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VITAMIN D- STATUS AND RECEPTOR EXPRESSION PATTERNS IN PEDIATRIC SOLID TUMORS
A POTENTIAL PROGNOSTIC BIOMARKER

PhD Thesis

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Abbreviations

(H3)-K27: Histone 3 K27
25(OH)D3: 25-hydroxyvitamin D3
ACT: Adrenocortical tumor
Akt: Protein kinase B
ALL: Acute lymphoid leukemia
AML: Acute myeloid leukemia
APL: Acute promyelocytic leukemia
B-ALL: B cell acute lymphoblastic leukemia
BMI: Body mass index
c-Src: Cellular proto-oncogene, non-receptor tyrosine kinase
CerK/C1P: Ceramide kinase/Ceramide - 1 – phosphate
CI: Confidence interval
CNS: Central nervous system
CpG: 5' - Cytosine - phosphate – Guanin - 3'
CR: Complete remission
CYP24A1: cytochrome P450 family 24 subfamily A member 1
CYP27B1: cytochrome P450 family 27 subfamily B member 1
CYP2R1: cytochrome P450 family 2 subfamily R member 1
DA: Dopamine
DHCR7: 7-dehydrocholesterol reductase
DIPG: Diffuse intrinsic pontin glioma
DNA: Deoxyribonucleic acid
EPM: Ependymoma
ERK 1/2: Extracellular signal-regulated kinases
EWS: Ewing's sarcoma
EX: Exitus
HBL: Hepatoblastoma
HCC: Hepatocellular carcinoma
HRE: Hypoxia response element
IACR: International Association of Cancer Registries

IARC: International Agency for Research on Cancer

IICC-3: International Incidence of Childhood Cancer, volume 3

IU: International unit

MAPK: Mitogen-activated protein kinases

MBL: Medulloblastoma

MEK 1/2: Mitogen-activated protein kinase

mRNA: Messenger ribonucleic acid

NBL: Neuroblastoma

NCI: National Cancer Institute

NGF: Neurotrophic growth factor

NR: Nuclear receptor

NTRK: Neurotrophic tyrosine receptor kinase

OPC: Oligodendrocyte precursor cell

pACT: Pediatric adrenocortical tumor

PCR: Polymerase chain reaction

PD-L1: Programmed death-ligand 1

PFA: Posterior fossa ependymoma group A

PNET: Primitive neuroectodermal tumor

PR: Partial response

PTC: Papillary thyroid carcinoma

PTEN: Phosphatase and tensin homolog

RCT: Randomized controlled trial

RNA: Ribonucleic acid

RXR: Retinoid X receptor

SD: Stable disease

SH-SY5Y cells: Triple-subcloned NBL cell line derived from the SK-N-SH cell line

SNP: Single-nucleotide polymorphism

TAD: Topologically associated domain

TF: Transcription factor

TH: Tyrosine hydroxylase

TMA: Tissue microarrays

TNM: Classification of Malignant Tumors (Tumor size, lymph Nodes, distant Metastasis)

TSS: Transcription start site

US: United States of America

UV-B: Ultraviolet B (radiation)

UV: Ultraviolet (radiation)

VDR: Vitamin D receptor

VDR: Vitamin D receptor

VDRE: Vitamin D response element

ViDA: Vitamin D Assessment trial

VITAL: VITamin D and Omega -3 TriAL

Vitamin D: Vitamin 1,25(OH)2D3 (strong affinity ligand of the receptor)

WGS: Whole genome sequencing

WHO: World Health Organization

WT: Wilms tumor

WT1: Wilms tumor 1 gene

1. Introduction

Increasing data demonstrate the beneficial impact of vitamin D supplementation on the reduction of cancer-related mortality (1) and, recently, the correlation between vitamin D receptor (VDR) polymorphisms and cancer risk and progression, prompting the hypothesis that vitamin D and its metabolites could play a role in cancer treatment and prevention (2).

The literature on the potential pathomechanisms and precise etiology of childhood tumor development is expanding, and simultaneously, diagnostic, and therapeutic biomarkers are being discovered. These could be beneficial in the future to evaluate and enhance the prognosis more accurately, and to treat more effectively even those pediatric solid tumors that currently have a poor prognosis. In parallel, while countless studies have been published in the literature on the anti-tumor effects of vitamin D via VDR, the childhood aspect of these is very limited. Due to these facts, we decided on to investigate the role of vitamin D and VDR receptor patterns in pediatric solid tumors as potential prognostic biomarkers.

1.1. Pediatric oncological aspects

According to a multinational assessment, the incidence of most forms of childhood and adolescent cancer has increased significantly in the majority of regions over the last 40 years (3). Despite outstanding mortality rates, cancer is the second major cause of death from disease in children beyond infancy, after accidents, in high-income nations, responsible for more than 5000 potentially avoidable deaths annually in people below 15 years of age (4).

1.1.1. Childhood cancer incidence

The most recent and comprehensive incidence data currently accepted and published by the World Health Organization (WHO) is provided by the International Incidence of Childhood Cancer, volume 3 (IICC-3) study. In collaboration with the International Agency for Research on Cancer (IARC) and the International Association of Cancer Registries (IACR), this international summary was produced using data from 2001-2010, for the first time looking at the age group 0-19 years. In the final analysis, 153 registries

in 62 countries were able to provide sufficient quality data, totaling 2.64 billion person-years. Based on the included data, 385 509 new childhood cancer cases are registered annually worldwide which equates to about 140.6 per million person-years (5). According to data from the Hungarian National Childhood Tumor Registry for the years 2001 to 2015, this data represents 161 million person-years, which translates to approximately 230 new cases per year in Hungary (6). Both Hungarian and international surveys show a slightly increasing trend in childhood cancer incidence compared to the last decades. This is mainly attributable to more precise data recording by comprehensive registries and can be attributed to an actual increase, the exact cause of which is difficult to ascertain due to a complex and partially unknown etiology.

From the perspective of genders, in every part of the globe, males had a higher incidence than females (incidence ratios by gender varied from 1:1 to 1:2 in the 0–19 age group) (5). Comparable to international data, male preponderance is observed in the Hungarian patient population (boy/girl ratio: 1.22/1) (6).

The diagram below shows that the frequency distribution of tumor types is different in each age group (Figure 1 (5)).

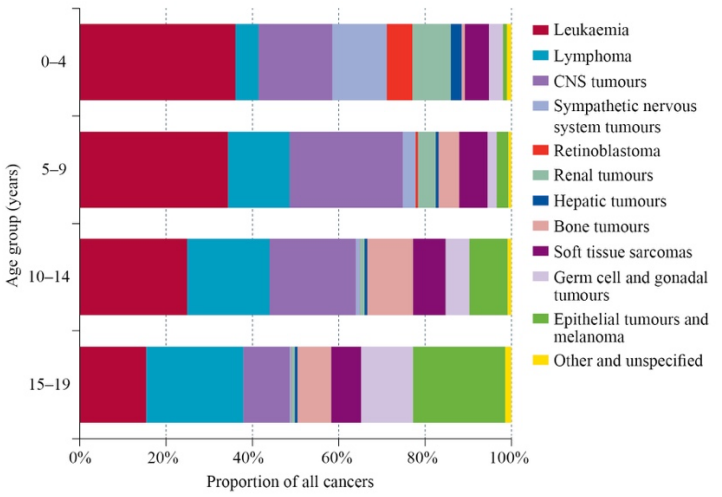

FIGURE 1 | Cancer types proportionally distributed by age group (2001–2010). Tumors defined by the International Classification of Childhood Cancer (volume 3.6).

Leukemia, followed by central nervous system (CNS) tumors, and then lymphomas, are the most prevalent malignancies in children aged 0–14 years according to the WHO data. Lymphomas are the most prevalent malignancy among 15–19-year-olds in all regions, followed by epithelial malignancies and melanoma. Leukemia accounted for 36.1% of all

cases in 0–4-year-old children, but only 15.4% of cases in 15–19-year-old youth. CNS tumors are the second most common type of tumor in children with ages 0–4 years (17.2%), 5–9 years (26.3%), and 10–14 years (20.0%), after leukemia. Epithelial tumors and melanoma accounted for 0.9% of all cancers among children aged 0–4 years, but they are second-most prevalent type of tumor in adolescents aged 15–19 (21.3%). Neuroblastoma is the most prevalent sympathetic nervous system tumor. It is prevalent among 0–4-year-olds (12.5%) but extremely uncommon among 15–19-year-olds (0.02%). Renal tumors are prevalent in children aged 0–4 (8.9%), and their occurrence declines with age, reaching 0.7% in adolescents at ages 15–19. 4.7% of all malignancies in children aged 0–14 years were bone tumors, compared to 7.8% among individuals aged 15–19 years. Identical proportions of 0–14-year-old and 15–19-year-old children are diagnosed with soft-tissue sarcomas (5).

In our home country, data from the Hungarian National Childhood Tumor Registry show a similar distribution between tumor types to the international tendency. The relative distribution of childhood malignancies shows the prominence of leukemias (28%) and central nervous system tumors (26%) (Figure 2) (6).

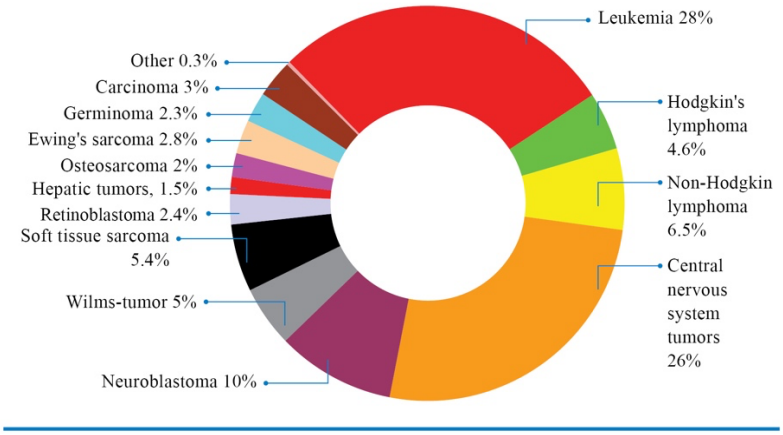

FIGURE 2 | Relative distribution of childhood cancer in Hungary (0–14-year-olds, 2001-2015, n=3450 cases, National Childhood Tumor Registry).

1.1.2. Survival data

Due to improvements in oncology and supportive therapies in the last decade, the survival rates for pediatric cancers have increased dramatically in developed countries (7).

The 5-year survival rate for all childhood malignancies in Europe improved from 54% (1978–1982) to 75% (1993–1997) (8) and exceeded 80% in 2005–2007 (9). The 5-year

survival rate in the United States (US) increased from 58% (1975–1977) to 85.3% (2009–2015) (7). Before the 1960s, leukemia was considered an incurable disease. Nowadays in Europe and North America, the five-year survival of acute lymphoblastic leukemia (ALL) approaches 90% (9, 10). In some high-income countries, the five-year survival rate for AML approaches 70%, outweighs 90% for Burkitt and Hodgkin lymphoma, retinoblastoma, and nephroblastoma, but remains below 60% for certain types of hepatic and CNS malignancies (7, 10-13).

Survival data in Hungary correlate with the international trend. According to the latest data, the overall survival cure rate is close to 80%; in ALL 83.1% (Figure 3 (6)) and in solid tumors 72.5% five-year asymptomatic survival. In Hungary, survival is over 90% for retinoblastoma, Hodgkin's disease, Wilms' tumor and neuroblastoma of infancy, while for some CNS tumors and advanced neuroblastoma of older children it is less than 50% (14).

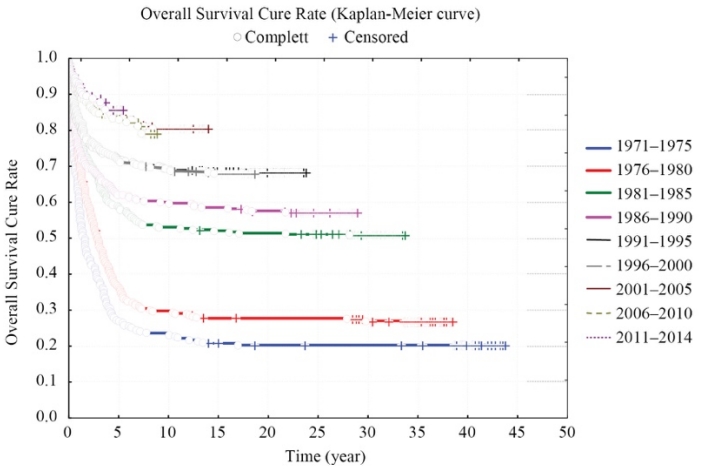

FIGURE 3 | Improvement in long-term survival of childhood ALL in Hungary since the 1970s (diagnosis: 1971-2014, n=2476 cases, Kaplan-Meier analysis, Statistica 7.0, p=0.001 Hungarian National Childhood Tumor Registry)[6]

1.1.3. Developmental origins for childhood cancer

The precise origin of childhood malignancies is still unknown in many cases. With advances in science, the deregulatory mechanisms that lead to cancer are being uncovered and may even provide new therapeutic targets for the future and even the present (15). There is evidence from genetically modified rodents that multiple pediatric malignancies arise from stem or progenitor cells during specific stages of development (16-18). The

literature mentions several mechanisms of deregulation in the development of childhood cancers.

One of the deregulation mechanisms is point mutations. Childhood cancer is characterized by the accumulation of point mutations in genes responsible for epigenetic processes, which usually display disease-specific patterns.

Other well-known way of deregulation is the presence of fusion genes which is common etiological factor for several types of pediatric tumors in several entities that may even act as therapeutic targets. Frequently, these fusion genes activate development-critical genes, such as the neurotrophic growth factor receptor family (e.g., neurotrophic tyrosine receptor kinase; NTRK) genes. Table 1 below provides a sampling of the fusion genes described in pediatric oncology (15).

TABLE 1 | The presence and prevalence of some fusion genes in pediatric cancers. B-ALL: B cell acute lymphoblastic leukemia, APL: Acute promyelocytic leukemia.

Pathology/Diagnosis	Gene Fusion Partner 1	Gene Fusion Partner 2	Prevalence (% of total cases)
Embryonal tumor (with multilayer rosettes)	C19MC	TTYH1	100
High grade glioma	NTRK1	TPM3	3
Low grade glioma	KIAA1549	BRAF	66
Medulloblastoma	PVT1	MYC	8
Supratentorial ependymoma	RELA	C11orf95	72
Alveolar rhabdomyosarcoma	Pax3	FOXO1	65
Clear-cell sarcoma	EWS	ATF1	90
Congenital fibrosarcoma	ETV6	NTRK3	90
Ewing's sarcoma	EWS	FLI1	85
Inflammatory myofibroblastic tumor	ALK	TPM3	18
Inflammatory myofibroblastic tumor	ROS1	TFG	5
Papillary thyroid cancers	RET	PTC3	4
Renal cell carcinoma	ALK	VCL	2
Synovial sarcoma	SS18	SSX1	60
B-ALL	BCR	ABL	3-5
B-ALL	ETV6	RUNX1	6-35
APL	PML	RARA	95

Other than deregulation, a conspicuous characteristic of pediatric cancers is epigenetic dysregulation, with growth attributable to extensive dysregulation of gene expression. For example, the point mutations in histone genes dysregulated histone post-translational changes, influencing gene expression (19-21). Possibly the most researched of these is the histone-3 (H3)-K27M mutation described in diffuse midline gliomas, including diffuse intrinsic pontine glioma (DIPG), thalamic, and spinal cord gliomas (15, 22). Irregular variations in H3-K27 methylation are known in ependymomas (23) and medulloblastomas (24). This confluence of K27 genetic and epigenetic changes in H3 variant cancers may indicate a crucial function for H3-K27 in both normal hindbrain development and carcinogenesis. In a recent pan-cancer analysis of pediatric cancers, mutations in epigenetic modifiers - including histone modifying enzymes (so called SETD2, KDM6A) and chromatin complexes (BRG1-associated factors complex and polycomb repressive complex 2) - have surfaced as the most prevalent and most extensive class of mutated genes (25, 26).

10% of pediatric tumors in two major whole genome sequencing (WGS) analyses, comprising more than 2500 cases and representing two dozen molecularly characterized tumor categories, did not find any underlying mutations or structural- or copy changes (25, 26). This raises the possibility that tumorigenesis is caused by an extra, non-genetic layer of dysregulated epigenetics. For instance, a subtype of posterior fossa group A ependymomas (PFAs), have a relatively low frequency of recurrent deoxyribonucleic acid (DNA) mutations. When compared to other types of ependymoma, these so called genetically silent ependymomas show CpG-island hypermethylation, suggesting extensive epigenetic changes in their development (27).

Another theory mentions that the rich developmental setting of actively growing tissues is related to the idea that the microenvironment plays a vital role in controlling cancer progression. When children malignancies emerge from the same tissue stem- and precursor cell populations that these processes are intended to support, intercellular connections that regulate tissue development and growth are distorted to favor malignancy (15). The effect of neuronal activity on the growth of both juvenile glioma cells and oligodendrocyte precursor cells (OPC) serves as an example of this concept. As an aspect of an adaptive reaction of myelin-forming cells that regulates myelin development and serves as an indicator of experience-dependent brain plasticity, neuronal

activity promotes resilient proliferation of OPCs and pre-OPCs (28, 29). Neuronal activity similarly encourages glioma proliferation, growth, and progression in glial cancers like pediatric glioblastoma and H3-K27M-driven diffuse midline glioma through activity-regulated secretion of growth factors (brain-derived neurotrophic factor, neuroligin-3) (30, 31). In many tissues, neuronal activity is a potent regulator of the stem cell niche, which is crucial for homeostasis, organogenesis, renewal, and cancer (15, 32, 33).

Consequently, diverse pre- and postnatal developmental phases, as well as their unique microenvironmental and transcriptional states, will need to be taken into consideration in future attempts toward mechanistic comprehension and, eventually, treatment for childhood malignancies (15).

1.1.4. Biomarkers

WHO has defined biomarkers as "almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction" (34).

The National Cancer Institute (NCI) defined biomarkers in the NCI Dictionary of Cancer Terms as "a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease. A biomarker may be used to see how well the body responds to a treatment for a disease or condition. Also called molecular marker and signature molecule".

There is an enormous selection of biomarkers, which may include a group of modifications, such as proteomic, metabolomic, and gene expression profiles. Moreover, a biomarker among other things, could be nucleic acids (e.g., a micro-ribonucleic acid (RNA) or other non-coding RNA), antibodies, peptides or even proteins (such as an enzyme or receptor). A biomarker could be identified in the circulation (whole blood, serum, or plasma), excretions or secretions (stool, urine, etc.), and may therefore be evaluated non-invasively and serially. Alternatively, it may be tissue-derived and demand a biopsy or imaging. Cancer biomarkers are beneficial for patient evaluation in risk assessment, screening for occult primary cancers, differentiating benign compared to malignant results, separating two forms of malignancies, establishing prognosis.

Biomarkers could also be beneficial in prediction for individuals with cancer and following the condition of the malignancy to either identify recurrence or assess response or progression to therapy. The latter are usually indicated by clinicopathological findings, which have recently been complemented using various biomarkers, such as gene expression signatures (35, 36), circulating tumor cells as predictive survival markers (37), or even analyzing the receptor pattern of a tumor tissue (38). Some receptors, signaling pathways, and genetic biomarkers are capable of serve as therapeutic targets, thereby functioning as therapeutic biomarkers (39-41).

The research and development of cancer biomarkers and the identification of ever-more-recent biomarkers are increasingly frequent subjects in the scientific literature, as is the identification of prognostic markers and therapeutic targets. This seems to be particularly true for childhood malignancies, whereby specific genetic abnormalities in pathogenesis contribute to tumor development. The identification of a growing number of biomarkers is resulting in the availability of further targeted and complementary therapies every day.

1.2. Oncological aspects of vitamin D

According to the landmark epidemiological research published almost 40 years ago (42), the incidence of colorectal cancer is reduced by living at lower latitudes and getting more sun exposure, both of which result in higher endogenous 1,25(OH)2D3 (vitamin D) production. Since roughly the same time, it has been known that vitamin D can inhibit melanoma cell growth in vitro (43). Both discoveries suggested that vitamin D deficiency could be a risk factor for cancer (44).

A vitamin D intervention trial for colorectal cancer confirmed this hypothesis. In the so-called Sunshine randomized clinical trial (RCT), 139 patients with advanced and/or metastatic colorectal carcinoma were included, comparing the association of lower and higher doses of d-vitamin supplementation with survival. The supportive risk ratio was significantly improved in those who received higher doses of vitamin D supplementation in parallel with first-line oncotherapy, with a multivariate odds ratio (OR) of 0.64 (1-sided 95% CI, 0-0.90; $p = 0.02$) for progression-free survival or death, which was statistically significant (45). Numerous in vitro studies determined that vitamin D would also be beneficial against prostate and breast cancer, as well as lymphoma and leukemia.

Consequently, it was anticipated that randomized control trials would offer more conclusive evidence for the theory. Both the so-called VITAL trial in the US (46), and the so-called ViDA trial in New Zealand (47) failed to demonstrate any prevention of cancer and no appreciable decrease in cancer mortality in these clinical settings. The study, known as VITAL, conducted with a randomized, double-blind, placebo-controlled design with a two-by-two factorial arrangement. Its objective was to investigate the potential advantages and drawbacks of administering cholecalciferol (at a daily dosage of 2000 international units (IU)) and marine omega-3 fatty acids (at a daily dosage of 1 g) for the primary prevention of cancer and cardiovascular disease. The study participants consisted of 25,871 individuals (specifically men aged 50 years or older and women aged 55 years or older) with a five-year intervention phase (study pill taking median: 5.3 years). The incidence of invasive cancer, regardless of its form, was found to be equivalent between the vitamin D group and the placebo group. Specifically, there were 793 individuals in the vitamin D group and 824 people in the placebo group who developed cancer, resulting in an OR of 0.96 (95% confidence interval (CI), 0.88-1.06; p = 0.47). Regarding the incidence of breast, prostate, and colorectal cancer, no significant differences between the two groups were observed. During the follow-up period, 154 participants in the vitamin D group and 187 participants in the placebo group died (OR: 0.83; 95% CI, 0.67-1.02) (46). The Vitamin D Assessment (ViDA) experiment was a randomized, double-blind, placebo-controlled study. The trial included patients between the ages of 50 and 85, and the intervention phase lasted for a median duration of 3.3 years. A total of 5108 individuals were randomized to either the vitamin D group (n = 2558) or the placebo group (n = 2550). The first oral dose of vitamin D was 200,000 IU, and subsequent monthly doses were 100,000 IU. The administration of high-dose vitamin D supplements monthly for a duration of up to four years, in the absence of calcium supplementation, does not seem to have a preventive effect against the occurrence of cancer. In a randomly selected sample of 438 people, the average follow-up serum vitamin D level was higher by more than 20 ng/mL in the group receiving vitamin D compared to the group receiving a placebo. A total of 328 cancer cases were registered as primary events during the follow-up period (among the participants, 165 individuals (6.5%) in the vitamin D group and 163 individuals (6.4%) in the placebo group). The adjusted OR was calculated to be 1.01 (95% CI, 0.81–1.25; p = 0.95) (47). Moreover, three additional randomized controlled trials

(48-50) found no effect of vitamin D supplementation. A meta-analysis including 4 randomized controlled trials (partly including the above referred publications) also found no significant association in cancer incidence with vitamin D supplementation but suggests a significantly lower cancer mortality (51). The data of the above mentioned randomized controlled trials is summarized in the table below (Table 2 (46-52)), partly based on the table of the meta-analysis (51).

TABLE 2 | Principal characteristics of randomized controlled trials investigating the association between vitamin D supplementation and cancer incidence and mortality (partly based on the above-mentioned meta-analysis). OR: Odds ratio, CI: Confidence interval, UK: United Kingdom, US: United States of America, IU: International unit, Ca: Calcium.

Authors, year, country	Trial name, type, participants	Age of participants	Trial duration	Contrast for OR	Incidence: OR (95 % CI) (n case/ n total)	Mortality: OR (95% CI) (n case/ n total)
Trivedi et al 2003 UK	- Pilot community trial - general population	65–85 years	5 years	vitamin D (100000 IU/4-month (~833 IU/day) vs placebo	0.09 (0.86-1.36) (188/1345) vs (173/1341)	0.86 (0.61-1.20) (63/1345) vs (72/1341)
Wactawski-Wende et al 2006 US	- WHI - randomized, double blind, placebo-controlled trial - postmenopausal women	50-79 years	7 years	vitamin D (400 IU/day) +Ca carbonate (1000 mg/day) vs placebo	0.98 (0.91-1.05) (1634/18 176) vs (1655/18 106)	0.89 (0.77-1.03) (344/18 176) vs (382/18 106)
Lappe et al 2007 US	- randomized, double blind, placebo-controlled trial - postmenopausal women	66.7 years (7.3)	4 years	vitamin D (1100 IU/day) + Ca vs Ca (carbonate 1500/day or citrate 1400 mg/day)	0.76 (0.38-1.55) (13/446) vs (17/445)	NA
Avenell et al 2011 UK	- RECORD - randomized, placebo-controlled trial (two-by-two factorial design) - general population	77.2 years (6)	2-5.2 years	vitamin D (800 IU/day) vs no vitamin D	1.07 (0.92-1.25) (338/2649) vs (315/2643)	0.85 (0.68-1.06) (151/2649) vs (178/2643)
Manson et al 2018 US	- VITAL - randomized, placebo-controlled trial (two-by-two factorial design) - general population	67.1 years (7.1)	5 years	vitamin D (2000 IU/day) vs placebo	0.96 (0.88-1.06) (793/12927) vs (824/12944	0.83 (0.67-1.02) (154/12927) vs (187/12944)
Scragg et al 2018 New Zealand	- ViDA - randomized, double blind placebo-controlled trial - general population	50-85	4 years	vitamin D (20000 IU in the first month, then 10000 IU/month) vs. placebo	1.01 (0.81-1.25) (165/2558) vs (163/2550)	0.97 (0.64-1.47) (44/2558) vs (45/2550)

By now, based on RCTs and meta-analyses including large numbers of participants, it can be assumed that there is no correlation between vitamin D supplementation and the incidence of cancer. However, it is still controversial in the literature whether pre-disease administration of vitamin D and thus an adequate vitamin D supply can be related to cancer mortality. This warrants further investigation.

According to a Mendelian randomization analysis based on 74 single nucleotide polymorphisms related to vitamin D blood levels (53). These findings showed that vitamin D may not have universally evident cancer-prevention benefits. Two studies about vitamin D intervention (54, 55), interestingly suggests that 1 in 4 people are so-called low vitamin D responders, which possibly implies that these people need to increase the dosage of their daily vitamin D intake to achieve full clinical advantages (56). On the other hand, so-called high vitamin D responders, appear to tolerate even low serum vitamin D levels, which means that they are less prone to illnesses like cancer, which the lower incidence and better prognosis of which have been described as being associated with higher serum vitamin D levels (57).

1.2.1. Vitamin D homeostasis and supplementation

The normal vitamin D level is specified as 30 ng/ml (75 nmol/l). A value below this level indicates subclinical vitamin D deficiency that is not necessarily manifested in musculoskeletal disorders but increases the probability of cardiovascular diseases, diabetes mellitus, autoimmune conditions, and tumors. According to current scientific literature, a serum vitamin D level of 20 to 29 ng/ml is insufficient (58), 10 to 19 ng/ml indicates deficiency (59), and less than 10 ng/ml means severe deficiency (60, 61).

According to the published data 25-75% of the global population has an insufficient or deficient vitamin D status (62), in Hungary, almost three-quarters of the population have a low vitamin D status (63).

Endogenous vitamin D is created by keratinocytes and released into the circulation where it is carried to the liver by serum so called glycoprotein GC (GC vitamin D binding protein) (64). Likewise, exogenous vitamin D acquired by diet or supplements travels from intestinal enterocytes to the circulation via chylomicrons, where it is transported to the GC protein. Vitamin D is metabolized into 25-hydroxyvitamin D3 (25(OH)D3) by the liver enzyme cytochrome P450 family 2 subfamily R member 1 (CYP2R1) (Figure 4

(65)). Since 25(OH)D3 is the most prevalent and stable form of vitamin D and has a half-life of approximately 3 weeks, serum levels of this metabolite are frequently used as a biomarker for the status of vitamin D (66). 25(OH)D3 is then hydroxylated in the kidneys via the enzyme cytochrome P450 family 27 subfamily B member 1 (CYP27B1) to become 1,25-dihydroxyvitamin-2 D3 (1,25(OH)2D3), which binds to vitamin D receptor (VDR) with high affinity (67) (Figure 4 *(65)*).

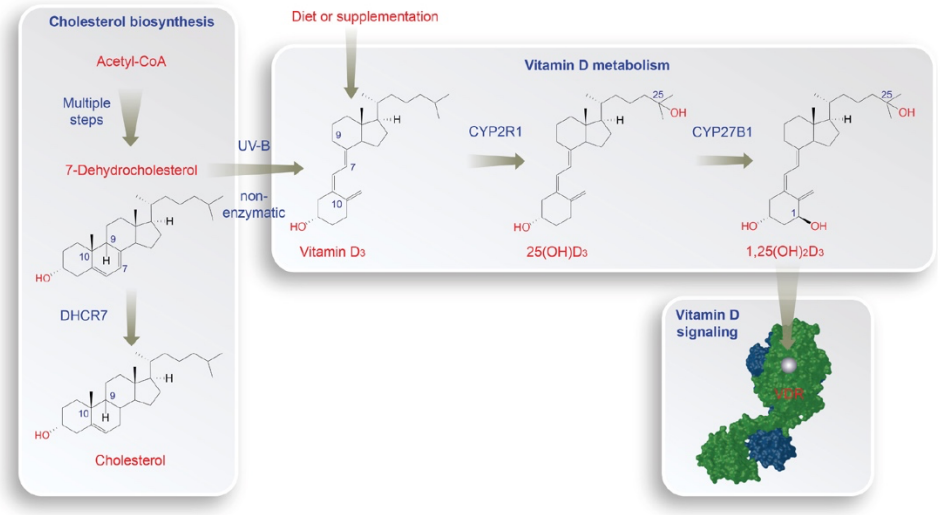

FIGURE 4 | The relationship between cholesterol, vitamin D synthesis, and VDR. Vitamin D3 is derived from 7-dehydrocholesterol, the second-to-last precursor of the cholesterol biosynthesis pathway. UV-B radiation provides the necessary energy for the non-enzymatic synthesis of previtamin D3 (not demonstrated) in the epidermis, which swiftly isomerizes into vitamin D3. The liver enzyme CYP2R1 transforms vitamin D3 to 25(OH)D3, whereas the kidney and other cell types express the enzyme CYP27B1, which produces 1,25(OH)2D3. Vitamin D signaling is centered on the activation of the transcription factor VDR (green) by its strong-affinity ligand 1,25(OH)2D3.

As a member of the nuclear receptor superfamily of transcription factors, the VDR provides vitamin D with a direct route to regulate genes through vitamin D. VDR has been seen in a variety of tissues and mesenchymal and epithelial cell types (68). Studies utilizing radiolabeled vitamin D indicate that although VDR has been discovered in the cellular membrane and cytoplasm, VDR localization is mostly nuclear (69). A well-known signaling route involves vitamin D binding to VDR in the cell, the resulting complex forming a heterodimer with the retinoid X receptor (RXR). This complex can bind to vitamin D-responsive sites located in the promoter regions of target genes (Figure 5 (70)). Numerous downstream effects of this interaction stimulate differentiation and inhibited proliferation, angiogenesis, invasiveness, and metastatic potential (71).

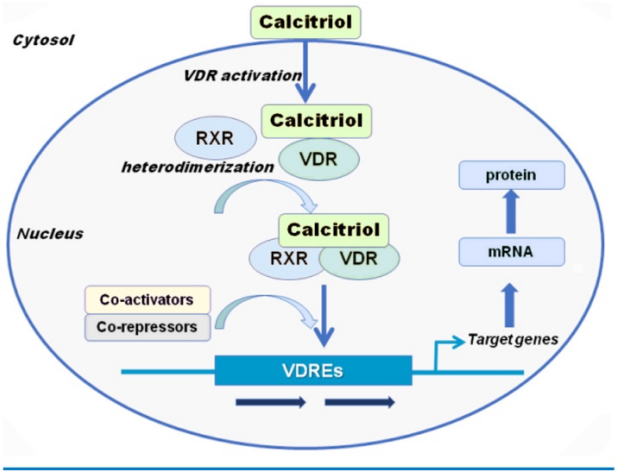

FIGURE 5 | Target cell Vitamin D receptor (VDR) action. Intracellular calcitriol [1,25(OH)2D] binds to the VDR, resulting in the dimerization of the VDR with the retinoid X receptor (RXR). The ligand-bound VDR-RXR complex links to uniquely structured vitamin D response elements (VDREs) in numerous, broadly separated vitamin D-responsive regions. This alters the induction of co-activators or co-repressors, resulting in either positive or negative regulation of gene expression.

Our hominin ancestors threw the majority of their body hair some 2 million years ago (72), which allowed them to sweat more effectively and hence perform better physically (73). To defend themselves from sunburn and ultraviolet (UV)-induced cancer at these tropical latitudes in East Africa, where homo sapiens eventually emerged some 300,000 years ago, their initially pale skin had to develop intense pigmentation (74). Dark skin pigmentation depends on eumelanin-laden melanosomes in keratinocytes of the basal stratum, whereas cells of the upper layer of skin (stratum spinosum) contain the greatest concentration of 7-dehydrocholesterol reductase (DHCR7) and are the primary site of vitamin D synthesis (75). The activity of the enzyme DHCR7 is crucial for the non-enzymatic process that converts 7-dehydrocholesterol to vitamin D3 in the skin (76). Because they have more 7-dehydrocholesterol in their skin, people with low DHCR7 activity produce vitamin D even with a reduced ultraviolet B (UV-B) exposure (Figure 4). Thus, the quantity of endogenously provided vitamin D is determined by the genotype of the DHCR7 gene rather than skin tone. Indeed, a genome-wide association study with almost 80,000 people of European ancestry found that variations in the genes that regulate vitamin D metabolism and transport, including DHCR7, GC, CYP2R1, and cytochrome P450 family 24 subfamily A member 1 (CYP24A1; plays role in degradation of vitamin D), clarify some of the genetics underlying the variation in intra- and inter-individual vitamin D status (77).

Because the 7-dehydrocholesterol is relatively widespread in human skin, UV-B exposure is the primary limitation for endogenous vitamin D production. Despite this, at higher

latitudes, UV-B reaching the earth exhibits pronounced seasonal variations, which means, beginning at 38 degrees latitude, the further north people live, the more winter months there are in which the endogenous vitamin D synthesis is insufficient. (78). Moreover, a predominance of domestic activities, the wearing of protective textiles outdoors, and the application of UV-blocking sun cream reduce the opportunity to produce vitamin D even during the summer months (65). After all, as excessive UV-B exposure may contribute to various types of skin cancer (79, 80), it is prudent to exercise caution when relying on the natural method of vitamin D production. However, few diets contain significant quantities of vitamin D, so dairy products such as milk and margarine are fortified with vitamin D2 or vitamin D3 in some countries (81). Recent literature suggests that vitamin D supplementation on a weekly or daily basis is equally effective in achieving adequate vitamin D intake. The recommended daily intakes for each age group are shown in Table 3 below (82).

TABLE 3 | The recommended daily intakes for each age group. BMI: Body mass index, IU: International unit.

Age	**Recommended intake-normal BMI (IU/day)**	**Recommended intake-obese patient (IU/day)**	**Upper level (IU/day)**
0-12 month	400-1000	400-1000	2000
1-18 years	600-1000	600-1000	4000
>18 years	1500-2000	3000-6000	10 000

1.2.2. VDR as a transcription factor

It is known from the literature that vitamin D can increase or decrease the expression of genes related to cellular growth, differentiation, and mineral homeostasis.

VDR involves 11 exons on the reverse side of chromosome 12q12-q14 and holds a significant non-coding region contains exons 1F-1C and 2-9 and coding a protein formed by 424 amino acids (Figure 6a). VDR is member of the steroid hormone receptor superfamily and serves as a transcription factor (TF) related to the nuclear receptor (NR) family. NRs utilize cyclical gene regulation involving TFs alternate across active and inactive states. VDR can be found in the nucleus regardless of the presence of the ligand,

but the VDR ligand's presence or absence determines whether or not it recruits activator or repressor complexes. VDR heterodimerizes using RXR to generate the VDR-RXR complex, that binds either repressor or activator complexes based on its liganded or unliganded status (Figure 6b) (83).

The liganded VDR as a TF links to target genes promoter hypoxia response element (HRE) regions. The strongly conserved DNA-binding domains of steroid hormone receptors include two zinc-finger patterns that identify HREs. The separation and direction of the HRE half-sites, as well as the HRE sequence, are essential for correct receptor recognition. Further TFs (e.g., fos and jun), may impact gene expression through vitamin D (84).

It is demonstrated that VDR ligand status modifies proteasomal degradation and regional chromatin profile via enzymatic regulation of histone changes (83).

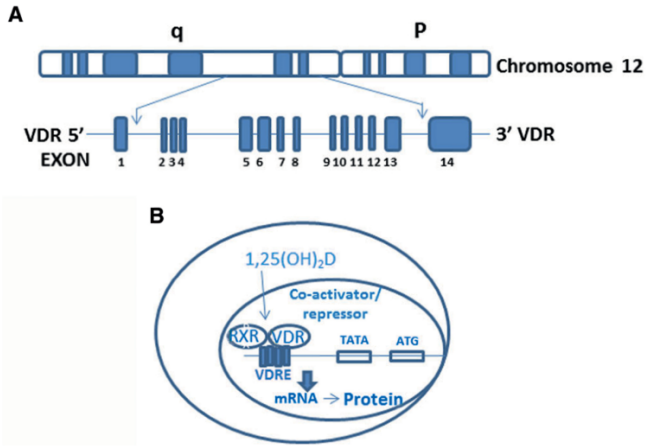

FIGURE 6 | The structure and function of VDR gene its gene targets. The genomic organization of the VDR gene on 12q13 which contains 14 exons (a). Vitamin D attaches VDR in the cytoplasm, promoting its translocation into the nucleus, where it heterodimerizes with RXR. VDR-RXR complex connects to vitamin D response elements (VDREs) on targeted genes, causing coactivator (liganded VDR) or repressor (unliganded VDR) formation (b).

Given the wide range of proteins that interact with VDR, it may be a dynamic component of a large nuclear protein complex (85). A recognized expert on the subject, Carlberg has created a more detailed model of vitamin D signaling based on the hypothesis that VDR is part of a broader, variably constructed protein complex that also includes RXR, other co-receptors, pioneer factors (PU.1, CEBP, GABP, ETS1, RUNX2, BACH2), co-factors, chromatin- modifiers, and remodelers (Figure 7) (86).

According to the concept, firstly, using chromatin-remodeling proteins, pioneer factors improve the accessibility of VDR to bind the enhancer region, such as demethylation of

genomic DNA. Secondly, chromatin modifiers create traces on chromatin regions, such as H3K27ac (the histone, the mutation of which has already been discussed in the context of gliomatogenesis; chapter 1.1.3.). Even though VDR might not be the first protein of the complex that interacts with the enhancer region, its activation by vitamin D promotes the actions of the remaining complex members. The above could clarify the epigenetic impacts (which we have already discussed, may play a major role in the development of childhood cancers; chapter 1.1.3.) caused by vitamin D, including chromatin opening, pioneer factor induction, and histone markings. In the next step DNA loops processes to transcription start site (TSS) regions throughout the same topologically associated domain (TAD) region that are linked with a transcriptional apparatus have stabilized. Through vitamin D-induced impacts on chromatin organizing protein-dependent TAD structure, this alters the TAD's overall formation. The epigenome-modulating effects of vitamin D will either increase or decrease the enzyme activity of RNA polymerase II, resulting in a modification in the messenger RNA (mRNA) expression of the appropriate genes (86).

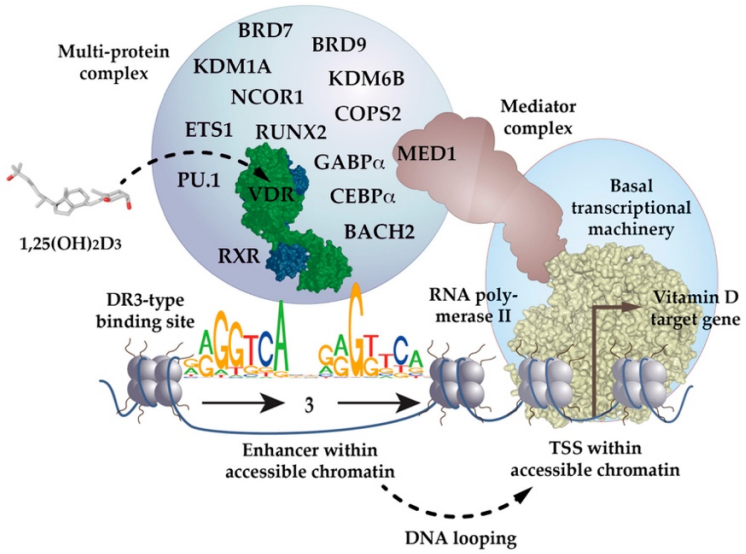

FIGURE 7 | VDR as the principal ligand-inducible subunit of a multiprotein complex. Detailed description in the text.

1.2.3. The possible role of vitamin D in tumorigenesis and its inhibition

The earliest forms of life appeared on Earth approximately 3.9 billion years ago (87). A significant driver of evolution is a reasonable levels of UV radiation which have been acted as a trigger of mutations in genomic DNA that could result in advantageous phenotypic alterations (88). However, excessive UV concentrations can damage sensitive

macromolecules such as DNA and proteins (89). In order to protect these molecules from unnecessary mutagenesis and destruction, sun-exposed species have evolved a variety of protective mechanisms, such as the synthesis of vitamin D (90). Approximately 1.2 billion years ago, when cholesterol-producing species evolved, vitamin D production began (87).

Numerous aspects of the epigenome, including chromatin accessibility, histone alterations, and VDR binding, are significantly influenced by vitamin D in both healthy and malignant cells. Changes in the transcriptome require epigenome modifications induced by vitamin D (65). Furthermore, several cancer driver gene expression changes that take place during carcinogenesis may be influenced by vitamin D-triggered modifications of the epigenome (91).

Administration of vitamin D has a major impact on the transcriptomes of both healthy and neoplastic cells and regulates the expression of thousands of target genes, to regulate a variety of cellular processes, including growth, differentiation, and immune modulation. Oncogene expression is frequently increased and tumor suppressor gene expression is decreased as a result of epigenetic modifications occurring throughout tumorigenesis (92). A portion of these cancer driver genes possess up-regulated targets for vitamin D, such as the tumor suppressor cell cycle inhibitor CDKN1A (93, 94) or the down-regulated oncogene MYC (MYC proto-oncogene, BHLH TF) (95). Through the oncogenes MYC, JUN (an AP-1 transcription factor subunit), JUNB, JUND, and FOS and the tumor suppressor genes CCNC (cyclin C), CCND1, CDKN1A, CDKN1B, and G0S2 (G0/G1 switch 2), vitamin D regulates proliferation and cell cycle (96-100). Moreover, vitamin D target genes regulate a wide range of other cellular processes, including adhesion, migration, extracellular matrix organization, blood vessel development, immune functions, apoptosis, cell communication, steroid metabolism, and oxidative stress (65, 97, 98, 101).

695 vitamin D target genes (102) are most strongly associated with the cellular events of neutrophil activation and degranulation, positive regulation of cytokine secretion in T helper 1 cells and negative regulation of T cell proliferation and also appears to play role in monocytes (103). In a gene ontology investigation of 662 vitamin D target genes indicated similar roles, moreover, in cytokine-mediated signaling pathway, extracellular matrix organization, and positive regulation of angiogenesis. Vitamin D seems to

downregulate 10 human leukocyte antigen class II genes and 5 S100 calcium-binding protein A genes, in addition to modifying the functioning of the CXCL gene family expressing chemokines (104). The immune tolerance induced by vitamin D results in the activation of regulatory T cells, which suppresses the activity of additional immune cells (86, 105, 106).

Maybe the strongest cancer-preventing impact of vitamin D via immune system modification is the prevention of the development of tumors rather than the management of already present tumors. Every day, hundreds of normal cells in each of us undergo the process of becoming cancer cells, but the great majority of these abnormal cells are quickly identified by cytolytic T cells and destroyed (107). In this approach, vitamin D's stimulation of cytolytic T cells serves as a potent strategy for delaying the development of cancer (65, 108).

Certain cancer prevention benefits of vitamin D may stem from on a control of the microenvironmental immune component that is averse to tumor growth (109, 110), moreover, its significance is even larger in the case of pediatric cancers (chapter 1.1.3. (15). For instance, vitamin D can increase the cytotoxicity by antibodies of macrophages and natural killer cells during monoclonal antibody-based cancer therapy (111, 112).

Overall, vitamin D via the VDR performs several processes that are antagonistic to tumorigenesis, including, among numerous others, protecting against the mutagenetic effect of UV-B radiation, modifying the epigenome, influencing the cell cycle, and promoting immune functions.

1.3. Beginning of the own investigation

As researchers have sought novel prognostic and therapeutic biomarkers over the past several decades, the number of studies examining the relationship between vitamin D and cancer has increased dramatically. However, the literature on the childhood aspect of the issue is extremely limited; articles examining the possible role of vitamin D in childhood malignancies are the first to point out that this is a substantially understudied area.

This research represents the first investigation into serum vitamin D levels among pediatric cancer patients as well as the examination of VDR expression patterns in childhood tumor samples and entities. The main objective of this study is to assess the potential prognostic significance of these results.

2. Objectives

2.1. Assessment of serum vitamin D status

2.1.1. Serum vitamin D level of patients compared to average children population

The first goal was to assess whether the initial serum vitamin D level (measured at time of diagnosis) of children with solid tumors is lower than in the average population, to compare retrospectively the serum vitamin D level of our patients with the average childhood vitamin D level calculated for the given period.

2.1.2. Initial vitamin D status and prognosis in children with solid tumors

We aimed to investigate whether higher initial vitamin D levels are statistically associated with a more favorable prognosis and whether the initial vitamin D levels are statistically distinct between the favorable and unfavorable prognostic groups.

2.1.3.1. Efficacy of vitamin D supplementation

The next goal was to assess the efficacy of vitamin D supplementation given to the children diagnosed with solid malignancies, i.e., whether vitamin D supplementation given along with the oncological treatment had increased serum vitamin D levels.

2.1.3.2. Supplemented Vitamin D status and prognosis

In addition, we investigated whether the higher vitamin D level measured after vitamin D supplementation was associated with a more favorable prognosis and whether the vitamin D levels after supplementation are statistically distinct between the favorable and unfavorable prognostic groups.

2.2. Assessment of the VDR pattern of solid tumors with immunohistochemistry

2.2.1.1. VDR expression profile and prognosis

In the second phase of the research, we aimed to detect the potential presence of VDR in samples of solid childhood tumors and to determine whether an association between higher VDR expression and better prognosis can be detected.

2.2.1.2. Difference in VDR patterns in different tumor types

We aimed to investigate whether there is a difference in VDR expression patterns between childhood solid tumor types.

2.2.2. Additional pathological observation

We also aimed to record any additional observations seen during the pathological evaluation.

3. Methods

3.1. *Patient and Sample Selection*

3.1.1. Assessment of serum vitamin D status

3.1.1.1. Characterization of patient and control groups

In the retrospective phase of our study, we included 173 children aged 2 weeks to 19 years (mean = 6.11, SD = 5.28) with solid tumors treated at Semmelweis University, 2nd Department of Pediatrics (between January 1, 2009. and December 1, 2013.) whose serum vitamin D level was measured at the time of diagnosis. The examined group included 96 male (55.5%) and 77 female (44.5%) patients. BMI data were available in 130 cases to calculate age- and gender- specific comparisons by calculating BMI percentile values statistical models (Figure 8).

The cancer patient group contained 46 neuroblastoma (NBL) (26.6%), 24 medulloblastoma (MBL) (13.9%), 21 Ewing-sarcoma (EWS) (12.1%), and 10 ganglioneuroma (5.8%) cases. Less frequent tumor types were nephroblastoma (8, Wilms tumor; WT) (4.6%), retinoblastoma (6, 3.5%), astrocytoma (5, 2.9%), ependymoma (5 EPM) (2.9%), glioblastoma (5, 2.9%), papillary thyroid carcinoma (5 PTC) (2.9%), atypical teratoid rhabdoid tumor (4 AT/RT, 2.3%), hepatoblastoma (4 HBL) (2.3%), teratoma (4, 2.3%), primitive neuroectodermal tumor (4 PNET) (2.3%), hemangioma (2, 1.1%), adrenal cortex carcinoma (2, 1.1%), histiocytosis (2, 1.1%), optic glioma (2, 1.1%), other brainstem tumor (1, 0.5%), AML (1, 0.5%), choroid plexus carcinoma (1, 0.5%), corpus pineal tumor (1, 0.5%), desmoplastic carcinoma (1, 0.5%), other endocrine tumor (1, 0.5%), testicular tumor (1, 0.5%), juvenile granulosa cell tumor (1, 0.5%), medullar thyroid carcinoma (1, 0.5%), myofibroma (1, 0.5%), pineoblastoma (1, 0.5%), plexus papilloma carcinoma (1, 0.5%), rhabdoid renal tumor (1, 0.5%), and sinus ethmoidalis tumor (1, 0.5%) (Table 4). All diagnoses were based on histopathology. Patient with the same pathological diagnosis received identical treatment recommended by the Hungarian National Protocols.

TABLE 4 | Number and proportion of histopathological diagnoses in the studied population (PTC: papillary thyroid carcinoma, AT/RT: atypical teratoid rhabdoid tumor, PNET: primitive neuroectodermal tumor).

Diagnosis	Number of patients	Proportion of total (%)
Neuroblastoma	46	26.6
Medulloblastoma	24	13.9
Ewing sarcoma	21	12.1
Ganglioneuroma	10	5.8
Wilms tumor	8	4.6
Retinoblastoma	6	3.5
Astrocytoma	5	2.9
Ependymoma	5	2.9
Glioblastoma	5	2.9
PTC	5	2.9
AT/RT	4	2.3
Hepatoblastoma	4	2.3
Teratoma	4	2.3
PNET	4	2.3
Other, less frequent tumors	22	12.7

Prognosis of the disease was classified either favorable complete response (CR, n=116), partial response (PR, n=11), and stable disease (SD, n=15) or unfavorable progressive disease (PD, n=5) and death (EX, n=26) based on the 5-year overall survival data.

As the reference average vitamin D level, we used the mean of serum vitamin D values measured in the 0–19 age group at Semmelweis University (between 01. April 2009. and 31. March 2010.) and published in the literature.

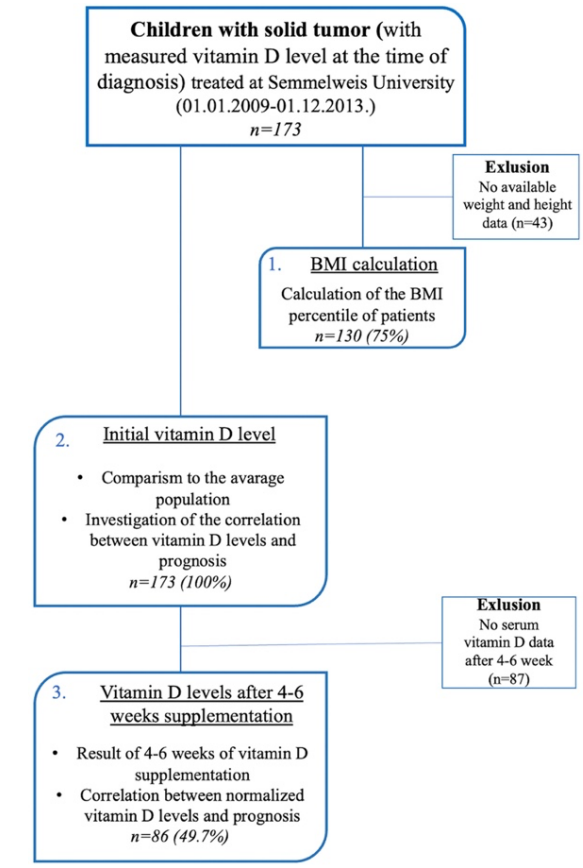

FIGURE 8 | Patient selection flowchart for the assessment of serum vitamin D status in children with solid tumors.

3.1.1.2. Samples, classification of vitamin D levels and prognostic groups

We collected vitamin D values taken at the time of diagnosis, 1-3 days before the initiation of the oncological treatment, and parallel vitamin D supplementation. According to the Hungarian National Protocols, all cancer patients received vitamin D supplementation from the beginning of the treatment. Vitamin D supplementation was given at a dose of 500 IU/day for people under 20 kg of body weight, 1000 IU/day for people weighing 20–40 kg, and 2000 IU/day for people above 40 kg. Since it is known that with regular intake serum vitamin D level reaches a plateau in 4 to 6 weeks (113), we collected data of vitamin D values measured after 4-6 weeks after the supplementation initiation. In this phase of the study, patients who did not have vitamin D measurements four to six weeks after treatment initiation were excluded (n = 87) (Figure 8).

3.1.2. Assessment of VDR pattern of solid tumors with immunohistochemistry

3.1.2.1. Patients for assessment of VDR pattern

For the immunohistochemistry examination 177 children with solid tumor were selected whose biopsies and tumor tissue formalin-fixed, paraffin-embedded (FFPE) tissue blocks were available for immunohistochemical analysis. The patients were previously treated at the 2nd Department of Pediatrics, Semmelweis University.

For investigating the association between the presence of VDR in the tumor tissue and outcome, tumor samples and the survival datasets of 110 children were available aged 2 weeks to 17 years (mean = 5.6, SD = 4.67) (Figure 9). Among these 110 children, 62 (56%) were boys, and 48 (44%) were girls.

The core data on the children were provided by the Hungarian National Childhood Cancer Registry and the electronic medical record system of the Semmelweis University. The full anonymity of the children was kept.

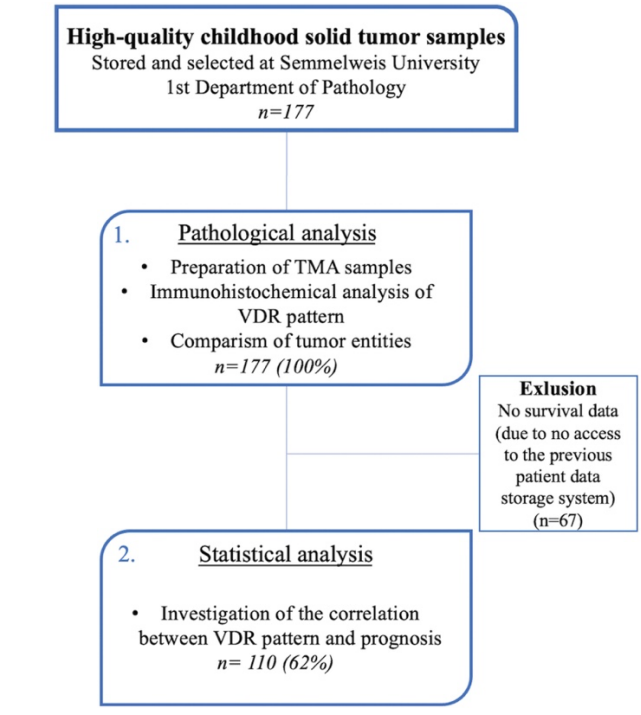

FIGURE 9 | Patient selection flowchart for the assessment of VDR pattern of solid tumors with immunohistochemistry.

3.1.2.2. Samples for assessment of VDR pattern

The samples were stored, prepared, and analyzed at the Department of Pathology and Experimental Cancer Research, Semmelweis University. In our study, we examined the

presence of VDR in 177 childhood tumor samples and classified each tumor entity based on their VDR pattern. The analysis included 37 ependymoma, 10 Ewing sarcoma, 6 ganglioneuroma, 2 hepatoblastoma, 68 medulloblastoma, 27 neuroblastoma, 5 papillary thyroid carcinoma, 12 PNET, 1 rhabdoid renal tumor, 2 teratoma and 7 Wilms tumor samples (Table 5). Diagnoses were based on histopathologic, immunophenotypic and molecular analyses according to the current WHO classification.

TABLE 5 | Number and proportion of histopathological diagnoses in the studied samples (PNET: primitive neuroectodermal tumor, PTC: papillary thyroid carcinoma).

Histopathological diagnosis	Number of samples	Proportion of total (%)
Medulloblastoma	68	38.4
Ependymoma	37	20.9
Neuroblastoma	27	15.3
PNET	12	6.7
Ewing sarcoma	10	5.6
Wilms tumor	7	3.9
Ganglioneuroma	6	3.4
PTC	5	2.8
Teratoma	2	1.1
Hepatoblastoma	2	1.1
Rhabdoid renal tumor	1	0.6

3.2. Methodology

3.2.1. Investigation of serum vitamin D levels

3.2.1.1. Measurement of serum vitamin D levels

The Department of Laboratory Medicine of Semmelweis Inuversity calculated the 25-(OH)-D3 vitamin levels from the serum samples by using immunoanalytical methods and electrochemiluminescence immunoassay (ECLIA). The laboratory used the Cobas 601 kit (Roche) and the Liaison kit (DiaSorin).

A serum vitamin D level under 30 ng/ml (vitamin D deficiency) was considered as low level that requires supplementation and above 30 ng/ml considered as normal serum vitamin D level.

3.2.1.2. Statistical analysis

We performed one sample t-test, paired sample t-test, two sample t-test and analysis of variance (ANOVA) using Microsoft Excel 2013 and logistic regression models using Stata 18 statistical software on the retrospective dataset at significance level of 5%.

3.2.2. Investigation of VDR pattern of solid tumors with immunohistochemistry

3.2.2.1. TMA samples

After selecting representative tumor areas on the hematoxylin-eosin-stained slides, tissue microarrays (TMA) were created with a computer-driven semi-automated instrument (TMA Master, 3D HISTECH Ltd., Budapest, Hungary). Duplicate or triplicate cores of 2 mm diameter were arrayed from the tumor samples into the recipient blocks. In the case of smaller tumor samples, whole slides were used.

3.2.2.2. Immunohistochemical analysis

For immunohistochemical analysis the TMA blocks were cut into 3 μm sections and then applied to silane-coated adhesive microscope slides. Immunohistochemical staining was performed on the Dako Agilent Autostainer System (Agilent, Santa Clara, CA, US). Antigen retrieval was performed using Target Retrieval Solution, Low pH. TMA sections were labeled with VDR antibody (Santa Cruz, mouse monoclonal antibody, 1:600 dilution). External controls (cerebral cortex, cerebellum, lymph node, liver, kidney, uterus, and skin) were used as positive and negative controls. TMA slides were scanned using a Panoramic scan instrument (3D HISTECH) equipped with a 20× Carl Zeiss objective (NA = 0.83; Carl Zeiss MicroImaging Inc., Jena, Germany).

The relative proportion of positive cells was determined on the scanned slides at 400× magnification by counting 200 cells in three different areas on each TMA core by two independent pathologists, evaluating a minimum of a 0.1 mm^2 area. According to the expression level of the VDR protein, the following scores were assigned: negative < 10% (1 - low), 11-50% (2 - intermediate) and 51-100% (3 - high). Negative and low were considered as minor-, intermediate (11-50%) and high (51-100%) were considered as major VDR expression in the samples.

All tissue samples were handled in a coded fashion, according to the Dutch and Hungarian National Ethical guidelines (National Ethical Review Board approval: TUKEB no. 7/2006).

3.2.2.3. Statistical analysis

Fisher's exact test was conducted to analyze the difference between groups. The result was considered significant if the p-value was less than 0.05. For comparison of two groups, the odds ratio was calculated with Haldane–Anscombe correction. The proportion of significant VDR expression was calculated by diagnoses. Confidence intervals of 95% were assigned to the proportions, for which Wilson score intervals were calculated. All analyses were carried out by R version 4.1.0 statistics software.

4. Results

4.1. *Assessment of serum vitamin D status*

In the first phase of the study, we compared retrospectively the serum vitamin D level of the patient group with the average childhood vitamin D level calculated for the given period. We investigated the possible association between the initial- and supplemented serum vitamin D levels of patients and prognosis.

4.1.1. Serum vitamin D level of patients compared to average children population

Firstly, we compared the average vitamin D level of our patients with the average vitamin D level of the pediatric population using a one sample t-test. Based on the relevant literature the mean serum vitamin D value of the average children population was 28.4 ng/ml (SD = 6.07) (114). The patient group had a mean vitamin D value of 24.3 ng/ml (SD = 11.17) at the time of diagnosis, before the start of the oncological treatment and parallel vitamin D substitution. The mean of the initial vitamin D levels of children with solid tumors was significantly lower than the mean value of the non-cancerous, average population ($t(172) = -4.97$, $p < 0.001$).

4.1.2. Initial Vitamin D status and prognosis in children with solid tumors

A serum vitamin D level under 30 ng/ml was considered as low vitamin D level (n= 126), above 30 ng/ml considered as normal vitamin D level (n=47). To describe prognosis, two categories were created: the favorable category consisted of complete response (CR, n=116), partial response (PR, n=11), and stable disease (SD, n=15), the unfavorable category consisted of progressive disease (PD, n=5) and death (EX, n=26) based on the 5-year overall survival data (Table 6). We investigated whether higher initial vitamin D levels are statistically associated with a more favorable prognosis. We performed a logistic regression model in which we also included the BMI percentile value as a variable, which includes the children's age, gender, weight, and height. We found that the odds of a favorable prognosis occurring increased by 95 % (OR= 1.95 95% CI (1.21-3.13) p=0.006) for a ten ng/ml increase in the initial serum vitamin D level. (It means that the odds of a favorable prognosis occurring increased by 7 % (OR= 1.07 95% CI (1.02-1.12) p=0.006) for a one ng/ml increase in the initial serum vitamin D level.) We

investigated whether the initial vitamin D levels are statistically distinct between the favorable and unfavorable prognostic groups.

The initial vitamin D level showed a significant difference in the favorable and unfavorable prognostic groups (ANOVA-1 p= 0.009).

TABLE 6 | Number of patients in different serum vitamin D- and prognostic categories initially.

Initial vitamin D level (ng/ml)	Complete response	Partial Response	Stable disease	Progressive disease	Exitus
	Favorable prognosis			**Unfavorable prognosis**	
>30 (normal)	32	3	9	0	3
	44			3	
<30 (low)	84	8	6	5	23
	98			28	
Total	116	11	15	5	26
	142			31	

4.1.3.1. Efficacy of vitamin D supplementation

With regular intake, the serum vitamin D level reaches a plateau phase after 4 to 6 weeks as mentioned in chapter 3.1.1.2 (113). To examine the efficacy of vitamin D supplementation - administered in parallel with the oncological treatment at our hospital - we compared the initial vitamin D levels with the values measured after 4 to 6 weeks, using a paired T test. In this phase of the study, only patients were included who did have vitamin D measurements 4 to 6 weeks after treatment initiation (n=86). Vitamin D supplementation in fact increased the serum vitamin D levels of children; the difference between initial (mean = 24.44, SD = 10.7) and supplemented values (mean = 30.23, SD = 12.2) was significant ($t(85)= -4.13$, $p <0.001$).

4.1.3.2. Supplemented vitamin D status and prognosis

We investigated whether the higher vitamin D level measured after vitamin D supplementation was associated with a more favorable prognosis. We performed a logistic regression model in which we also included the BMI percentile value as a variable, which includes the children's age, gender, weight, and height. There was no significant difference in the odds of a favorable prognosis for ten ng/ml increase in the serum vitamin D level by supplementation (OR = 1.48 95 % CI (0.91-2,41) p = 0.114).

Moreover, we investigated whether the vitamin D levels after supplementation are statistically distinct between the favorable and unfavorable prognostic groups. Vitamin D levels measured after supplementation showed no significant difference in the favorable and favorable prognostic groups (ANOVA-1 $p = 0.09$).

4.1.4. Seasonal fluctuation of serum vitamin D levels

The vitamin D level's seasonal fluctuations might be explained by changes in the length of daylight hours and the angle of the sun's rays. As a result, the vitamin D level generated by UVB radiation on the skin fluctuates (115). According to the data (counted from the measurements between 1991 and 2020) published by the National Meteorological Service (116) in Hungary, the number of daylight hours shows a seasonal fluctuation between 60 and 280 hours per month, with the minimum in December and the maximum in July. Based on this distribution, we divided our patients' data into two categories - measured in the months of low (October–March) and high (April–September) numbers of daylight hours. We compared the patient group's vitamin D values that were measured in the months with low and high numbers of daylight hours, using a two sample t-test. No significant difference ($p=0.30$) was found in our patient population at a 5% significance.

4.2. *Assessment of VDR pattern of solid tumors with immunohistochemistry*

At the second phase of our study, we examined the VDR expression on childhood solid tumor samples with immunohistochemistry. We determined the percentages of the appearance of VDR-expressing tumor cells on TMA blocks and scored them as mentioned in chapter 3.2.2.2. Tumor samples with high (>50%) expression (Figure 10a) and intermediate (10–50%) expression (Figure 10b) were considered as major VDR expression, while low (<10%) expression (Figure 10c) and non-expressing, so-called negative cells (Figure 10d) were considered as minor VDR expression during the later statistical analysis.

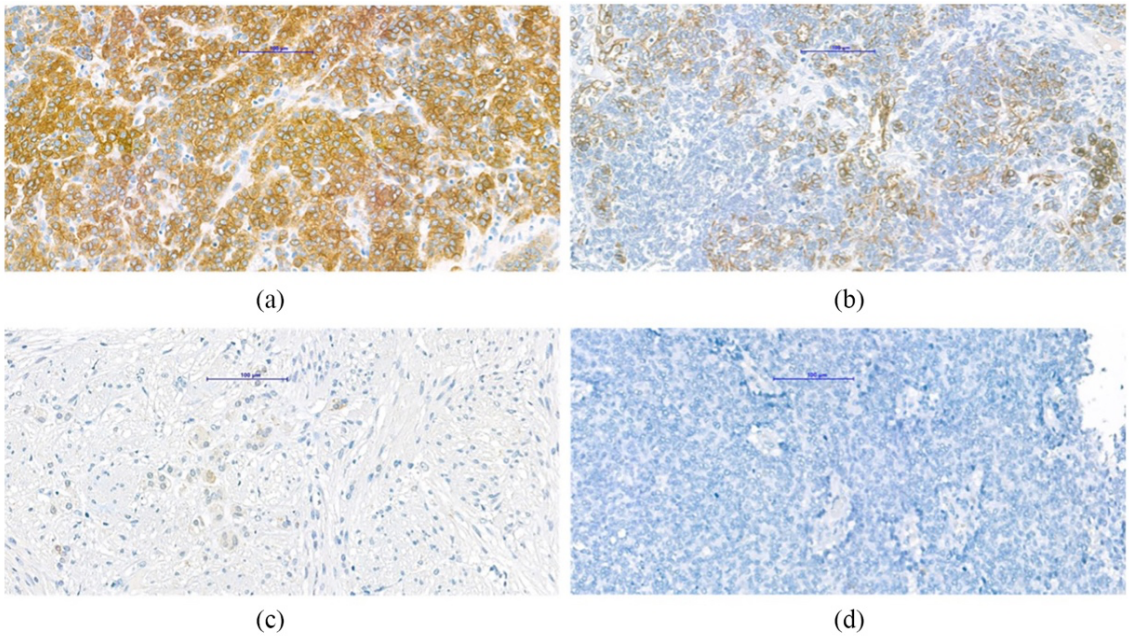

FIGURE 10 | Representative vitamin D receptor (VDR) immunohistochemistry samples according to expression levels. Tumor cells showed membrane/cytoplasmic VDR expression, no nuclear positivity was observed (a) Score 3, high expression (VDR >50%), (b) Score 2, intermediate expression (10%<VDR<50%), (c) Score 1, low expression (VDR <10%), (d) Score 0, considered negative.

4.2.1.1. VDR expression profile and prognosis

Disease outcomes were divided into favorable (complete remission, partial remission, stable disease) and unfavorable (progressive disease, exitus letalis) prognostic groups for subsequent statistical analysis as mentioned in the earlier phase of our studies.

We investigated whether lower (minor) VDR expression is associated with a worse (unfavorable) prognosis. We found by conducting a Fisher's exact test, that minor VDR expression was associated with a significantly less favorable prognosis ($p = 0.0061$) in the examined childhood solid tumors. Due to the presence of zero values in the contingency table, the Haldane–Ansoff correction was required to calculate the OR. Moreover, given its exceptionally high value, its clinical relevance could be assumed; we found that there is a clinically significant association; minor VDR expression has more than 14-fold odds of an unfavorable prognosis (OR=14.74) (Table 7).

TABLE 7 | In the table below the number of samples belonging to each VDR expression and the prognostic group is shown. A significant association (p=0.0061) is described between lower VDR expression in tumor samples and poor outcomes.

	Favorable Prognosis	Unfavorable Prognosis
Major VDR Expression	18	0
Minor VDR Expression	66	26

4.2.1.2. Difference in VDR patterns in different tumor types

We aimed to evaluate whether there is a difference between the solid tumor entities examined based on the VDR expression. We found that the rate of VDR expression differed significantly between various tumor types ($p < 0.001$). Numerically, the attached table shows the VDR expression ratios for each tumor type (Table 8).

TABLE 8 | The VDR expression percentage distribution for each tumor type is shown in the table below. The first column shows the number of samples tested for each diagnosis and the corresponding tumor types. The second column shows the number of samples with major VDR expression, and the third column shows the percentage of minor expression with corresponding 95% confidence intervals (CI) (PTC: papillary thyroid carcinoma, PNET: primitive neuroectodermal tumor).

Tumor Types (number of samples)	Number of Samples with Major VDR Expression	Proportion of Minor VDR Expression with CI
Neuroblastoma (n=27)	2	7.4 % (2.1-23.4)
Ewing sarcoma (n=10)	2	20.0 % (5.7-51.0)
Wilms tumor (n=7)	2	28.6 % (8.2-64.1)
PTC (n=5)	5	100.0 % (56.6–100)
Ependymoma (n=37)	2	5.4 % (1.5-17.7)
Medulloblastoma (n=68)	6	8.8 % (4.1–17.9)
PNET (n=12)	4	33.3 % (13.8-60.9)

The figure shows the proportion of major and minor expression with 95% confidence intervals according to tumor types (Figure 11).

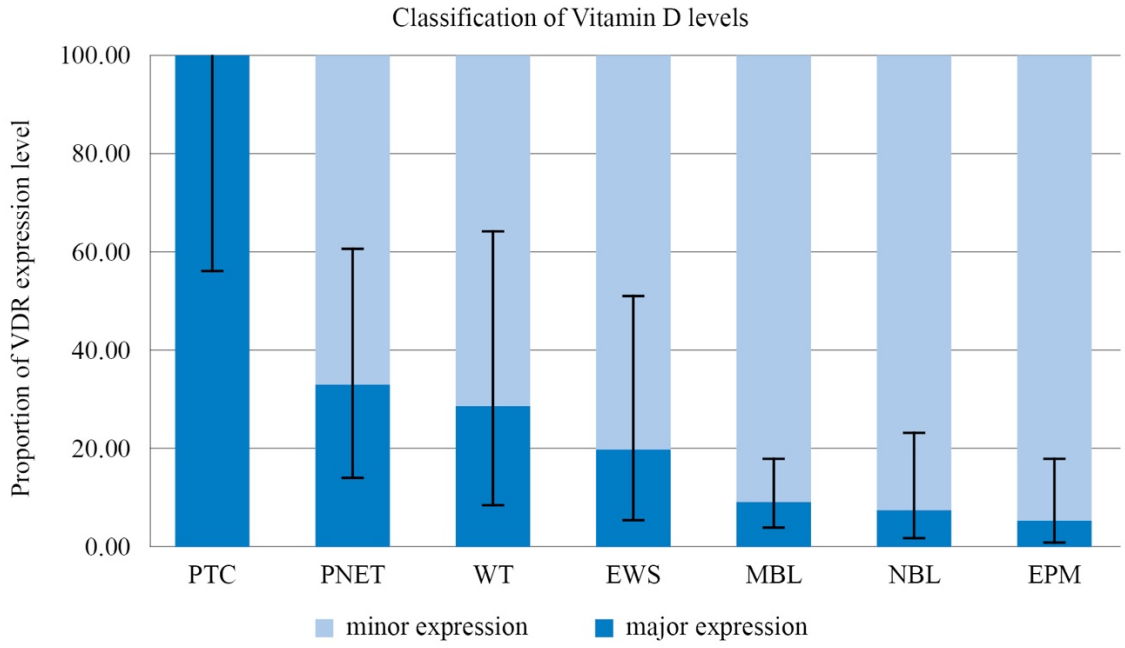

FIGURE 11 | Proportion of vitamin D receptor expression levels for different diagnoses with 95% confidence intervals (VDR: vitamin D receptor; PTC: Papillary thyroid carcinoma; WT: Wilms-tumor; PNET: primitive neuro-ectodermal Tumors; EWS: Ewing sarcoma; NBL: neuroblastoma; MBL: medulloblastoma; EPM: ependymoma).

4.2.2. Additional pathological observation

Pathological observations were made during the examination regarding the VDR characteristics of the tumor types.

VDR expression of Wilms tumor

Wilms-tumor (nephroblastoma) was generally characterized by significant VDR expression, which was seen mainly on the epithelial cells of the tubules (Figure 12a).

VDR expression of CNS tumors

In case of central nervous system tumors, based on the observations made during the evaluation, minor VDR expression was characteristic for ependymoma and medulloblastoma. Interestingly, paranuclear dot-like positivity was observed in some ependymoma samples (Figure 12b). In contrast, major VDR expression was observed in

PNET. Given that the current WHO tumor classification has already broken down the previous PNET category into several different entities (22), a detailed examination of these will be considered later.

VDR expression of hepatoblastoma

Relatively few (n=2) hepatoblastoma samples were available. Due to statistical correctness, the statistical analysis does not include the results of hepatoblastoma VDR expression measurement. However, immunohistochemistry of a total of 4 TMA samples from 2 tumor blocks revealed major, diffuse VDR expression of the tumor cells (Figure 12c).

VDR expression of neuroblastoma

In the case of neuroblastoma, it was observed that the mature ganglion cells in the tumor showed mainly major VDR expression (Figure 12d).

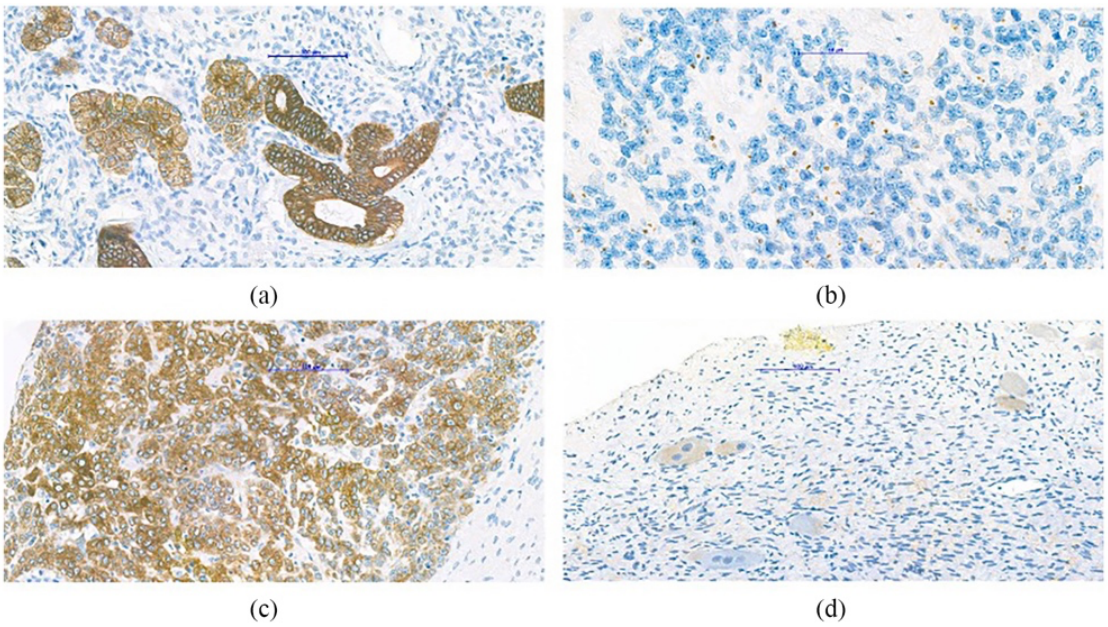

FIGURE 12 | Representative VDR protein expression patterns in specific tumor subtypes. (a) Wilms tumor samples showed strong membrane/cytoplasmic expression, seen predominantly in the epithelial component. (b) Few ependymoma samples showed paranuclear dot-like positivity. (c) Hepatoblastoma samples showed strong, diffuse membrane/cytoplasmic VDR expression. (d) Ganglioneuroma samples showed positive ganglion cells.

5. Discussion

5.0.1. Awareness of childhood cancer

Approximately 400 000 children and adolescents ages 0 to 19 are diagnosed with cancer annually. Every three minutes, a family across the globe receives the devastating news that their child has been diagnosed with cancer (5, 117) . Cancer is the leading cause of non-accidental death among children and adolescents. The probability of pediatric cancer survival exhibits significant variability across countries; in high-income countries, the survival rate for children with cancer exceeds 80%, whereas in numerous low- and middle-income nations, the successful treatment rate for children with cancer is less than 30% (118, 119).

Recent sequencing studies have demonstrated that the mutational burden in the majority of pediatric malignancies is significantly lower than in adult malignancies (25, 26). Compared to adult cancers, fusion genes (120), histone mutations (16, 121), and germline mutations are more prevalent, and several mutations observed in pediatric tumors are uncommon in adult cancers. According to evidence from genetically modified mouse models of childhood cancer, it is suggested that many pediatric malignancies arise from stem or progenitor cells during specific developmental periods (122). Furthermore, there is an increasing awareness that epigenetic dysregulation is fundamental to numerous types of childhood cancer, as opposed to the multiple mutational "hits" typically reported for adult cancers (27).

Based on what is known so far and current above-mentioned research confirming this, environmental factors cannot be held responsible for the development of childhood cancers, thus setting a barrier to prevention. Moreover, it is also a fact that the prognosis is influenced by the stage of the cancerous disease at the time of diagnosis, which is often unfavorable in the case of childhood tumors partly due to the absence of screening. Therefore, the need for additional prognostic and therapeutic biomarkers arises.

5.0.2. The idea behind the investigation

As researchers seek for novel prognostic and therapeutic biomarkers the number of studies examining the relationship between vitamin D and cancer has increased dramatically in the literature over the past several decades. However, the literature on the

childhood aspect of the issue is very poor; articles investigating the possible role of vitamin D in childhood malignancies are also the first to highlight that this is a vastly under-represented area in the literature.

This hiatus has attracted our attention to the question of whether there is a potential association between the development and prognosis of childhood malignancies and serum vitamin D levels and VDR expression. Could it be considered as a possible prognostic and therapeutic biomarker?

Firstly, we assessed whether the initial serum vitamin D level (measured at time of diagnosis) of children with solid tumors is lower than in the average population, then, we investigated whether higher initial vitamin D levels are statistically associated with a more favorable prognosis. Afterwards, we investigated whether there was an association between adequate vitamin D supplementation along with oncotherapy and prognosis.

In the next phase of the study, the VDR expression pattern of pediatric tumor samples were investigated by immunohistochemistry. In addition to the correlation between the VDR expression pattern of tumor samples and prognosis, we looked for differences and characteristics of the VDR pattern in different tumor types, thus examining the role of the VDR expression pattern as a possible biomarker in childhood solid tumors.

5.1. *Assessment of serum vitamin D status*

5.1.1. Serum vitamin D level of patients compared to average children population

Residence at lower latitudes and receiving more sunlight, both of which increase the body's production of vitamin D, reduces the risk of colorectal cancer, according to a significant epidemiological study conducted a few decades ago (42). Scientists have known for roughly the same amount of time that vitamin D can inhibit the proliferation of melanoma cells in vitro (43). These two facts led to the hypothesis that inadequate vitamin D intake may be a cancer risk factor. Numerous in vitro studies and clinical trials demonstrating the efficacy of vitamin D against prostate cancer (123), breast cancer (124), lymphoma (111), and leukemia all corroborated this hypothesis. On the other hand, in three randomized controlled trials, vitamin D supplementation had no impact (48-50),

while their meta-analysis suggested noticeably decreased cancer mortality (51), there has been no decrease in the incidence of cancer (125).

Observational epidemiologic research has demonstrated that inadequate vitamin D levels increase the risk of developing various malignancies and adequate vitamin D supplementation can aid in cancer prevention. Randomized controlled trials were unable to confirm these findings in the general population, indicating the need for segmentation into more sensitive subpopulation (65).

Childhood cancer patients are an extremely understudied subpopulation in this area worldwide (126). In Hungary, we were the first to compare the serum vitamin D levels of childhood solid tumor patients to those of a healthy population. We conducted a retrospective study about the possible role of subclinical vitamin D deficiency as a risk factor in tumorous diseases developing among children.

Consistently, the results of our investigation also showed significantly lower levels ($t(172) = -4.97$, $p < 0.001$) among the children with cancer comparing with the data of the average, non-cancerous children population. The procedure used and the fact that the serum vitamin D level is a result of numerous factors raise the question of whether a causal relationship could be found, or a reverse causality exists. There are several potential risk factors for vitamin D deficiency along with childhood cancer at diagnosis. For example, loss of appetite, resulting in a decrease in oral vitamin D consumption; malaise and fatigue, leading to an increase in indoor activities and a decrease in solar exposure. In certain cases, low levels of active vitamin D due to renal or hepatic disease involvement. In addition, genetic factors (dark skin color, lactose intolerance) and developmental disorders (accelerated growth in childhood and adolescence, obesity) can play a role (127). On the other hand, the VDR can be detected in various cells of the body that as a nuclear receptor reduces cell proliferation and stimulates differentiation through the complex network of genomic and non-genomic mechanisms, which are adverse processes of tumor genesis (65).

5.1.2. Initial Vitamin D status and prognosis in children with solid tumors

Numerous cohort studies were conducted among adults to evaluate the correlation between initial vitamin D values and the prognosis. Lower mortality rates were registered with higher initial vitamin D values, while worse prognostic data were paired with lower

values in cases of breast, lung, and colorectal cancers (128, 129); lymphoma (130); and gastric carcinoma (131). UV radiation exposure on the skin causes DNA damage in melanocytes, which can result in mutations and tumor development such as melanoma. Interestingly, Vitamin D, which is also produced by UV radiation, is widely recognized for its anti-proliferative effects against melanomas. The impact was absent in cells deficient in both the tumor suppressor phosphatase and tensin homolog (PTEN) deleted on chromosome 10 and the VDR. In melanoma, PTEN is frequently absent or mutated. A substantial increase in PTEN levels and downregulation of the protein kinase B isoform (Akt) pathway and its downstream effectors were observed in selected melanoma cell lines following incubation with vitamin D, which indicates that vitamin D might decrease the viability of melanoma cells by targeting PTEN (132).

During our retrospective research, we could describe an association between the favorable prognosis and higher vitamin D values. We found that the odds of a favorable prognosis occurring increased by 95 % (OR= 1.95 95% CI (1.21-3.13) p = 0.006) for a ten ng/ml increase in the initial serum vitamin D level.

5.1.3. Supplemented Vitamin D status and prognosis

Several studies suggested the supplementation of vitamin D analogs as an optional component of the treatment and, in some cases, it is already part of the standard treatment protocol (125). A number of investigations found that vitamin D enhanced the efficacy of chemotherapy and bolstered the fight against certain malignancies (133). However, in our patient population, no significant difference was described in the odds of a favorable prognosis for ten ng/ml increase in the serum vitamin D level by supplementation (OR = 1.48 95 % CI (0.91-2,41) p = 0.114). In contrast to our results, one study has shown that a vitamin D analogue (EB1089) induces apoptosis in chronic lymphoid leukemia cells (134). In mouse models of retinoblastoma, two vitamin D analogs (16,23-D3 and 1a-OH-D2) suppressed tumor growth with reduced toxicity. Increased apoptosis caused by increased p53-related gene expression appears to be the mechanism of action (135). In research on human breast cancer cells, significant data suggests that vitamin D's potent antiproliferative action is due to its targeting of multiple important cell cycle regulators. In this study, EB1089, which has considerable anticancer effects from both in vitro and

in vivo trials, is one of the most promising analogs of vitamin D in terms of fighting cancer (136).

There is a growing literature on tumor research on potentially effective complementary therapies to oncotherapy, such as the role of vitamin D through its antitumor gene-modifying effect on VDR. In a study involving leukemia cells, treatment with vitamin D significantly decreased cell viability. Interestingly, the results of this research suggest a correlation between vitamin D cytotoxicity and VDR expression. The increased expression of VDR correlates with a higher level of vitamin D cytotoxicity, indicating that vitamin D has antitumor properties (137). According to a review on glioblastoma, Vitamin D and its analogs may induce cell cycle arrest, apoptosis, anti-migratory and anti-invasive effects, and repression of stemness, while upregulating VDR to further enhance the anti-tumor response (138).

5.2. *Assessment of VDR pattern of solid tumors with immunohistochemistry*

5.2.1. VDR expression profile

Comparing the literature with our own data, we wondered why there was no association between adequate vitamin D supplementation along with oncological treatment and prognosis in our patient population. Given that in vitro studies have shown that vitamin D and its analogues exert their anti-tumor effects through the VDR, we became interested in investigating the VDR pattern of childhood solid tumors. We wondered whether the VDR pattern of childhood solid tumors was already associated with prognosis and whether there might be a difference between the different entities so that they could be examined separately in terms of VDR pattern and thus vitamin D supplementation.

5.2.1.1. VDR expression profile and prognosis

Vitamin D and its analogues has been established that suppress the proliferation of human cancer cells via the VDR. VDR is a nuclear receptor superfamily ligand-dependent TF (139). In a study, the expression of VDR and CYP24A1 mRNA was examined in a cohort of human breast, lung, colon, and ovarian tumor samples using real-time reverse transcription polymerase chain reaction (PCR). In human malignancies, the expression of CYP24A1 mRNA and, to a lower extent, VDR mRNA appears to be altered. The function

and metabolism of vitamin D and its growth inhibitory effect may be compromised in the pathogenesis of solid tumors to promote tumorigenesis (140).

In a well-known signaling pathway, VDR binds vitamin D in the cell, and the resulting complex connects with the RXR to form a vitamin D-VDR*RXR heterodimer. The VDR is translocated into the nucleus and attaches to the VDRE, triggering the transcription of specific genes when it connects to its ligand, vitamin D. VDREs in the promoter regions of target genes can be bound by this complex. Activated VDR regulates several genes involved in a wide range of cellular functions and processes, for example, this interaction results in several downstream effects, including the stimulation of differentiation and inhibition of proliferation, angiogenesis, invasiveness, and metastatic potential (71, 141), so it is understandable, that low vitamin D levels have been associated with cancer in humans (142, 143).

In our study, lower VDR expression was associated with a worse prognosis in various childhood solid tumors ($p = 0.0061$). The fact that an inverse association between VDR expression and tumor aggressiveness has been found suggests that VDR may be a target subjected to downregulation or ablation along the cancer progression cascade into more aggressive stages. VDR expression and activity degradation may be a common molecular change observed in a variety of tumor types, including breast, prostate, and colon cancer (140, 144, 145).

In a study on pancreatic adenocarcinoma correlating with our results, the role of VDR as a potential prognostic biomarker was similarly suggested (146). According to a systematic-review and meta-analysis among breast cancer patients, the level of VDR protein expression in whole breast carcinoma cells identified by immunohistochemistry correlates with the overall survival of cancer patients. Based on the VDR expression, it was anticipated that a more individualized supplementation with vitamin D and a more accurate prognosis evaluation can be suggested for patients with breast cancer (147). Another research evaluated VDR expression in human colorectal malignancies as a potential indicator of the disease's biological behavior. A high level of VDR expression was associated with a favorable prognosis, the outcomes of their studies support the hypothesis that VDR may play a role in the pathogenesis of colorectal cancer (148).

5.2.1.2. Difference in VDR patterns in different tumor types

Given that our study group was heterogeneous in terms of solid tumor types, we investigated whether VDR protein expression levels differed by entities. We found that the rate of VDR expression varied significantly among various tumor types ($p < 0.0001$) (chapter 4.2.1.2. Figure 11).

Major VDR protein expression was detected in 100% of the papillary thyroid carcinoma (PTC) samples we examined. In a study on PTC, elevated protein, and mRNA expression of VDR was demonstrated compared to normal and benign tissues. However, in high TNM stage PTC, nuclear VDR protein expression was lower, which was associated with low nuclear p21 (a cell cycle regulator protein) expression. These results provide additional support for the putative anti-proliferative effects of VDR in PTC, which are attenuated in aggressive thyroid cancer. In addition, lesser VDR mRNA expression in PTC was associated with decreased serum 25(OH)D levels in this study. Overall, the possibility of a positive correlation has been identified between decreased VDR expression, low serum vitamin D level, and aggressiveness of thyroid cancer (149). According to a systematic review, the role of vitamin D in the pathogenesis of thyroid neoplasms has been the subject of a large number of reports. Several in vitro investigations utilizing vitamin D, or its analogues appeared to be promising. It was anticipated that gene variants associated with vitamin D may also play a significant modulatory role in thyroid tumorigenesis (150).

In our studies, 20% of the available Ewing's sarcoma (EWS) samples showed major VDR expression. The role of vitamin D in bone and calcium homeostasis in bone-derived Ewing's sarcoma has been investigated in a limited number of studies (151), and one study has attributed the height difference between EWS patients and the general population to polymorphisms in VDR (152), but to the best of our knowledge, the prognostic implications of the disease have not been investigated yet.

5.2.2. Additional pathological observation

VDR expression varied depending on the type of tissue examined. Pathological observations on the pattern of VDR expression in each tumor tissue were also made.

VDR expression of Wilms tumor

In cases of Wilms tumor, major VDR expression was observed in nearly 30% of our samples. A similar pattern of VDR expression to Wilms' tumor (WT) has been described in healthy kidneys. In both nephroblastoma and intact kidneys, VDR expression is characteristic of tubular epithelial cells (Figure 14a) (153). This phenomenon raises the possibility that the presence of VDR is already characteristic in the early stage of differentiation so that nephroblasts also carry this property. It is also suggested that the favorable prognosis for nephroblastoma may be related to the VDR pattern characteristic of mature renal tissue and the signaling pathway that facilitates maturation. An interesting data point to the possibility of the opposite cause-and-effect relationship. The results of a study showed that Wilms tumor 1 gene (WT1) induces cellular VDR, which implies a new molecular mechanism by which WT1 may be participating in cell differentiation. The finding that induction of VDR by WT1 raises the hypothesis that loss of WT1 may impact the response to vitamin D, which may also affect tumorigenesis, pointing to a novel pathway for WT1's antioncogenic function (154).

VDR expression of CNS tumors

During the immunohistochemical examination of the VDR pattern on CNS tumors, our results were variant.

Ependymoma (EPM) samples showed VDR positivity only in 5.4% of the examined cases which we have not considered as significant expression. Some EPM samples presented an interesting paranuclear dot-like positivity (Figure 14b). A phenomenon comparable to the localization of VDR has not, however, been described in the literature.

Major VDR expression was found in only 8% of medulloblastoma (MBL) samples which cannot be considered significant either. In a research project, the co-localization of VDR and Programmed death-ligand 1 (PD-L1) in ovarian and MBL tissues was described, vitamin D/VDR has been found to stimulate PD-L1 expression. In addition, PD-L1 is inhibited by the targeting of VDR by the small molecule antagonist of vitamin D (MeTC7) (155).

Our observation of negativity of VDR expression does not correlate with the previous results in the literature. A review found that VDR plays a role in the development of the

central nervous system (156). It has been described that primary cortical neurons produce less neurotrophic growth factor (NGF) when VDR expression is inhibited (157).

On the other hand, the results of immunohistochemical investigations of PNET correlate with data from the known literature; we found significant VDR expression. In our studies, 33% of PNET samples showed major positivity. Given that the current WHO tumor classification has already broken down the previous PNET category into several different entities (158), a detailed examination of these will be considered later.

Regarding the central nervous system from this point of view, the literature suggests that VDR is expressed in the CNS, which may play a role as a neurosteroid both in development and function (156, 157). According to a group's previous research, developmental vitamin D deficiency alters the ontogeny of dopaminergic neurons in the developing mesencephalon. In their recent study, they demonstrated that vitamin D binding to VDR substantially increased the production of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine (DA) synthesis, in VDR-expressing SH-SY5Y cells (triple-subcloned neuroblastoma cell line derived from the SK-N-SH cell line). On the basis of the recent finding that N-cadherin plays a role in the direct differentiation of dopaminergic neurons, they pointed out that vitamin D deficiency reduces N-cadherin expression in the embryonic mesencephalon. On an in vitro model, they confirm that vitamin D increases TH expression, decreases proliferation, and elevates N-cadherin, a potential mediator of these processes (159).

Moreover, the presence of VDR may have therapeutic implications for CNS malignancies. A review of several studies suggests that vitamin D and its analogues exhibit synergy when combined with other treatments for brain cancer (160).

All in all, our results and the literature are partly contradictory, although most of the available data on the subject describe in vitro studies. Overall, VDR expression in pediatric CNS tumors requires further investigation to provide a clear position statement.

VDR expression of hepatoblastoma

The diffuse, significant VDR expression we saw in hepatoblastoma (HBL) samples (Figure 14c) is correlated in some studies, where significant VDR expression was detected in healthy liver tissue and in hepatocellular carcinoma (HCC). In a study on HCC which also investigated the surrounding non-tumorous tissue they found that in HCC,

compared to the intact liver tissue, lower levels of CYP27B1 (25(OH)D3 hydroxylase enzyme) and VDR-expression was detected in HCC cells which could have been indicative of a reduction in the vitamin D antitumor effect of vitamin D antitumor activity. In parallel, the positivity of the inactivating CYP24A1 enzyme (plays role in degradation of vitamin D) indicated the ability of tumor cells to degrade vitamin D. According to the authors opinion, this may be due to one of the reasons for the findings in previous clinical trials, that a large proportion of hepatocellular carcinoma patients do not respond to the vitamin D treatment. Based on these data, it is hypothesized that a more effective anti-HCC effect of vitamin D would be more effective in the future on well chosen, selected patients. Furthermore, CYP24A1 inhibitors may enhance the antitumor effect of vitamin D (161). Another research group used freshly isolated cells from human, rat and mouse normal livers and biliary and epithelial hepatic cell lines. They found that hepatocytes express extremely low levels of VDR mRNA and protein. On the contrary, sinusoidal endothelial, Kupffer, and stellate cells of normal rat livers, as well as the mouse biliary cell line biliary duct cells and rat hepatic neonatal epithelial cells, expressed both VDR mRNA and protein in a significant manner. It was hypothesized that hepatocytes were negative for the receptor, whereas the small hepatic sinusoidal and ductular cell populations should express the receptor. An interesting addition was that human hepatocarcinoma and intrahepatic metastases of colon adenocarcinoma were found to express the VDR gene transcript. They also described that Kupffer, stellate, and endothelial cells exhibited a considerable increase in CYP24A1 in response to vitamin D, indicating that the VDR is entirely functional in these cells (162). In a different research project, a human hepatoblastoma cell line was cultured for varying amounts of time with vitamin D or one of its two analogues (EB 1089, CB 1089). Vitamin D and two of its analogues were characterized in the report as antiproliferative agents in the in vitro growth of the HBL cell line. The results indicated that vitamin D and its analogues are effective inhibitors of proliferation of hepatoblastoma cells, as demonstrated by the fact that a significant number of treated cells killed, and that this effect could be amplified with prolonged treatment (163).

Based on our new findings on removed hepatoblastoma tissues and the above-mentioned literature, it is presumed that immunohistochemical examination of the tumor VDR pattern in hepatoblastoma is recommended, and in cases of significant VDR expression

in a well-selected group of patients, the administration of vitamin D or its analogs may be reasonable as a part of oncotherapy.

VDR expression of neuroblastoma

Neuroblastoma samples showed VDR major expression only in 7.4% of the examined cases, which all in all was not considered significant. More interesting results were obtained from pathological observations on the samples. Our observation of the tumor samples of the sympathetic nervous system where VDR expression was observed in mature components, such as ganglion cells, is consistent with previous data in the literature. An immunofluorescent immunohistochemistry experiment was conducted on rat satellite glial cells, which are glial cells that cover the surface of neuron cell bodies in the ganglia of the peripheral nervous system. They discovered that satellite glial cell neurons could respond to vitamin D and were capable of both active vitamin D biosynthesis and following calcitriol degradation (164). The results and the phenomenon known from literature that vitamin D as a transcription factor plays a role in maturation raise the effect of vitamin D via VDR on the development of the peripheral nerve's glial system, so it may be possible that, like the healthy peripheral sympathetic nervous system, cells within the tumor mature that have VDR.

Neuroblastoma cell lines (e.g., SH-SY5Y, SK-N-SH) are often used in in vitro procedures to study the development of the dopaminergic neurons. It serves as a model of neurodegenerative disorders since the cells can be converted to various types of functional neurons. Secondarily, those information about the behavior of neuroblastoma cells can be obtained. In a paper which was a previous work of an above-mentioned research team, ontogeny of dopaminergic systems was investigated. Neuroblastoma (SH-SY5Y) cells were engineered to overexpress VDR in order to observe the biological processes by which the vitamin D hormone, via its receptor VDR, modulates DA production and turnover. This in vitro neuronal model identified the role of vitamin D in DA neuronal development and maturation, demonstrating that vitamin D via VDR is directly involved in regulating the expression of dopaminergic-associated genes (165). A respected study of the human neuroblastoma cell line identified a signaling pathway through which VDR exerts its antiproliferative effect. Ceramide kinase/Ceramide - 1 - phosphate (CerK/C1P) axis is an essential effector of vitamin D/VDR and its structural analogues in the

regulation of human neuroblastoma cell proliferation, as demonstrated by the authors of this study. Knowledge of the molecular mechanisms underlying the anti-proliferative action of the hormone and its analogues, involving the CerK/C1P axis and the so called VDR/COUP-TFI/HDACs complex, could also aid in the identification of novel disease-specific biomarkers in vitamin D-deficient patients (166).

In the NBL tumor samples we examined, VDR expression was detected in the mature components. This suggests that expression may have been involved in facilitating maturation in those components. However, following this logic, further maturation in immature components is presumably not expected under vitamin D supplementation, as they do not have the receptors to achieve this effect. Overall, vitamin D supplementation is nevertheless recommended for NBL patients, as it has also been shown to contribute to this degree of maturation, which, although modest, is a factor in reducing tumor aggressiveness.

Our above-mentioned results and the data of the current literature led us to consider that it might be reasonable to identify groups of patients who would benefit from VDR protein expression patterning. Besides that, VDR protein expression profile, with our easily and inexpensively available method could be used as a prognostic biomarker, and it would highlight patient groups in whom personalized, elevated doses of vitamin D and/or its analogues could be part of adjunctive therapy through their anti-tumor effect via VDR.

5.3. The current state and direction of the literature - VDR as biomarker

The literature on vitamin D and its antitumor effects via VDR is endless. Initially, decades ago, epidemiological studies drew attention to this possible link, the possible anti-tumor effect of vitamin D. These were followed by experiments to investigate the VDR pattern, the role of the VDR as a transcription factor, and the identification of signaling pathways that promote maturation and inhibit proliferation. Now the literature has come to investigate the polymorphism of the VDR gene as a prognostic factor. All of these play an equivalently important role in the emerging use of VDR status as a prognostic and therapeutic biomarker in malignancies.

5.3.1. An overview of epidemiological findings

A recent assessment of 20 meta-analyses of observational studies on cancer outcomes concluded that circulating vitamin D concentrations are uncertain to be associated with cancer incidence (167). Another meta-analysis found no association between vitamin D supplementation at the dose used in the research and cancer incidence. In contrast to cancer incidence, vitamin D supplementation was statistically associated with a 13% reduction in cancer mortality. In addition, in contrast to for cancer incidence, a consistent inverse relationship was observed in all studies included in the meta-analysis on cancer mortality (51). Based on the findings of another meta-analysis, initiatives to attain vitamin D levels between 20 and 50 ng/ml may contribute to a reduction in cancer mortality (168). In a meta-analysis of ten randomized controlled trials which all together pooled in 81362 participants the results confirm the beneficial effect of vitamin D supplementation on decreasing cancer-related mortality, as opposed to cancer incidence. Importantly, supplementation with vitamin D will be more effective and merit recognition as a prospective intervention in the subpopulation with no history of cancer, extra use of vitamin D, or calcium supplementation. Based on the suggestion of the authors, the clinical implication of these findings could be that intake of vitamin D can be recommended for cancer patients in current medical practice in order to provide the potential for the future development of mortality-reduction strategies (1).

In the epidemiological phase of our studies, we measured significantly lower serum vitamin D levels in the tumor patient group than in the control population in our retrospective study, which is in contrast with the current literature data summarized above. One reason for this may be the statistical difference due to the small number of elements. On the other hand, reverse causality, such as depressed general condition, fatigue, restricted food intake, cachexia and the resulting lower per oral vitamin D intake and UVB exposure. Indeed, there was no significant difference in the odds of a favorable prognosis for increased serum vitamin D levels by vitamin D supplementation in parallel with oncotherapy. However, in a later phase of our studies, we found an association between major VDR expression and a more favorable prognosis in the pediatric solid tumors we examined. Although clarification of this assumption merits additional research, it attracted our attention to the possibility that only in the presence of the appropriate target receptor will vitamin D presumably perform its antitumor effect.

5.3.2. Role of VDR in tumorigenesis, potential therapeutic target

In a recent study, the VDR expression pattern of 156 ovarian cancer samples was examined with immunohistochemistry. A significant cytoplasmic VDR expression has been described, one that correlates with clinical and pathological data. In addition, cytoplasmic VDR and clinical and pathological parameters were independent prognostic variables. Interestingly, in this patient group, high levels of cytoplasmic VDR expression were associated with decreased survival. The authors explained this phenomenon by the fact that VDR exerts its molecular effect via two distinct mechanisms. While the classic nuclear pathway involves genes with VDRE-containing promoters and sequentially regulates gene expression, the non-nuclear VDR-mediated pathway follows different mechanisms. VDR interacts with a cellular proto-oncogene, non-receptor tyrosine kinase, so called c-Src in the plasma membrane, activating a proto-oncogene serine/threonine-protein kinase, so called c-RAF and the mitogen-activated protein kinase (MEK1/2)/ extracellular signal-regulated kinases (ERK1/2) pathway. The ERK pathway is one of the primaries signaling cascades of the mitogen-activated protein kinases (MAPK) signaling pathway, which is essential for cancerogenesis, including cell proliferation, differentiation, migration, apoptosis, and chemoresistance, specifically, preclinical and early clinical studies demonstrate the importance of the ERK/MAPK pathway in ovarian cancer. In addition, based on previous studies, high MAPK activity in malignancies expressing cytoplasmic VDR was linked to increased cell growth and a poorer prognosis, in contrast, MAPK inhibition induced VDR nuclear migration and decreased in vitro cell viability. Overall, the results suggested cytoplasmic VDR as a possible predictive biomarker that could differentiate between tumors that are sensitive to MAPK inhibitors and those that are not in case of ovarian cancer patients. Thus, cytoplasmatic VDR could be utilized to determine which patients would benefit from a MAPK inhibitor treatment (169).

These findings raise the possibility that the localization of VDR within the cell may have a prognostic value, determining the signaling pathway and, along with it, the anti-tumor or tumorigenic effect. However, it is also known from the literature that VDR, located in the cytoplasm, after binding its ligand (vitamin D), forms a complex with the RXR and translocates to the nucleus, where it is responsible for antitumor effects, and thus the localization of the receptor alone is not sufficient to predict subsequent effects. In our

samples, we observed diverse VDR receptor expression, with VDR mostly detected in the cytoplasm.

Controversially, a systematic review and meta-analysis, based on the data of 2503 patients studied the relationship between VDR protein expression level investigated by immunohistochemistry and breast cancer improved prognosis. The results revealed that breast cancer patients with major total VDR expression in the nucleus and cytoplasm had a higher overall survival. It is expected that VDR expression will become a standard immunohistochemical examination item in breast cancer pathology diagnosis as part of a more accurate prognosis assessment, and based on that, an individualized vitamin D supplementation is reasonable (147).

Consistent with this, we have also been able to associate major VDR expression with a favorable prognosis.

Latest publications parallel to protein expression studies, research on VDR polymorphisms has risen to the forefront to ascertain the VDR profile of tumors as a possible new biomarker.

In the first study to investigate the association among VDR expression of human melanoma cells in excised patient tissues and VDR polymorphisms, researchers discovered a correlation between the two. Melanoma cells showed VDR expression predominantly in the cytoplasm, with limited tumors expressed VDR positivity in the nucleus. Data analyzed regarding the immunohistochemical detection of cytoplasmic VDR. When the cytoplasmic VDR links with the vitamin D ligand and an adequate coreceptor protein (RXR), it translocated to the nucleus and, by recruiting coactivators and corepressors, modulates the transcription of target genes. Therefore, the absence or down-regulation of VDR expression may had an effect on vitamin D resistance in melanoma and could influence the impact of vitamin D supplementation on melanoma patients. Four VDR polymorphisms were analyzed in this investigation; individual single-nucleotide polymorphisms (SNPs) of so called FokI, BsmI, and TaqI did not correlate with VDR expression in melanoma; only the so called ApaI genotype was associated with VDR expression. These results indicated that the identification of VDR expression in melanoma tissues and/or the determination of VDR genotype transmission can be utilized as a personalized tool for precision medicine when evaluating melanoma patients (170).

Numerous studies have investigated the relevance of VDR gene restriction fragment length polymorphisms to various types of cancer. A systematic review of the literature identified 176 independent studies aiming to determine the significance of additional VDR polymorphisms (BsmI, TaqI, FokI, ApaI, and Cdx2) for specific malignancies, with ethnicity as a key factor for heterogeneity. Prostate (Fok1, Bsm1, Taq1, Apa1, Cdx2), breast (Fok1, Bsm1, Taq1, Apa1, CdX2), colorectal (Fok1, Bsm1, Taq1, Apa1), and cutaneous cancer (Fok1, Bsm1, Taq1) have been associated with VDR polymorphisms (171).

A meta-analysis assessed the relationships between four VDR polymorphisms (BsmI, TaqI, FokI, ApaI*)* and cancer risk, a total of 126 eligible studies were included in the collective analysis. The VDR BsmI polymorphism was a risk factor for cancer susceptibility, particularly for colorectal cancer and cutaneous cancer, in the 'b' allele-carrying Caucasian population. In case of VDR TaqI polymorphism, carriers of the 't' allele were at higher risks of breast cancer, oral cancer, and basal cell cancer, but a lower risk of prostate cancer and cutaneous cancer. Carriers of the 'f' allele of the VDR FokI polymorphism have been identified to have increased risks for ovarian cancer and cutaneous cancer but a decreased risk for glioma. The VDR ApaI polymorphism was a risk factor for basal cell carcinoma in Asians who carried the 'a' allele (172).

A systematic review also included articles that investigated and defined the relationship between VDR polymorphisms and cancer risk and incidence. Multiple studies indicate that VDR polymorphism (BsmI, TaqI, FokI, ApaI) plays a crucial role in tumorigenesis of various cancer types by influencing vitamin D metabolism and the cellular response to vitamin D. It is also evident from the studies that the relationship between VDR polymorphism and tumorigenesis varies with age, gender, race, and ethnicity. This review also contained research which were conducted among pediatric oncology patients; the case-control studies indicated that VDR polymorphism (FokI) was associated with a lower risk of pediatric solid tumors, had no association with Hodgkin's lymphoma, and may have influenced bone mineral density, patient height, and overall survival (173).

5.3.3. Literature of vitamin D, VDR and childhood cancer

The literature examining the association of vitamin D deficiency, supplementation, VDR-expression and gene polymorphisms with childhood solid tumors remains limited.

There is only one systematic evaluation in the current literature of international evidence regarding the prevalence of vitamin D deficiency in pediatric cancer patients and its potential causes. Since only 19 studies could have been identified with predominantly small sample sizes and highly variable quality, there is currently insufficient evidence to determine the prevalence of vitamin D deficiency and insufficiency in the pediatric oncology population. Moreover, due to the considerable diversity of the investigated variables and the limited number of reported results in the majority of studies, the authors were only able to conduct a meta-analysis of studies analyzing correlations between age and vitamin D status. Nonetheless, the systematic review raises the possible scenario of a high prevalence of vitamin D deficiency and insufficiency in older adolescents with cancer, which could deteriorate during treatment and persist after therapy has concluded (174).

In a single-center study involved 51 patients, comparing the vitamin D levels of children with cancer and controls from a healthy population, a higher incidence of vitamin D insufficiency and a significantly lower mean vitamin D value were discovered in cancer children compared to controls. It was additionally found that children with cancer older than 6 years had a higher risk of developing vitamin D deficiency, as did children with hematological malignancies. In conclusion, the study revealed an increased prevalence of vitamin D deficiency in children with cancer; consequently, routine measurement of vitamin D levels in these children, followed by supplementation was recommended (175). Consistent with these results, we found similar results in our own patient population; serum vitamin D levels at diagnosis were significantly lower than in the control population. Our results also support the suggestion that vitamin D supplementation is reasonable in children.

Two tertiary referral institutions participated in an intriguing retrospective cross-sectional study of pediatric patients with adrenocortical tumors (ACT). In this study, 108 pediatric patients' ACT samples were evaluated for clinicopathological characteristics, VDR mRNA (qPCR) and protein (immunohistochemistry) expression, and VDR-wide methylation. Particularly the carcinomas, the majority of pediatric adrenocortical tumors (pACT) lacked nuclear VDR expression and had decreased mRNA levels. Patients with advanced disease and decreased disease-free and overall survival were associated with tumors with high VDR methylation levels. Based on the results, VDR hypermethylation

and underexpression could serve as predictive and prognostic biomarkers for pACT (176).

In contrast with the above mentioned data investigated on adult cancer patients, in a research of 95 pediatric patients with Hodgkin's lymphoma (HL), no association was observed between HL development and the VDR polymorphisms (FokI, BmI, TaqI, Apa1, and Cdx2) (177).

In a pilot study conducted at a single institution, the effect of selected SNPs in the VDR region on prediagnostic levels of 25(OH)D3 and 1,25(OH)2D3 (vitamin D) and overall survival in a cohort of pediatric solid cancer patients was evaluated. Regardless of the underlying disease, multiple significant associations between the alleles of the investigated polymorphisms and pediatric solid cancer as well as the effect of vitamin D on overall survival were observed (178).

Overall, there are still few data available on the childhood aspect of the topic, and data from a small population of children is provided. Epidemiological studies in children with childhood cancer are consistent, and our data correlate with this; vitamin D deficiency is more common in children with tumors, suggesting the need for vitamin D supplementation. This may be due to a variety of reasons, with few studies so far addressing VDR expression and gene polymorphism.

To the best of our knowledge, our study is the first to investigate VDR expression patterns in solid tumor samples from multiple pediatric entities by immunohistochemistry. Consequently, there is no previous data that examines the relationship between VDR expression and prognosis in such a large number of pediatric patients, and the differences between tumor types. Based on our results, namely that there is a significant correlation between low VDR expression and unfavorable prognosis, and that tumors with known aggressive behavior have lower VDR expression, we propose a personalized, accurate pathological diagnosis including the VDR expression profile. In particular, both the performance of the test and the use of vitamin D and its analogues are simple and inexpensive methods.

5.4. Strength and limitations

The study we conducted is the first one that examines VDR expression patterns in solid tumor samples from multiple pediatric entities using immunohistochemistry.

The strength of the study is that it examines solid tumor specimens from childhood malignancies. On one hand, it adds to the poor childhood literature; on the other hand, it is one of the few studies that has been done on excised tumor samples, not cell lines.

An outstanding strength of the study is the large population of elements examined in both the epidemiological and immunohistochemical phases.

A limitation of our study is the retrospective nature of the epidemiological phase. We suggest that supplying vitamin D is reasonable and a prospective study of vitamin D in pediatric patients with cancer is recommended.

Throughout the extensive duration of the study, one case of acute myeloid leukemia remained in the cohort, which although not a solid tumor, it did not have a statistically significant impact on the overall results or clinical significance.

Current literature suggests that some polymorphisms in the VDR gene affect the prognosis of certain tumor diseases. Our study is limited to the expression of the VDR protein by immunohistochemistry and does not alone determine with complete certainty the signaling pathway of vitamin D in the cell (transcription factor in the nucleus vs other signaling pathways in the cytoplasm). In the future, it is proposed to complement the VDR profile studies with gene polymorphism studies and associated prognostic factor value analyses.

Although overall a large number of data was involved in the immunohistochemical study, in some cases a small subset of tumor types was created, which could not be included in the statistical analysis, so an extension of this is planned for the future.

6. Conclusions

Our study enriches the existing limited literature on the association between vitamin D, VDR patterns and the prognosis of childhood solid tumors and is one of the first to investigate these questions in multiple childhood solid tumor entities simultaneously.

6.1. Assessment of serum vitamin D status

6.1.1. Serum vitamin D level of patients compared to average children population

The mean value of the initial vitamin D levels of children with cancer were significantly lower than the average population mean. Although there is conflicting literature on the association between cancer incidence and vitamin D deficiency in adult cancers, and insufficient data is available in the childhood aspect, overall preventive vitamin D supplementation in the pediatric population is recommended.

6.1.2. Initial vitamin D status and prognosis in children with solid tumors

Our result indicated that higher initial serum vitamin D levels were associated with a favorable prognosis and patients in the favorable prognostic group had a significantly higher initial vitamin D levels. The extensive literature and our data agree on the argument for vitamin D supplementation in the currently healthy child population. Based on the most recent literature, daily doses of 400–1000 IU recommended, and for children aged 0–18 years, with daily doses of 600–1000 IU.

6.2. Assessment of VDR pattern of solid tumors with immunohistochemistry

6.2.1. VDR expression profile and prognosis

Higher VDR expression in childhood solid tumor cells is associated with a favorable prognosis, which raises the potential role of the VDR profile as a prognostic biomarker.

6.2.2. Difference in VDR patterns in different tumor types, pathological observation

VDR expression differed significantly by tumor type. Although the number of elements is small for some entities, further investigation of these may be useful. For Wilms' tumor

and hepatoblastoma, VDR profiling with immunohistochemistry is recommended as an adjunct to histopathology. In case of neuroblastoma, it would be beneficial to investigate the role of vitamin D analogues in further maturation through the presence of VDR.

6.3. Final conclusion

All in all, VDR profiling of individual pediatric solid tumors may have a potential prognostic biomarker role. On this basis, it may be worthwhile to identify groups of patients for whom its incorporation into a personalized, accurate histological diagnosis may also act as a therapeutic biomarker through the maturation-promoting and proliferation-inhibiting effects of vitamin D and analogues through VDR.

6.4. Implication for practice and research

In the future, our work may contribute to complementing the histological diagnostic methodology with this new prognostic and therapeutic biomarker, the VDR expression profiling, in certain priority patient groups.

In the future, a tumor specific VDR expression profiling study on a larger number of subgroups of elements would be advisable. Furthermore, based on the literature trends, a study investigating VDR gene polymorphisms will be explored to clarify the prognostic biomarker association of VDR polymorphisms in specific tumor types.

6.5. New statements

1. This study is the first in Hungary to investigate subclinical vitamin D deficiency as a potential risk factor for childhood cancers and to investigate VDR expression patterns in solid tumor samples from multiple pediatric entities by immunohistochemistry.
2. The mean value of initial vitamin D levels of children with cancer were significantly lower than the mean of the average children population.
3. Higher initial serum vitamin D levels are associated with a favorable prognosis.
4. Elevated VDR expression in childhood solid tumor cells is correlated with a favorable prognosis, suggesting that the VDR profile may serve as a potential prognostic biomarker.
5. Previously unreported dot-like VDR expression has been detected in ependymoma samples.

7. Summary

The literature on the potential pathomechanisms and precise etiology of childhood tumor development is expanding, and simultaneously, diagnostic, and therapeutic biomarkers are being discovered. In parallel, countless studies have been published in the literature on the anti-tumor effects of vitamin D via VDR, but the childhood aspect of these is very limited. Due to these facts, we decided to investigate the role of vitamin D and VDR receptor patterns in pediatric solid tumors as potential prognostic biomarkers.

Firstly, the correlation between serum vitamin D levels at diagnosis in children with solid tumors and children without tumors was evaluated; the difference was found to be statistically significant (p=1.34E–08). Secondly, we investigated any potential correlations between vitamin D levels and cancer patients' prognoses and found that vitamin D values measured at the initial and the prognostic categories were dependent variables (p=0.016) with a high OR (51.33).
In the next phase of the study, the VDR expression pattern of pediatric tumor samples were investigated by immunohistochemistry. A significant correlation between the minor VDR expression pattern of tumor samples and unfavorable prognosis (p=0.0061, OR=14.74) was described. Afterwards, we looked for differences and characteristics of the VDR pattern in different tumor types, thus examining the role of the VDR expression pattern as a possible biomarker in childhood solid tumors. The rate of VDR expression differed significantly between various tumor types ($p<0.0001$).

To the best of our knowledge, our study is the first to investigate VDR expression patterns in solid tumor samples from multiple pediatric entities by immunohistochemistry. In conclusion, VDR profiling of individual, carefully selected pediatric solid tumors may have a potential prognostic biomarker role. It could be worthwhile to identify patient groups who could benefit from the administration of personalized, elevated doses of vitamin D and/or its analogues. In this manner, vitamin D could be part of adjunctive therapy through their anti-tumor effect via VDR, in which way VDR may also act as a therapeutic biomarker.

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9. Bibliography

9.1. Original articles written in English that serve as the basis for the thesis

Examining the Vitamin D Status of Children with Solid Tumors
Orsolya Juhász, Zsuzsanna Jakab, András Szabó, Miklós Garami
Journal of the American College of Nutrition (2019)

Immunohistochemical Detection of the Presence of Vitamin D Receptor in Childhood Solid Tumors
Orsolya Juhász, Noémi Jákob, Hajnalka Rajnai, Marcell Imrei, Miklós Garami
Cancers - Pediatric Special Issue (2022)

9.2. Original articles written in Hungarian that serve as the basis for the thesis

D-vitamin-státusz vizsgálata szolid tumoros gyermekekben
Orsolya Juhász, Zoltán Erdélyi, Miklós Garami
Gyermekgyógyászat (2016)

D-vitamin jelentősége a gyermekonkológiai megbetegedésekben (etiológia, terápia)
Orsolya Juhász, Noémi Jákob, Hajnalka Rajnai, Marcell Imrei, Edit Brückner, Miklós Garami
Gyermekorvos továbbképzés (2022)

9.4. Publications in Hungarian not directly related to the theses

AVPR2 gén teljes deléciója okozta nephrogén diabetes insipidus – Esettanulmány
Orsolya Juhász, Tamás Bense, Kálmán Tory, Ágnes Sallai
Gyermekgyógyászat (2019)

Az I-es típusú neurofibromatosishoz társuló idegrendszeri daganatok diagnózisa és korszerű kezelése
Edit Brückner, Orsolya Juhász, Péter Hegedüs, Viktória Sági, Anita Pfeffer, György Fekete, Miklós Garami
Gyermekgyógyászati továbbképző szemle (2021)

A gyermekkori mellékvesekéreg-tumorok ellátása: út a diagnózistól a gyógyulásig
Anita Pfeffer, Nikolett Beniczky, Orsolya Juhász, Dóra Török, Miklós Garami
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Klinikai ajánlások az NF I. betegek kezeléséhez: új terápiás lehetőségek
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