cancer cell lines are dependent on the expression of the calcium sensing receptor [190]. Forkhead box O (FOXO) proteins are transcription factors that control proliferation. $1,25(OH)_2D_3$ promotes the interaction between several of the FOXOs with VDR and FOXO regulators such as SIRT1 and protein phosphatase 1, keeping FOXO dephosphorylated and in the nucleus in order to suppress genes involved with proliferation. Levels of other genes linked to proliferation such as *MYC*, *FOS*, and *JUN* are also decreased by $1,25(OH)_2D_3$ [191]. Insulin like growth factor (IGF) is a growth promoter in several tumours. $1,25(OH)_2D_3$ stimulates the expression of IGF binding protein 3 (IGFBP3), which binds IGF I and II, limiting their proliferative effects. TGFβ2 is an antiproliferative factor in epithelial cells. $1,25(OH)_2D_3$ stimulates the expression of TGFβ2 as well as the TGFβ receptors in a number of cell types including breast and prostate cancer cells [192]. Hedgehog (HH) signalling promotes proliferation, and its overexpression is a major cause of basal cell carcinoma. Expression of components of the HH pathway such as SHH and GLI1 is suppressed by $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ inhibits EGF promotion of proliferation by targeting the EGF/EGFR complex to endosomes and inhibiting the expression of EGFR [178]. Mutations in APC, leading to over activation of the Wnt/β-catenin pathway, are the cause of most colorectal cancers. When β-catenin binds to TCF/LEF sites in the nucleus, proliferation is stimulated. $1,25(OH)_2D_3$/VDR competes

with TCF/LEF for binding to β-catenin so, binding of β-catenin to VDR may promote differentiation. Moreover, $1,25(OH)_2D_3$/VDR stimulates the expression and translocation (with calcium) of E-cadherin to the cell membrane where it forms a complex with β-catenin and other catenins again promoting differentiation. The ability of $1,25(OH)_2D_3$ to increase intracellular calcium contributes to these actions of $1,25(OH)_2D_3$ by increasing E-cadherin expression and reducing the induction of cyclin D1 [164].

Apoptosis

$1,25(OH)_2D_3$ promotes the apoptosis of a number of cell types. These actions are accompanied by increased expression of the pro-apoptotic genes G0S2 (G0/G1 switch gene 2) and Bax with suppression of the proapoptotic genes Bcl2 and Bcl-XL [193]. Other pro-apoptotic genes induced by 1,25(OH)2D include death-associated protein-3 (DAP-3), caspase 8 apoptosis-related cysteine peptidase (CFKAR), Fas-associated death domain (FADD), and a number of caspases (i.e., caspase 3, 4, 6, and 8). $1,25(OH)_2D_3$ sensitizes cells to apoptosis induced by reactive oxygen species (ROS) and cytokines (i.e., TNF-α). By promoting both calcium influx and release from intracellular stores, $1,25(OH)_2D_3$ induces apoptosis by activating the calcium-dependent μ-calpain and calcium/calpain-dependent caspase 12. Normal cells are protected by the presence of the calcium-binding protein $CaBP_{28k}$, which acts as a calcium buffer in cells and that are less abundant in a number of cancer cells. By inducing PTEN (phosphatase and tensin homolog), $1,25(OH)_2D_3$ inhibits PI3K and so reduces Akt activation, increasing BAX activity and so promoting apoptosis. Finally, $1,25(OH)_2D_3$ has been shown to stimulate autophagy in some cancer cells in part by inhibiting the anti-autophagy gene mTOR and increasing the levels of the pro-autophagy gene *BECLIN-1* [138,178,194].

DNA repair

Oxidative stress is the major cause of DNA damage in tissues other than the skin, which is exposed to the direct damage by UVB radiation that induces the formation of cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4)pyrimidone photoproducts (6-4PP). Preventing DNA damage from producing DNA mutations is the role of DNA damage repair (DDR), operating through mechanisms involving damage recognition, repair, and signal transduction. $1,25(OH)_2D_3$ topically applied protects the skin from UVB-induced photodamage including increased clearance of CPDs, decreased apoptosis, increased survival, and increased expression of p53 [178,195]. In keratinocytes

$1,25(OH)_2D_3$ reduces Poly-ADP-ribose polymerase (PARP) activity, a protein involved with DNA breaks. In various tissues, vitamin D deficiency is associated with increased frequency of chromosomal damage due to oxidative and other stresses. In humans, vitamin D deficiency is associated with increased levels of 8-OH-20-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage, whereas vitamin D supplementation reduces these levels. Moll et al. found that $1,25(OH)_2D_3$ induces two genes important for DDR: XPC (xeroderma pigmentosum complementation group C) and DDB2 (damage-specific DNA binding protein 2 also known as XPE) [196]. VDR agonists also induce the DNA repair protein GADD45 (growth arrest and DNA-damage inducible). Other anti-oxidative enzymes induced by $1,25(OH)_2D_3$ include thioredoxin reductase 1, superoxide dismutase, glucose-6 phosphate dehydrogenase, and glutathione peroxidase [147,178].

Prostaglandin metabolism

Prostaglandin (PG) production is associated with cancer growth and metastasis. The enzymes responsible, cyclo-oxygenase (COX) 1 and 2 are induced by a variety of tumour promoters. $1,25(OH)_2D_3$ at least in some cancer cell lines suppresses COX2 expression synergistic with NSAIDs and that of PG receptors, while increasing the expression of 15-PGDH (hydroxyprostaglandin dehydrogenase 15-NAD), the enzyme that inactivates PGs [164,197].

Angiogenesis

Angiogenesis is critical for tumour growth and metastasis. Vascular endothelial growth factor (VEGF) is the major stimulator of angiogenesis. VEGF production is generally induced in hypoxic states by hypoxia-induced factor-1α (HIF-1α). $1,25(OH)_2D$ reduces hypoxia-induced expression of VEGF in a variety of cancer cell lines at least in part by reducing the expression of HIF-1α. In addition, $1,25(OH)_2D_3$ inhibits the proliferation of endothelial cells and reduces VEGF-induced endothelial cell sprouting and elongation resulting in tumours with decreased vascularization [137,198].

Inhibition of metastasis

In addition to reducing the blood supply to the tumour, $1,25(OH)_2D_3$ reduces the migration and invasion capacity of tumour cells [137,164]. This is facilitated by down regulation of the matrix protein laminin and its receptors α6 and β4, and induction of E-cadherin that contrast the ability of cancer cells to bind to endothelial cells, necessary for

metastasize. $1,25(OH)_2D_3$ also reduces the expression of (carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) by endothelial cells again reducing metastasis. $1,25(OH)_2D_3$ degradation of the matrix by cancer cells, limiting the activity of matrix metalloproteinases and cathepsins by inducing their inhibitors [138,199].

Regulation of miRNA and lncRNA

VDR binding sites in the genome are numbered in the thousands and VDR coding transcripts number in the hundreds. A relevant level of regulation occurs via microRNAs (miRNAs) and long noncoding RNAs (lncRNAs), a difference being that miRNAs are typically around 20 nucleotides in length, whereas lncRNAs are 200 or more nucleotides. A number of miRNAs have been identified to be regulated by $1,25(OH)_2D_3$–VDR and important for its antiproliferative actions. These include increased expression of miR145, which blocks the expression of E2F3, a key regulator of proliferation, or miR-32 that blocks the proapototic protein BIM, which paradoxically protects the cell (human myeloid leukemia) from AraC-induced apoptosis [138,200]. In VDR-null (*Vdr*−/−) keratinocytes, a number of oncogenic lncRNAs are increased, whereas tumour suppressor lncRNAs are decreased [201].

Alterations in VDR levels and vitamin D metabolism

Most tumours express the VDR, and mutations in the *VDR* are uncommon [164,202]. However, the expression of the VDR is often lost as a tumour undergoes progressive dedifferentiation, and loss of its expression in a tumour is a bad prognostic sign. Similarly, *CYP27B1* [$25(OH)D_3$ 1α-hydroxylase] is expressed in many tumours at early stages of development and mutations are uncommon but like the VDR, expression of CYP27B1 typically declines with progressive dedifferentiation [137,138]. On the other hand, *CYP24A1* [$25(OH)D_3$ 24-hydroxylase] expression is often increased in tumours, effectively reducing the levels of $1,25(OH)_2D_3$ in the tumour microenvironment, and thus explaining tumour resistance to $1,25(OH)_2D_3$ [137,178,194]. This increase is secondary to the *CYP24A1* gene being part of a region of gene duplication seen in some tumours; its overexpression is a poor prognostic sign [138].

PART 3: VITAMIN D, EPIGENOME, AND CANCER

Epigenetics is the study of heritable changes in gene expression that cannot be explained by changes in DNA sequence [203]. Epigenetics has become a topic of growing interest in recent years, since a body of evidence showed that epigenetic processes are responsible for global and local condensed or decondensed chromatin states that eventually determine gene expression, definitely resulting crucial for cell identity and functions. Epigenome, or 'epigenetic landscape', refers to the epigenetic status that determines the way a single eukaryotic genome may manifest itself [204].

The first identified mechanisms of epigenetic regulation included DNA methylation and covalent histone modifications. DNA methylation usually occurs at the 5′ position of the cytosine ring within CpG dinucleotides, and its consequence is the silencing of genes and noncoding genomic regions. Most CpG sites are concentrated either in CpG islands, short CpG-rich DNA regions located in approximately 60% of human gene promoters, or in regions of large repetitive sequences (i.e., centromeres and retrotransposon elements) [205]. Modifications in DNA methylation are performed mainly by DNA methyltransferases (DNMTs) and ten-eleven translocation (TET) proteins. A plethora of enzymes, such as histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), and histone demethylases (HDMs) regulate covalent histone modifications that may result in both gene activation or silencing [206].

The epigenetic regulatory system is often disturbed in cancer [207,208]. Global DNA hypomethylation and promoter-specific hypermethylation are typical features of epigenome disruption in cancer cells. Loss of global methylation may lead to chromosomal instability, loss of imprinting, and activation of transposable elements, thereby leading to disturbances in the genome [209]. Conversely, hypermethylation of promoter regions of tumour suppressor genes leads to loss of expression of key genes affecting pathways involved in maintenance of cellular functions, including cell cycle, apoptosis, and DNA repair [210,211] (Figure 8). Several bona fide tumour suppressor genes are silenced by promoter hypermethylation in tumours [194,209].

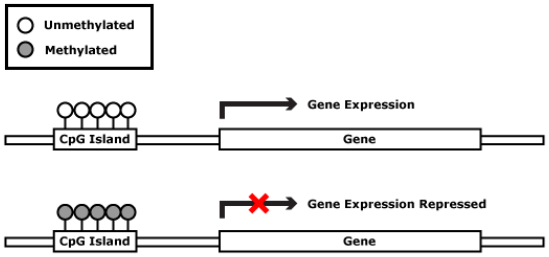


Figure 8. Simplified illustration of DNA methylation ant its main effect. Many genes in the human genome have upstream CG-rich regions called CpG islands. DNA methylation of a gene's CpG island represses gene expression.

Vitamin D can interact with the epigenome on multiple levels, with potential impact in health and disease [148]. Firstly, there is recent evidence that certain VDR ligands have DNA demethylating effects. Secondly, VDR protein physically interacts with coactivator and corepressor proteins, which in turn are in contact with chromatin modifiers, such as HATs, HDACs, HMTs, and with chromatin remodelers. Generally, HATs are defined as activators of transcription, whereas HDACs as transcription repressors. Thirdly, a number of genes encoding for chromatin modifiers and remodelers, such as HDMs of the Jumonji C (JmjC)-domain containing proteins and lysine-specific demethylase (LSD) families are primary targets of VDR and its ligands. Finally, critical genes in the vitamin D signalling system, such as those coding for vitamin D receptor (*VDR*) and the enzymes 25-hydroxylase (*CYP2R1*), 1α-hydroxylase (*CYP27B1*), and 24-hydroxylase (*CYP24A1*) have large CpG islands in their promoter regions and therefore can be silenced by DNA methylation [147].

3.1 Effect of vitamin D on DNA methylation

There is evidence that $1,25(OH)_2D_3$ is able to induce DNA demethylation, but it is not clear if vitamin D-induced demethylation is a passive or an active process. The fact that vitamin D can alter methylation of DNA in the promoter of certain genes is novel.

Tapp et al. found a negative association between serum $25(OH)D_3$ levels and CpG Islands (CGI) methylation of the adenomatous polyposis coli (*APC*) promoter region, a tumour suppressor gene often inactive in colorectal cancer [212]. In the same study they highlighted a weak positive correlation of vitamin D level with methylation of the mammalian autonomous retrotransposon *LINE-1* (genomic long interspersed nuclear element-1), increasing stability of this region.

In the setting of colorectal cancer, Rawson et al. reported that a high dietary vitamin D intake was associated with lower methylation of two Wnt-antagonists DIKKOPF1 (DKK1) and WNT5A, commonly deregulated in colorectal cancer; this relationship was observed only in early stage tumours [213].

Treatment of the triple negative breast cancer cell line DA-MB-231 with $1,25(OH)_2D_3$ reduced DNA methylation of the promoter of E-cadherin, a cohesive molecule that prevents invasion and distant spread of malignant cells [214].

3.2 Interactions of vitamin D with chromatin modulators and remodelers

The chromatin context can alter nuclear receptor binding, determining which epigenetic modifications will occur thereafter. Unliganded VDR is able to bind genomic DNA, where it usually forms complexes with corepressor proteins that either exert HDAC activity, e.g., ALIEN [215], or are associated with HDACs, such as NCOR1 and SMRT. Upon binding of $1,25(OH)_2D_3$ with VDR/RXR, the corepressors dissociate and are replaced by coactivator complexes, which include histone acetyltransferases (HATs). As known, acetylation of histones enables chromatin relaxation and gene transcription. Many of the coactivators recruited by the VDR, including p160 steroid receptor coactivator proteins (SRC1, 2, and 3), p300, or CBP have lysine acetyltransferase activity [147] (Figure 9).

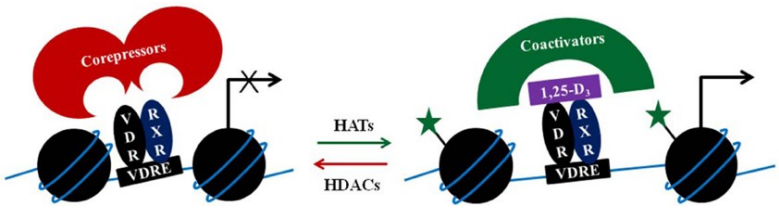


Figure 9. Simplified illustration of a two –step coregulatory model. Acetylation of histones (black circles) is indicated by the green stars [147].

Histone methylation can lead either to gene activation or repression, depending on the histone site that is methylated, the degree of methylation (e.g., mono-methylation, di-methylation, or tri-methylation), amino acid residues affected, and their position in the histone tail [211]. Methylation of histones depends on a dynamic process arising from the

actions of methyltransferases (HMTs) and demethylases (HDMs) [216]. So far, two protein families capable of demethylating lysines are known: the amine oxidases (whose major member is protein LSD-1/KDM1A) [217], and jumonji C (JmjC)-domain-containing proteins (whose first identified member was KDM2A/JHDM1A) [218]. There is a reciprocal regulatory effect between the activity of VDR and histone demethylases. In the colon cancer cell line SW480-ADH $1,25(OH)_2D_3$ increased expression of lysine-specific demethylase 1 and 2.

$1,25(OH)_2D_3$ treatment affected also the expression of a series of different JmjC histone demethylases, exerting inhibition of expression of KDM4 family members thus increasing genome stability, while upregulating members of KDM5 cluster responsible for gene repression. In other words, components of the machinery involved in DNA epigenetic regulation are primary targets of vitamin D, whose genome transcriptional activity can be affected by epigenetic factors.

In different pathologies, the expression pattern of the nuclear receptor cofactors is altered, compromising the effect of $1,25(OH)_2D_3$ [219]. The initial interactions between VDR and coactivators are critical for the assembly of intricate multiprotein complexes that remodel the chromatin structure, recruit the core transcriptional machinery, and induce expression of $1,25(OH)_2D_3$ target genes. Often, differences in responsiveness to $1,25(OH)_2D_3$ depend on the expression pattern of the coregulators of VDR [148].

3.3 Epigenetic regulation of the vitamin D system

The major regulators of $1,25(OH)_2D_3$ levels and signalling *CYP2R1*, *CYP24A1*, *CYP27B1*, and *VDR*, collectively so called "the vitamin D tool" genes, are prone to epigenetic regulation. CpG islands span the promoters of *CYP2R1*, *CYP24A1*, and *VDR*, while a CpG island is located within the *CYP27B1* gene locus (Figure 10). Therefore, DNA methylation and histone modifications in these genomic regions can modify the chromatin state from an open to closed conformation and lead to transcriptional repression of these genes [147].

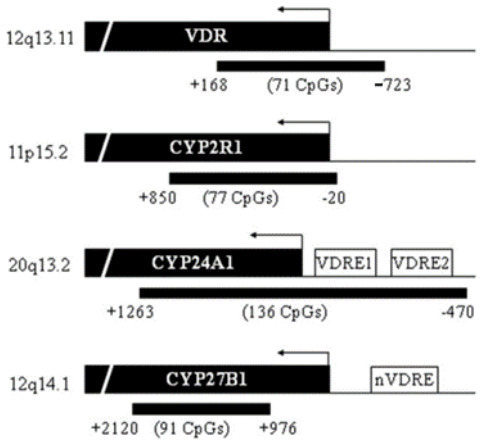


Figure 10. Location of CpG islands in the promoter region of vitamin D tool genes [147]**.**

Expression of vitamin D system genes resulted deregulated in various types of cancer, and these alterations can be partially attributed to epigenetic changes.

3.3.1 VDR

Epigenetic corruption of VDR signalling is suggested to be one of the main mechanisms that leads to the reduced responsiveness to $1,25(OH)_2D_3$ actions which is frequently observed in cancer cells. In fact, in cancer the VDR is not overtly disrupted by either genetic mechanisms, such as mutation, or appears to be the subject of cytogenetic re-arrangements [202].

Abedin et al. reported that VDR actions in solid tumours are retained, but inhibited by epigenetic mechanisms that disfavour selectively antiproliferative target gene promoter responses, i.e. through hypermethylation of CpG sites [220]. Furthermore, accumulation of VDR-associated corepressors in cancer cells could inappropriately sustain histone deacetylation around the VDRE or target gene promoter regions, so that transcriptional repression of antiproliferative VDR target genes can occur [220]. Targeting this molecular lesion with co-treatments of vitamin D_3 compounds plus HDAC inhibitors resulted in a more transcriptionally permissive environment to favour expression of antiproliferative genes.

In breast tumours, methylation of exon1a of the *VDR* gene was reported significantly higher (65% of CpGs methylated) compared with normal breast tissue (15% of CpGs methylated) [221]. *In vitro*, in breast cancer cell lines, three hypermethylated regions in exon1a became demethylated after treatment with the DNMT1 inhibitor 5-aza-2'-deoxycytidine (DAC) and VDR mRNA expression increased. These regions were in

proximity to the SP1 binding sites (approximately 790 bp from TSS [Transcription Start Site]), NFκB binding sites (approximately −480 from TSS), and the exon 1a TSS. Treatment with $1,25(OH)_2D_3$ had no effect on methylation of these regions [221]. To note, the same Authors reported an association between *VDR* promoter methylation and expression of 5' truncated variants of VDR, selectively found in cancer samples.

In contrast, no methylation of the VDR promoter region was observed in colon cancer cell lines, and treatment with the demethylating agent DAC did not increase gene expression [222].

In parathyroid tumours the expression of VDR is decreased [223]; however, no differences in DNA methylation of *VDR* were observed between parathyroid tumours and healthy controls [224]. Similar results were seen in parathyroid adenoma samples, which showed decreased expression of VDR, but showed no promoter methylation [225].

Epigenetic silencing of *VDR* has been reported in colorectal cancer metastasis. VDR becomes target of polycomb group protein enhancer of zeste homolog 2 (EZH2) which is overexpressed in tumours and has histone methyltransferase activity. EZH2 regulates H3K27 trimethylation in the *VDR* promoter, thus resulting in VDR downregulation [226].

3.3.2 CYP2R1

CYP2R1 is a microsomal P450 enzyme which hydroxylates both vitamin D_2 and D_3 at position C-25 to form the circulating storage form $25(OH)D_3$. *CYP2R1* promoter region is located within a CpG island. Increased *CYP2R1* promoter methylation was found in leucocyte DNA from subjects with vitamin D deficiency (defined by serum 25-hydroxyvitamin D [25(OH)D] ≤ 25 nmol/L) [227], but vitamin D supplementation may decrease *CYP2R1* promoter methylation [228].

3.3.3 CYP27B1

CYP27B1 converts $25(OH)D_3$ to its active form $1,25(OH)_2D_3$. *CYP27B1* gene coding sequence contains a CpG island. In breast cancer cells MDA-MB-231, CYP27B1 hypermethylation leads to gene silencing which can be reversed by treatment with deoxyC. Similar results came from studies with non-Hodgkin lymphoma. Combination of the DNMT1 inhibitor DAC and the HDAC inhibitor TSA resulted in increased activity of CYP27B1 in prostate cancer cell lines [147].

Interestingly, the promoter region of *CYP27B1* contains a negative VDRE (nVDRE), located approximately at 500 bp. This region is responsible for $1,25(OH)_2D_3$-dependent transrepression, which seems to be achieved through recruitment of both HDACs and

DNMTs by VDR/RXR to the promoter region of *CYP27B1* [229]. Taken together these findings are consistent with the VDR-mediated epigenetic regulation in the *CYP27B1* gene.

3.3.4 CYP24A1

CYP24A1 encodes 1,25$(OH)_2D_3$ 24-hydroxilase, an inner mitochondrial membrane P450 enzyme which catalyses both 25$(OH)D_3$ and 1,25$(OH)_2D_3$. *CYP24A1* gene promoter is spanned by a wide CpG island that includes responsive elements and SP1 binding sites.
In several human healthy tissues (kidney, skeletal muscle, blood, brain, skin fibroblast, sperm), CYP24A1 is expressed at variable levels and *CYP24A1* promoter is not methylated, or a it is methylated at low levels [147].
In prostate cancer cells, *CYP24A1* promoter methylation downregulates gene expression and in clinical studies progression from benign to malignant lesions was paralleled by increasing methylation levels of the CYP24A1 promoter. Demethylating agents showed the ability to restore CYP24A1 expression in prostate cell lines [230].
In colon cancer cell lines, CYP24A1 can be induced by treatment with DAC in a cell line specific manner. This effect did not correlate with the methylation state of the promoter and therefore must affect genes upstream of CYP24A1 [222].

KEY CONCEPTS AND AIM OF THE STUDY

Besides the classical role in calcium and bone homeostasis, 1α,25-dihydroxycholecalciferol D3 [1α,25$(OH)_2D_3$] (calcitriol), the active metabolite of vitamin D, has been recognized to have "non-calcaemic" effects in a variety of cells after binding to vitamin D receptor (VDR, NR1I1), a member of the nuclear receptor superfamily which includes receptors for steroids, thyroid hormones, and retinoic acid [145]. The VDR forms homodimers or heterodimers with the retinoid X receptor (RXR, NR2B), to allow specific DNA binding. The binding of 1α,25$(OH)_2D_3$ with VDR-RXR complex is followed by the attachment of this complex to vitamin D responsive elements, which then initiate transcription in the promoter of target genes [146,150]. The effect of liganded VDR depends on the epigenetic landscape of target gene [147]. There is evidence that 1α,25$(OH)_2D_3$ protects against tumour formation by several VDR-mediated mechanisms, including regulation of growth arrest, cell differentiation, migration, invasion, and apoptosis, making it a candidate agent for cancer regulation [164,178,231,232]. A relationship between the vitamin D system and the adrenal pathophysiology and growth has been recently highlighted [233].

Previous findings of the research group I attended during my Ph.D program showed a decreased expression of VDR mRNA and protein in a small series of human adrenocortical carcinomas (ACCs), suggesting the loss of a protective role of VDR against malignant cell growth, as reported for other cancer types [234,235]. An aberrant global and gene-specific DNA promoter methylation has been observed in human adrenocortical tumours, either benign or malignant, implicating dysregulation of steroid biosynthesis and adrenal growth [81-83,236,237]. Downregulation of *VDR* gene expression in adrenocortical carcinomas may result from epigenetic events, that is, methylation of cytosine nucleotides in CpG island of *VDR* promoter. In fact, promoter methylation is able to distort the transcription factor binding sites, causing transcriptional silencing. In continuity with our previous observations [235], the main aim of my study was to analyse methylation of CpG sites in the *VDR* gene promoter of a different and larger series of human adrenocortical tissues, comparing adrenocortical adenomas (ACAs) with ACC samples.

MATERIALS AND METHODS

Patients and Tissue Samples

The study was approved by the institutional review board of the University Hospital of Padova in accordance with the Declaration of Helsinki guidelines as revised in 1983. Informed consent was obtained from all individual participants included in the study. The preoperative diagnosis was based on the clinical history, symptoms, signs, endocrine evaluation, and imaging examination (e.g., MRI, CT). Archival microdissected paraffin embedded slides of the patients were used for histological examinations and molecular studies. Diagnosis of adrenal malignancy was performed according to the histopathological criteria proposed by Weiss et al. [46] and the modification proposed by Aubert et al. [47]. Three normal adrenal cortices from adrenal glands of kidney donors were also studied. Histopathological slides were classified by two pathologists independently, and no discrepancy existed between them.

VDR Promoter Methylation Analysis

DNA extraction, bisulfite conversion, and bisulfite sequencing PCR

Total genomic DNA was extracted from formalin-fixed paraffin embedded (FFPE) adrenocortical tissues using QIAamp DNA FFPE Tissue Kit (Qiagen, Milan, Italy). DNA samples underwent bisulfite conversion using EZ DNA Methylation-Gold Kit (Zymo Research Co., Milan, Italy). Bisulfite treatment produces a chemical conversion of unmethylated cytosine to uracil, which is detected as a thymine after PCR. Methylated cytosines are protected from chemical conversion (Figure 11).

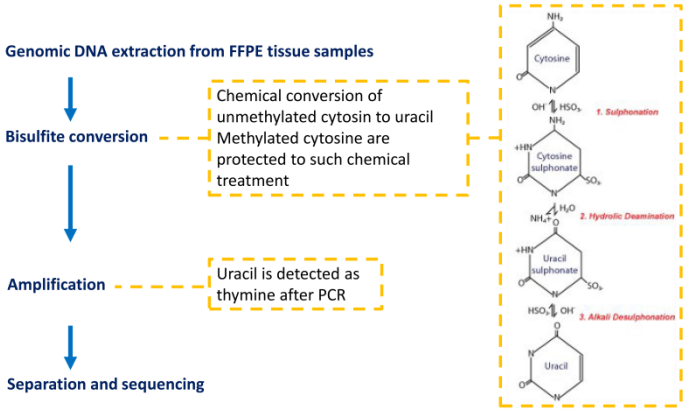


Figure 11. Steps of bisulfite-sequencing. Bisulfite converts DNA unmethylated cytosine to uracil.

Bisulfite-treated DNA was amplified using two sets of bisulfite sequencing primers designed by using MethPrimer (http://www.urogene.org/methprimer/index1.html) encompassing the region from −693 bp to −65 bp upstream VDR transcription start site. Primer sequences are as follows:

M1F 5′-GGAATTCGGGATTAGGGATTAGGGAAG-3′
M1R 5′ -AATACGTCACCCCCACCTAAACTAACCAAAC-3′
M2F 5′-GTTAGTCGCTAGGCGTTTTTTAGCGTTTCGC-3′
M2R 5′-TATAAAACAAAATTATCGATAATTATAAATA-3′
M3F 5′-GTAGAATTACGGTAGGAAGGGTGGGGGGTTG-3′
M3R 5′-CCCCGCCCACAAATCCAATCCTCTCTTACC-3′

PCR products were separated by electrophoresis and isolated using a Centri-Sep Columns (Princeton Separations, Milan, Italy). DNA was sequenced using the Reverse Primer (M1R and M2R) with an Applied Biosystems automated fluorescent sequencer (Applied Biosystems, Milan, Italy). In DNA sequence, methylated sites were visually counted.

Pyrosequencing reaction and analysis

PCR amplification of bisulfite converted DNA with specific methylation assay was performed using EpiTect PCR kit (Qiagen). Ready-made *VDR* assays were purchased from Qiagen (Qiagen). The primers were located within CpG islands in the promoter region of the target gene. Bisulfite treated PCR products were run in 2% agarose gel for quality. For pyrosequencing reactions, 15–20 μL of the bisulfite converted amplified DNA was added to a total reaction volume of 85 μL containing binding buffer and sepharose streptavidin. This reaction mix was agitated for 10 min at room temperature in order to facilitate the attachment of the beads to the biotinylated strands. The double stranded DNA was separated using the filter pump and this allowed having only single stranded (biotinylated) DNA as a template. This was released to the pyromark plate well each containing sequencing primer with annealing buffer in a reaction volume of 25 μL. The mixture was denatured for 2 min at 80°C and reannealed at room temperature for at least 5 min. The plate was put in the PyroMark Q24 machine and run with relevant run file. All sequencing primers contained at least one nucleotide (C not followed by G) to control for bisulfite conversion efficiency. A methylation standard (0%, 50 and 100% methylated) (EpiTect control set, Qiagen, Sweden, AB) and water were used as positive and negative controls, respectively. Data analysis was performed using the PyroMark

Q24 software (Qiagen, Sweden, AB). The program evaluates the target CpG sites and converts pyrograms to numerical values for peak heights and calculates the proportion of methylation at each base as a C/T ratio. Tumour methylation status was defined based on comparison to the methylation status of the normal reference samples. A difference of more than 5% in methylation for a particular assay between adrenal tumour and normal references was regarded as significant difference. Pyrosequencing technique was used in order to analyse methylation status of *VDR* promoter in one of the three carcinomas which resulted methylated with standard methylation analysis methods. *VDR* promoter sequence analysed was: ATTYGYGATAGGTYGGGAAYGTGGTTAGTYGYGGTTYG. In this sequence Y represents a cytosine, in which a methyl group may be added to form 5-methylcytosine. A sample of normal adrenal was used for control.

RNA Isolation/Quantitative Real-Time PCR (RT-qPCR)

Total cellular RNA was extracted from FFPE adrenal tissue slide samples using RNeasy Universal kit (Qiagen, Gaithersburg, MD) according to the protocol provided by the manufacturer. FFPE tissue was deparaffinized and treated with proteinase K, and genomic DNA was removed for total RNA extraction. Total RNA was quantified by Nano-Drop 1000 Spectrometer (ThermoScientific, Wilmington, DE). Quality of RNA was analyzed by Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). Evaluation of gene expression was performed by quantitative RT-PCR. Quantitative PCR for *VDR* and the housekeeping HMBS (hydroxymethylbilane synthase) gene primers were as follows:

5′-GAAGCCTTTGGGTCTGAAGTG-3′ (VDR forward),

5′-CCGCCATTGCCTCCATCC-3′ (VDR reverse),

5′-GGCAATGCGGCTGCAA-3′ (HMBS forward),

5′-GGGTACCCACGCGAATCAC-3′ (HMBS reverse).

The annealing temperature was 60°C for all genes. PCR was carried out using a DNA Engine (Opticon 2 continuous fluorescence detection system, MJ Research, Waltham, MA, USA). For each sample, results were normalized with the HMBS rRNA.

Western Blot and Densitometric Analysis

Adrenal tissue slides were deparaffinized using Xylene (Sigma) for 3 × 10 min and protein was extracted using Qproteome FFPE Tissue kit (Qiagen, Milan, Italy) and slides

were subjected to western blot analyses by 10% Tris-HCl polyacrylamide gel electrophoresis (PAGE) (Invitrogen Co., Eugene, OR, USA) in running buffer (Tris/Glycine/SDS). Membranes were probed at 4°C overnight with anti-VDR mouse polyclonal antibody (1 : 500, VDR-D6, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and mouse β-actin, Clone AC-15 (1 : 10 000) (Sigma–Aldrich, St. Louis, MO, USA). Primary anti-VDR and anti-β-actin antibodies were detected with a secondary goat anti-mouse fluorescent antibody (IRDye 800CW, Li-Cor Biosciences, Milan, Italy) (1 : 15.000). Signal was acquired by Li-Cor Odyssey Clx (Li-Cor Biosciences). Quantification of individual protein bands was measured by Li-Cor Image Studio Digits. For each sample, results were normalized with the housekeeping protein β-actin.

Immunohistochemistry

Immunohistochemical analysis for VDR protein expression was performed on 10% formalin-fixed and paraffin embedded adrenal tissue slides. Rat monoclonal antibody for VDR (9A7γE101.4) was purchased from Calbiochem (Darmstadt, Germany). Immunostaining was performed by the streptavidin-biotin amplification method using a Histfine Kit (Nichirei Co. Ltd., Tokyo, Japan). Antigen retrieval was performed by heating the slides in an autoclave for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0). The dilution of the primary antibodies was 1:50. The antigen-antibody complex was visualized with 3,3′-diaminobenzidine solution [1 mM 3,3′-diaminobenzidine, 50 mM Tris-HCl buffer (pH 7.6), and 0.006% H_2O_2] and counterstained with hematoxylin. Results were scored semiquantitatively based on percentage of positive cells and staining intensity. A human breast cancer specimen was used as a positive control. Negative controls were incubated with normal mouse antiserum instead of the primary antibody, which uniformly demonstrated no reaction.

Statistical Analyses

For two-sample comparison, differences between means were assessed by Mann-Whitney U test. Relationships between continuous variables were assessed calculating Spearman's rank correlation coefficient. All results are expressed as mean ± SD for continuous variables. P values <0.05 were taken as statistically significant. Statistical analysis was performed using the GraphPad Prism version 6.0 software (GraphPad Software).

RESULTS

Clinical Characteristics

Twenty-three patients (12 females, 11 males) who underwent adrenalectomy for sporadic adrenocortical tumours between 2006 and 2014 were classified as ACAs (n = 15) and ACCs (n = 8). The cohort was different from that described in the publication by Pilon et al. [235]. Fifteen patients with ACA included 2 cortisol-producing adenomas, 10 aldosterone-producing adenomas, and 3 non-functioning adenomas: 7 were females and 8 males, ranging from 31 to 67 years of age (mean age of 51.3 years at presentation). Eight ACCs consisted of 5 females and 3 males, ranging from 33 to 73 years of age (mean age of 52.7 years).

Clinical and tumour characteristics of the 8 ACC patients, including ENSAT stage at surgery [42], are depicted in Table 5.

Table 5. Clinical and tumour characteristics of the ACC patients analysed in the study.

Sample ID	Age	Gender	Stage at surgery (ENSAT)	Hormonal hypersecretion	Weiss score	Size (cm)	Outcome
C1	58	F	III	Cortisol + Androgen	6	18	Died for recurrence 4 years after surgery
C2	51	M	III	Cortisol	5	9	Alive, with recurrence
C3	36	F	III	Cortisol	9	11	Died for recurrence 6 months after surgery
C4	73	F	III	Non-functioning	9	15	Died for recurrence 1 year after surgery
C5	52	M	IV	Non-functioning	10	15	Died for recurrence 2 years after surgery
C6	33	M	III	Cortisol	8	14	Alive, with recurrence
C7	51	F	III	Non-functioning	6	6	Alive, with recurrence
C8	68	F	II	Cortisol	9	8	Died for recurrence 2 years after surgery

Five ACCs patients had endocrine symptoms and signs of excess cortisol secretion; three patients had non-functioning adrenal mass. All patients with ACCs were treated with the adrenolytic drug mitotane, 1,1-dichloro- 2-(o-chlorophenyl)-2-(p-chlorophenyl) ethane (o,p′-DDD), before surgery. Specifically, mitotane was given before surgery to the five patients with Cushing's syndrome because of hypercortisolism not amenable by other inhibitors of steroidogenesis; the remaining 3 patients were treated with mitotane as adjuvant therapy before second operation for recurrent disease. Mean adrenal tumour diameter in ACAs and ACCs group was 14 mm and 120 mm, respectively. Mean post-surgery follow-up of patients was 72 months (range of 12–120 months) for ACAs and 26 months (range of 6–48 months) for ACCs.

VDR Promoter Methylation Analysis

Methylation in the VDR promoter was observed in 3/8 ACCs specimens, which included two cortisol-producing and 1 non-functioning carcinoma patients (C3, C4, and C6 patients in Table 5). Two PCR products, including region from −693 to −65 bp, contained 42 CpG islands, and 27 of them (64%) were methylated. One representative case is presented in Figure 12. Methylation sites were identical in all 3 ACCs tissue specimens. No VDR promoter methylation was found in the other 5 ACCs, 3 normal adrenals, and the 15 ACAs.

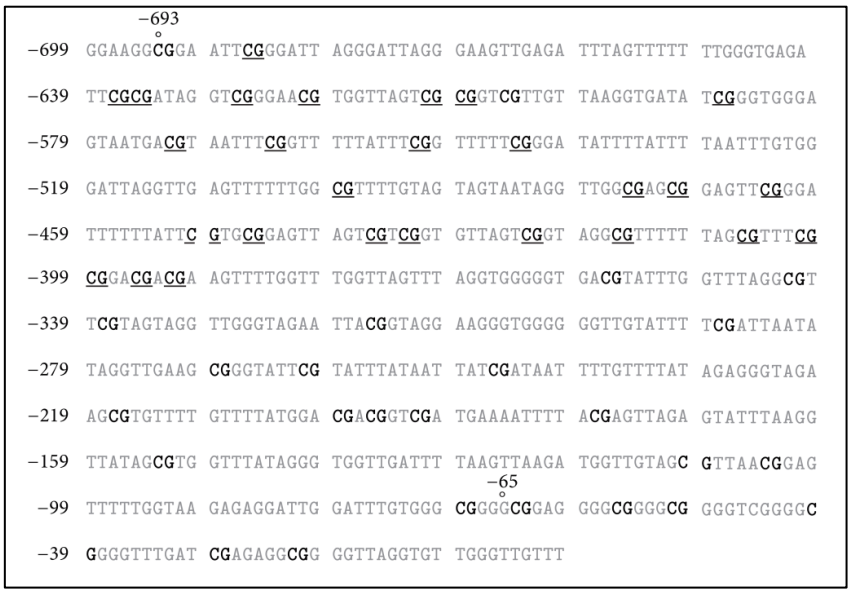
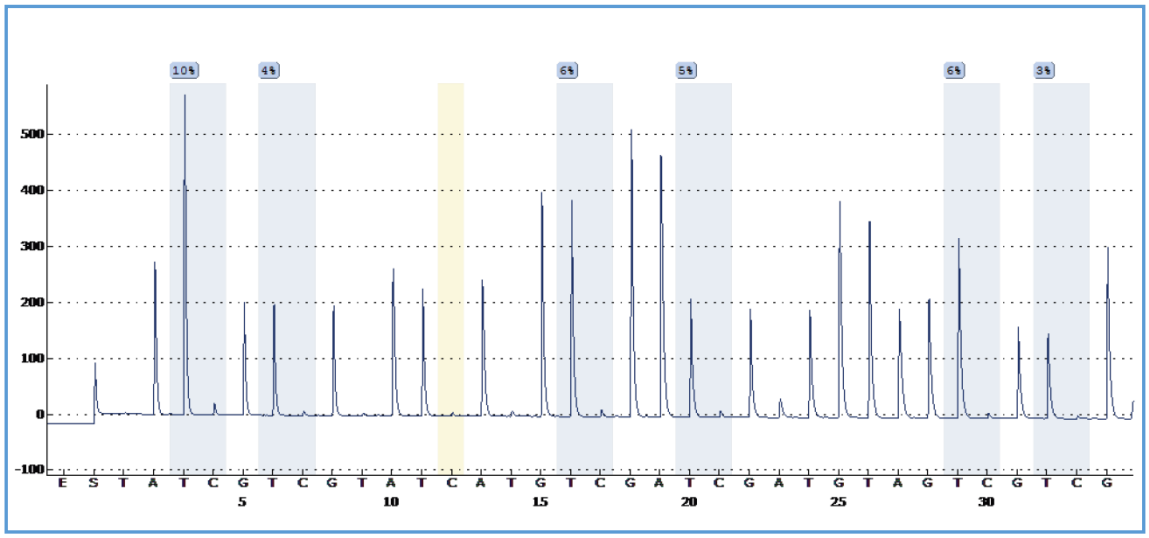


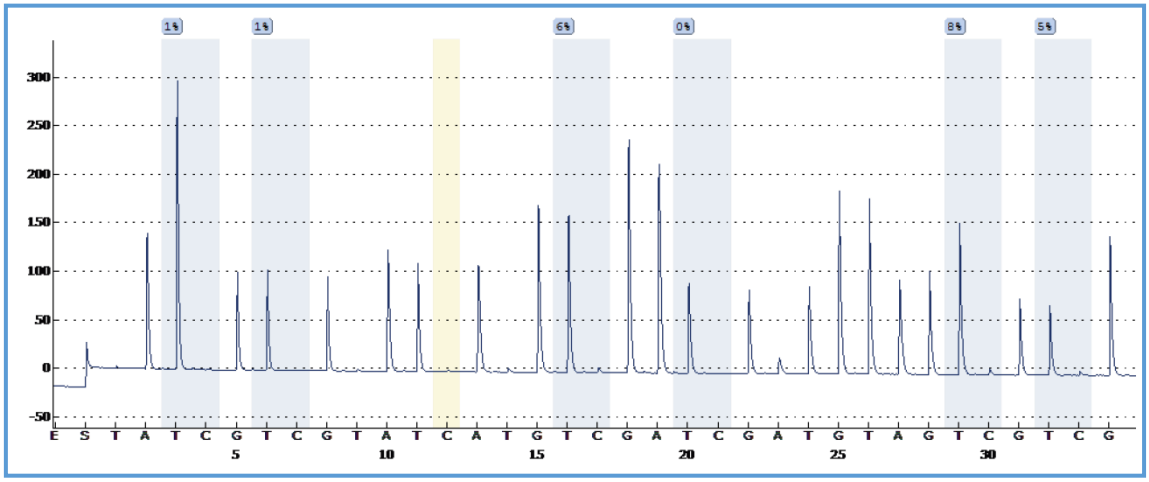
Figure 12. VDR methylated sites of VDR promoter region from −693 to −65 bp in one representative adrenocortical carcinoma, analysed by bisulfite sequencing. In DNA sequence, bold types indicate all CpG dinucleotides, and underlined CpG indicate methylated sites.

Pyrosequencing technique was performed in one carcinoma sample from the subgroup of the three carcinomas which resulted methylated with standard methods. Pyrosequencing provided quantitative evaluation of methylation in the analysed sequence and showed significantly higher methylation in two CpG sites (n. 1 and n. 4) of the promoter of *VDR* gene in the carcinoma sample compared to normal adrenal (Figure 13).

A] ACC (sample 3)



B] Normal Adrenal (N.A.)



Position	1*	2	3	4*	5	6
Quality	Passed	Passed	Passed	Passed	Passed	Passed
Meth (%) **ACC**	**10**	**4**	**6**	**5**	**6**	**3**
Meth (%) **N.A.**	**1**	**1**	**6**	**0**	**8**	**5**

Figure 13. Pyrograms of *VDR* methylation status analysis in the *VDR* gene promoter of the carcinoma sample (A) compared to normal adrenal (B). Methylation percentages are expressed for each of the six CpG sites included in the analysed sequence of *VDR* promoter region.

VDR Gene Expression

VDR RT-qPCR and Immunoblot

RT-qPCR analysis demonstrated variable levels of VDR mRNA in all adrenal tumours, with VDR mRNA expressed at higher levels in ACAs than in ACCs (0.41 ± 0.2 versus 0.11 ± 0.08 arbitrary units, $P < 0.01$) (Figure 14 A). VDR immunoblot in representative cases of a normal adrenal (NA), ACAs, and ACCs is shown in Figure 14 B. VDR/β-actin protein levels, measured in the entire series of tumour specimens, showed results similar to VDR mRNA in terms of difference between benign and malignant tumours (0.20 ± 0.2 versus 0.04 ± 0.06 arbitrary units, $P < 0.05$) (Figure 14 B). Low or absent VDR expression was observed in individual cases of either ACAs or in ACCs. A positive correlation between VDR mRNA and VDR/β-actin levels ($P < 0.003$) was observed (Figure 14 C).

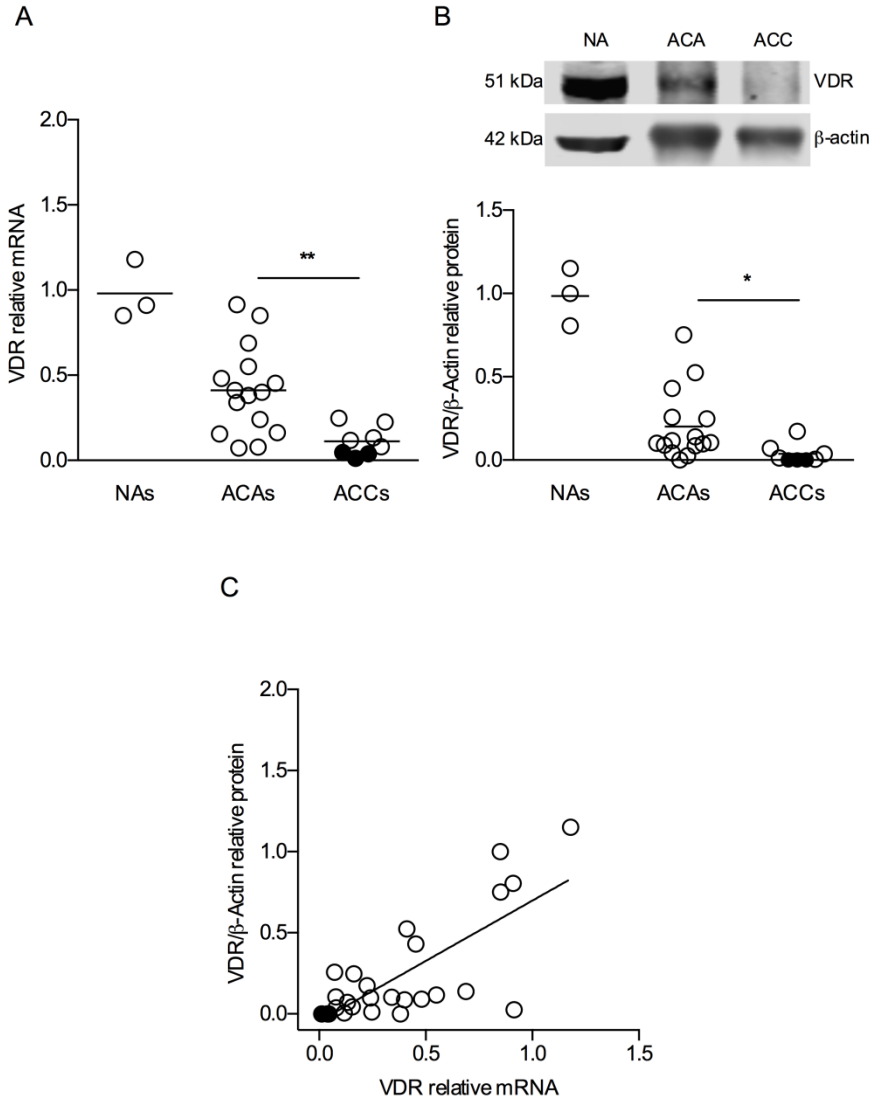


Figure 14. Expression of VDR in normal human adrenals, in adrenocortical adenomas (ACAs), and in adrenocortical carcinomas (ACCs). A) Individual mRNA levels and means (horizontal bars) of VDR, measured by qPCR, in normal adrenals (n = 3), ACAs (n = 15), and ACCs (n = 8). B) VDR immunoblot (above) in representative cases of a normal adrenal (NA), ACAs, and ACCs, and individual VDR/β-actin protein levels (below) in normal adrenals, ACAs, and ACCs. C) Correlation between VDR mRNA and VDR protein levels in all tissue samples, including normal and neoplastic adrenal. Black dots indicate methylated tissue samples. Mann-Whitney t-test ACAs versus ACCs. ** $P < 0.01$; * $P < 0.05$. Spearman correlation $r_s = 0.56$; $P < 0.003$.

VDR immunohistochemistry

Immunohistochemical staining for VDR of representative cases of one normal adrenal, one ACA, and one of the 3 methylated ACCs is reported in Figure 15. Both nuclear and cytoplasmic VDR immunostaining, consistent with translocation of VDR from cytoplasm to the nucleus after ligand binding [235], were observed in the 3 normal adrenals and in ACAs. At variance, expression of VDR was undetectable or very weak and limited to only scattered tumour cells in all ACCs, including the 3 methylated cases (Figure 15).

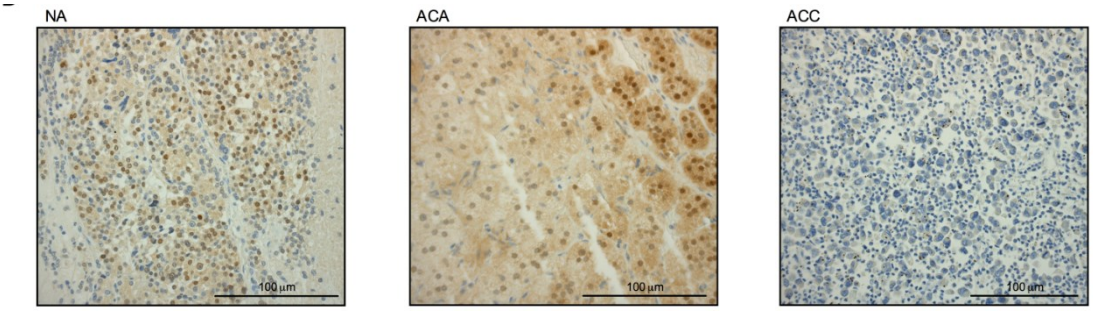


Figure 15. Immunohistochemical staining of VDR in a normal adrenal (left panel), one ACA (central panel), and one methylated ACC (right panel), showing clear VDR expression in NA, in a cortisol-producing ACA, in both the nucleus and predominantly the cytoplasm, and very weak VDR expression, limited to rare cells, in a cortisol-producing ACC. Sections were counterstained with hematoxylin.

DISCUSSION

Adrenocortical carcinoma (ACC) is a rare but aggressive malignancy with a very poor prognosis [1,59,125]. Complete surgical resection is virtually the sole hope of cure in ACC [3]. Recurrence occurs in approximately 60-80% of patients even after so-called radical resection, indicating a need for adjuvant treatment [120]. To date, adrenolytic drug mitotane, alone or combined with cytotoxic agents, remains the treatment of choice for the majority of patients [121]. Despite advances in diagnosis and treatment of ACC over the past years, molecular mechanisms underlying ACC development are still not fully elucidated.

Recent observations by Pilon et al. [235] reported a lower mRNA expression levels at RT-qPCR as well as weaker immunohistochemical expression of VDR in ACCs compared with adenomatous and normal adrenal tissues, suggesting a protective role of VDR against malignant adrenocortical cell growth.

Results of my study, performed in a different series of malignant and benign tumours, confirm a reduced expression of VDR in ACC samples, both at transcriptional and translational levels. Different molecular mechanisms might cause the reduced or nearly absent expression of VDR mRNA and protein observed in ACCs.

A somatic *VDR* gene mutation could occur in ACC, reflecting a mechanism implicated in the malignant transformation of adrenocortical cells concerning the loss of function of a tumour suppressor gene, similarly to what reported for *ZNRF3* and *p53* [94]. I did not analyse this possibility in this study. However, absence of evidence for *VDR* gene mutation in a recent whole-exome sequencing analysis of a very large number of ACCs [94] makes unlikely this event. Furthermore, *VDR* gene is rarely mutated during carcinogenesis [202]. Miller et al., looking for alterations in the *VDR* gene in a variety of cancers including 68 osteosarcomas, 23 other sarcomas, 34 non–small cell lung cancers, and 44 cell lines representing many tumour types, concluded that deletions, rearrangements and point mutations affecting the coding region of the *VDR* gene play a marginal role in the cancers they investigated.

Epigenetic mechanisms play a crucial role in regulating gene expression [211]. In my research, I hypothesized an epigenetic silencing of *VDR* gene in malignant adrenocortical tissues in order to provide an explanation for their low VDR expression. Epigenetic

inactivation of human *VDR*, reducing its mRNA and protein expression, has been shown in various cancer types [147,238], supporting the loss of an antiproliferative role of *VDR* [235].

Disruption of promoter activity by DNA methylation of CpG sites is a well-known epigenetic mechanism aimed at inactivating gene expression, an event which frequently affects tumour suppressor genes in cancer [211]. In general, cancer cells exhibit a global DNA hypomethylation with hypermethylation of tumour suppressor gene promoters [204]. Notably, the promoter of *VDR* gene lies in a CpG-rich island and contains strong regulatory sequences relevant for its transcriptional activity [239]. All these premises make *VDR* gene promoter methylation potentially involved in ACC.

Results from bisulfite sequencing performed with standard methods support a *VDR* gene silencing through this epigenetic mechanism in a subgroup of ACCs. Along with these results, pyrosequencing analysis, which is the most sensitive and accurate method to detect methylation at a single CpG [240], confirms an increase of methylated CpG in the ACC sample compared to the normal adrenal. Although pyrosequencing has not been performed in all tissue specimens, this result makes us more confident of the reliability of standard bisulfite sequencing. Whether methylation of *VDR* promoter is the mainly contributor of the reduced VDR expression in the three (hyper)methylated ACCs of entire series remains undemonstrated. Likewise, the contribution of *VDR* epigenetic silencing to the development of adrenal cancer requires further investigations. In fact, previous experimental studies using the H295R adrenocortical carcinoma cell line as a model corroborated that VDR is a key effector for proliferation in this human ACC cell line [235]. Nevertheless, the fact that the methylated CpG sites resulted the same in all the three methylated ACCs raises questions about the potential relevance of these specific sites with regard to the *VDR* promoter activity.

Different epigenetic mechanisms explaining the downregulation of VDR gene expression can be hypothesized for the remaining ACCs, and possibly for some of the adenoma cases with low VDR expression.

VDR corruption could be caused by a dysregulation of chromatin environment. In addition to aberrant DNA methylation in CpG islands, disruption of normal patterns of covalent histone modifications is another hallmark of cancer. One of the most characteristic example is the global reduction of the histone trimethylation of H4K20 (H4K20me3) and acetylation of H4K16 (H4K16Ac), along with DNA hypomethylation, at repeat

sequences in many primary tumours [204]. Furthermore, there are many examples of alterations in enzymes that add, remove or recognize specific modifications in specific types of cancer. Overexpression of individual histone deacetylases (HDACs), such as HDAC1, HDAC2 and HDAC6, among others, has frequently been recognised in tumours [241]. Despite growing evidence regarding the involvement of histone modification pattern in tumorigenesis, to date scarce data are available for adrenal cortex tumours. Recently, Davis et al. [242] reported that the oncogenic potential of two epigenetically distinct SW13 subtypes could be changed after treatment with HDAC inhibitors, involving different expression of two tumour suppressor genes. These findings are consistent with the hypothesis that chromatin remodelling and modulation can be involved in the epigenetic mechanisms underlying adrenocortical tumour growth and spread.

Insights into the complex network of chromatin remodellers involved in *VDR* gene control come from studies in colorectal cancer cell lines, suggesting that deregulation of the HDAC system [243], or EZH2-mediated trimethylation of H3K27 [147,226] in the *VDR* promoter can affect VDR expression. We cannot exclude that similar dysfunctions in these enzymes causing VDR downregulation could occur also in ACCs. Whether these alterations are present in the adrenocortical tumours examined in my study has not been investigated.

Unbalance of *VDR* corepressors in ACC cells could be an alternative mechanism concerning the reduced VDR expression. As known, these molecules can interact with a lot of nuclear regulatory cofactor complexes, therefore affecting nuclear hormone receptor binding to DNA responsive elements, therefore leading to gene functional inactivation [147]. To date, data about altered expression of *VDR* corepressors in adrenal cancer are however not available.

There is evidence that deregulation of short noncoding RNAs (miRNAs) could occur in adrenocortical tumours [244,245]. Additionally, a number of miRNAs may repress *VDR* post-transcriptionally in cancer [246]. Two miRNAs – miR-125b and miR-27b - regulating the amount of VDR were found, but both miRNAs can reduce VDR on the protein level with no effect on the mRNA level, indicating blockage of translation rather than increased mRNA degradation [247]. This is in contrast with the results obtained from VDR expression analysis in our series of ACCs, which showed VDR decreased at both mRNA and protein levels. Furthermore, miR-125b has been reported downregulated in

adrenocortical tumours [87,245], as mentioned for other cancers [246]. Taken together, these observations make unconvincing the role of these two miRNAs to explain the reduced VDR expression in our ACCs. However, other miRNAs might act as specific VDR downregulators in adrenocortical tumours although this has not been proven yet.

Since a growing body of evidence indicates that DNA promoter methylation can be a consequence rather than a cause of transcriptional inactivation, the hypothesis of *VDR* methylation as the result of malignant transformation cannot be definitely excluded. To note, methylation of *VDR* gene promoter has not been specifically reported in all genome-wide methylation studies on adrenocortical cancer [81-83]. As global methylation pattern of the ACCs included in my study is not known, it is difficult to establish if *VDR* epigenetic inactivation could have to some extent a causal role with regard to ACC development rather than being an epiphenomenon of a genome-wide hypermethylation.

Moreover, I cannot exclude that low expression of *VDR* gene in adrenocortical cancers, as well as in some adenomas, may rather be due to the effect of hormonal compounds, that is, estrogens, thyroid hormone, and glucocorticoids, which are likewise able to alter VDR mRNA/protein levels [146]. Interestingly, a critical role of estrogens and ERα in adrenocortical tumorigenesis has been reported [104].

Finally, mitotane, the drug used for preoperative treatment of all ACC patients enrolled the study, is known to stimulate CYP3A4 expression, potentially leading to reduced $1\alpha,25(OH)_2D_3$ bioavailability and thus influencing VDR regulation in adrenal cortex cells [235,248]. Furthermore, knowledge is lacking about the effect of mitotane *per se* on VDR expression in adrenocortical cancer cells [126], besides the potential effects exerted by glucocorticoid levels perturbation.

The main limitation of the study is the relatively small number of samples, and a larger ACCs study population is needed to confirm these results. The study could be enlarged using the adrenal tissue bank of ENSAT (European Network on Adrenal Tumors) collaborative group, which is dedicated to the study and treatment of adrenal tumours, providing study projects and enrolling research teams on this disease.

Notwithstanding, the results of my Ph.D research project represent the first evidence of an association between *VDR* gene promoter methylation and reduced VDR expression in ACC. This suggests a potential role of *VDR* epigenetic inactivation in malignant adrenocortical tumorigenesis. Adrenocortical carcinoma, either silent or hormonally

active, is a rare tumour with a dismal prognosis because of its highly invasive phenotype and marked resistance to radio- and chemotherapy [3,120]. The *VDR* promoter methylation might become a target for pharmacological agents to treat adrenal cancer in selected cases [100], i.e. ACC with differently methylated *VDR* promoter. In this regard, the human adrenocortical carcinoma H295R cell line, which provides the most appropriate model for ACC study [249], does not have *VDR* gene methylation (personal observation). The availability of adrenal cell models allowing the *in vitro* use of DNA methylation inhibitors should be addressed.

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