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Title: **Vitamin D in haematological disorders and malignancies**

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Running title: Vitamin D in haematological diseases

Abstract: Commonly known for its critical role in calcium homeostasis and bone mineralization, more recently vitamin D has been implicated in haematological cancer pathogenesis and shows promise as an anti-cancer therapy. Serum levels of $25(OH)D_3$, the precursor to the active form of vitamin D, calcitriol, are typically lower in patients with haematological disease compared to healthy individuals. This often correlates with worse disease outcome. Furthermore, diseased cells typically highly express the vitamin D receptor (VDR), which is required for many of the anti-cancer effects observed in multiple *in vivo* and *in vitro* cancer models. In abnormal haematological cells, vitamin D supplementation promotes apoptosis, induces differentiation, inhibits proliferation, sensitizes tumor cells to other anti-cancer therapies, and reduces the

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production of pro-inflammatory cytokines. Although the dosage of vitamin D required to achieve these effects may induce hypercalcemia in humans, analogs and combinatorial treatments have been developed to circumvent this side effect. Vitamin D and its analogs are well tolerated in clinical trials and thus further investigation into the use of these agents in the clinic is warranted. Here we review the current literature in this field.

Key words: vitamin D, leukemia, lymphoma, calcitriol, immune cells, vitamin D receptor, aplastic anemia, 25(OH)D3

I. Introduction

Vitamin D is a steroid that has long been implicated in calcium homeostasis and bone mineralization (1, 2). Vitamin D can be obtained through supplements, dietary intake, and ultraviolet radiation (UVR) exposure (3). UVR catalyzes the conversion of 7-dehydrocholesterol to pre-vitamin D_3 in the skin, which further undergoes modification to vitamin D_3 (4). This form of vitamin D can also be obtained in certain foods in the western diet but to a lesser extent (4). Vitamin D_3 is transported through the bloodstream to the liver through interaction with the vitamin D binding protein (4). 25(OH)D_3, the circulating form of vitamin D, is synthesized in the liver through hydroxylation by CYP2R1 (4). From there, 25(OH)D_3 travels to the kidney where CYP27B1 converts it to 1,25$(OH)_2D_3$, the active form of vitamin D, referred to as calcitriol (4). Certain immune cells also possess active CYP27B1, allowing them to convert the circulating 25(OH)D_3 to active calcitriol (3, 5). Once in its active form, calcitriol can exert its effects on various tissues and biological processes. Typically, calcitriol will bind the vitamin D receptor (VDR), to induce a conformational change in the protein, which permits binding to the retinoid X receptor (RXR) (6). The VDR-RXR heterodimer then acts as a transcription factor by binding vitamin D response elements (VDRE) in DNA, causing transcription or repression of target genes (6).

Aside from its classical roles in healthy individuals, vitamin D has been shown to exhibit numerous anti-cancer and immune-modulating properties (1, 4, 6). In multiple cancers and autoimmune diseases, calcitriol inhibits proliferation, induces apoptosis, decreases angiogenesis, and sensitizes cells to chemotherapy (6). Thus, considerable efforts have been dedicated to understanding the mechanism of vitamin D action and its full range of effects on tumor cells. Given that chemotherapy and other current anti-cancer agents have serious undesirable side effects, there is a push to investigate vitamin D use in the clinic since its main toxicity is hypercalcemia, which can be circumvented by using vitamin D analogs (6). Calcitriol and its analogs have been explored in Phase I and Phase II clinical trials for multiple cancers including prostate cancer, pancreatic cancer, and hepatocellular carcinoma (6).

Vitamin D actions on solid tumor cancers have been extensively characterized and reviewed (1, 4, 6). However, recently there has be a surge in vitamin D related studies on haematological disorders, both autoimmune and cancers, as there have been numerous *in vitro* and clinical studies with vitamin D. Clinical studies demonstrate that vitamin D analogs are well tolerated in patients with myelodysplastic syndrome (MDS) (7, 8), supporting their use in the clinic. Moreover, 11/19 MDS patients experienced a clinical response after treatment with vitamin D analogs or calcitriol (8). Thus, we will outline and summarize the current literature in this field in addition to identifying areas for future research and clinical development.

II. Vitamin D Serum Levels

$25(OH)D_3$ levels in haematological disorders and malignancies

Serum $25(OH)D_3$ levels are used as an indirect measure of vitamin D levels in patients (9). Often serum levels are categorized as sufficient, insufficient, and deficient, with the exact levels required for each determined by the diagnostic laboratory (9). Thus, there is some uncertainty about what levels of vitamin D are needed to maintain good health, although the Endocrine Society defines vitamin D deficiency as $25(OH)D_3$ serum levels below 20 ng/mL and

insufficiency as 25(OH)D_3 levels of 21-29 ng/mL (10). Vitamin D deficiency is relatively common in the healthy population with deficiency occurring at a higher rate in Native Americans, African Americans, and women with a body mass index greater than 30 according to one study in the United States (9). Interestingly, 72.8% and 19.6% of African Americans and Native Americans, respectively, had insufficient levels of 25(OH)D_3, while only 11.3% of healthy European-Americans were 25(OH)D_3 deficient (9).

Even with vitamin D deficiency occurring in the general healthy population, lower 25(OH)D_3 levels are associated with development of certain cancers and severity of cancer prognosis. Overall in haematological cancers, lower serum 25(OH)D_3 is associated with worse disease, higher malignant cell burden, and poor response to therapy based on a cohort of 105 patients with various leukemias, of which 72% were vitamin D deficient or insufficient (11). A meta-analysis of seven published studies encompassing 2,643 patients with haematological cancers showed that low serum 25(OH)D_3 levels were significantly correlated with shortened overall survival (OS) and relapse free survival (RFS) (12). Thus, the relationship between circulating 25(OH)D_3 levels and disease state in several hematological cancers and disorders is being investigated and will be discussed below.

The Philadelphia chromosome, which results from a reciprocal translocation of chromosomes 9 and 22, is characteristic of chronic myeloid leukemia (CML), and is also seen in some patients with acute lymphoblastic leukemia (ALL) (13). Sufficient 25(OH)D_3 levels were positively correlated with molecular response in Philadelphia chromosome positive leukemias (11). There was an observed protective effect of higher 25(OH)D_3 serum levels against development of chronic lymphocytic leukemia (CLL), although no correlation was detected between 25(OH)D_3 status and overall risk of lymphoid cancer development in this study (14). Recently Lee et al. determined the prevalence of serum 25(OH)D_3 deficiency in 97 newly diagnosed intensively treated AML patients (15). This study revealed that 65% of these patients did not have adequate circulating vitamin D levels, with 35% and 30% categorized as

insufficient and deficient, respectively (15). Furthermore, those patients that had serum levels below the accepted normal range had an increased association with worse RFS (15). This finding is supplemented by a different study that demonstrated a significant decrease in circulating 25(OH)D_3 levels in acute leukemia patients experiencing relapse compared to those in complete remission (11). Moreover, low vitamin D levels were associated with worse overall survival after azacitidine treatment in AML and more episodes of febrile neutropenia and hospitalization (16). Similarly, 30.5% of newly diagnosed CLL individuals were found to be 25(OH)D_3 insufficient, and there was a significant association between 25(OH)D_3 insufficiency and shortened time-to-treatment (TTT) and overall survival (17).

A study of 983 newly diagnosed non-Hodgkin's lymphoma (NHL) patients, comprised of NHL subtypes of diffuse large B-cell lymphoma (DLBCL) and T-cell lymphoma, found that 44% of these patients had insufficient circulating 25(OH)D_3 levels (18). These low 25(OH)D_3 serum levels were associated with shorter event-free survival (EFS) and OS in DLBCL and T-cell lymphoma, while there was no correlation with 25(OH)D_3 insufficiency and EFS in the other NHL subtypes (18). Vitamin D deficiency is also associated with shorter survival in follicular lymphoma, another type of NHL (19). When DLBCL patients were treated with rituximab, patients with vitamin D deficiency had shorter EFS (20). Rituximab-mediated cellular cytotoxicity was improved in vitamin D deficient patients supplemented with vitamin D (20). This suggests that vitamin D supplementation may improve patient outcomes in the clinic and should be further investigated.

Cutaneous T-cell leukemia (CTCL) patients with either Sézary syndrome (SS) or mycosis fungoides (MF) also have low 25(OH)D_3 circulating levels, defined as less than sufficient levels, comparable to the decreased levels in patients with other cancers (21, 22). Even when these patients were supplemented with vitamin D_2, there was no observed difference in response to treatment (21).

Serum 25(OH)D_3 levels have been determined in non-cancerous haematological disorders. In immune thrombocytopenic purpura (ITP), lower serum levels were negatively correlated with platelet count (23). In the case of primary autoimmune hemolytic anemia (AIHA), those patients that underwent multiple treatments had lower serum 25(OH)D_3 levels compared to those patients that only underwent one line of therapy (23). Across several haematological disorders, including AIHA, ITP, Evan's syndrome, and chronic idiopathic neutropenia, serum 25(OH)D_3 levels were decreased compared to healthy donors (23).

Since the main source of vitamin D is UV-B exposure, several studies have been conducted to determine early life sun exposure and development of certain cancers, including NHL, which has limited known risk factors. One study investigated whether early sun exposure could play a role in developing NHL and found that there was a significantly decreased risk of developing NHL in individuals with increased sun exposure between13 and 21 years of age (24).

Although there are ample studies regarding vitamin D status in haematological malignancies and disorders, the majority of these studies are retrospective. These studies provide valuable correlative information regarding current vitamin D status and disease state, but do no allow for assessment of causality. Thus, carefully designed prospective studies are necessary to more accurately define the role of vitamin D status in haematological disease pathogenesis.

III. VDR

Expression

VDR is necessary for anti-cancer effects of vitamin D (4), and therefore, its levels may predict response to vitamin D treatment, prompting investigations to determine VDR levels for various cancers. VDR was found to be highly expressed in 80% of B-cell Hodgkin's lymphoma

(B-HL), but only 17.4% of B-cell non-Hodgkin's lymphoma (B-NHL) (25). Of the subtypes of HL and B-NHL examined, nodular lymphocyte predominant Hodgkin's lymphoma (NSHL) and diffuse large B cell lymphomas (DLBCL) had the highest proportion of samples expressing VDR, respectively (25). B-CLL, CTCL with SS, and T-cell large granular lymphocytic leukemia (T-LGLL) cells were found to highly express VDR (22, 26, 27). On the contrary, VDR and CYP27B1 mRNA expression was decreased in tumor associated macrophages in Burkitt's lymphoma (BL) (28). In a small sampling of only four ALL patients, none had detectable levels of VDR (29).

Recent studies explored the role of vitamin D in the pathogenesis of acquired aplastic anemia (AA), an autoimmune cytopenia that can be associated with lymphoproliferative neoplasms, including LGLL (30). Surprisingly, untreated AA patients did not have altered circulating $25(OH)D_3$ serum levels compared to healthy donors, but had significantly decreased VDR mRNA expression levels (31). Thus, VDR downregulation is hypothesized to contribute to the hyperimmune status of AA (31). On the contrary, in other autoimmune cytopenias and haematological disorders, specifically AIHA, ITP, Evan's and CIN, VDR expression levels were higher in the disease state compared to normal donors (23).

Overall, the majority of cells involved in haematological diseases highly express VDR. This suggests that these cells may be responsive to vitamin D treatment, which may lead to anti-tumor effects. For those diseases that exhibit decreased VDR levels, the ability to restore VDR levels should be investigated in an effort to increase sensitivity of these cells to vitamin D treatment.

SNPs

Single nuclear polymorphisms (SNPs) have been found in VDR are often associated with different disease prognosis. A summary of the known SNPs is illustrated in Figure 1, created using the UCSC Genome Browser (32). One VDR SNP, rs10783219, was associated with a

significantly lower complete remission rate, shorter RFS, and decreased OS in AML (15). For NHL, SNPs with significant disease association have been discovered in VDR including rs3819545, rs2239186, and rs886441 (24). There was also a CYP24A1 SNP, rs2762939, associated with NHL (24). Furthermore, these SNPs were associated with development of specific subtypes of NHL. The VDR SNPs rs3819545 and rs2239186 correlated with the indolent types of NHL: follicular lymphoma (FL), chronic lymphocytic leukemia (CLL), and small lymphocytic lymphoma (SLL) (24). Interestingly, there were no reported polymorphisms associated with RXR or CYP27B1 for NHL (24). Although increased sun exposure was shown to decrease the risk of developing NHL, this trend was only true for those NHL individuals that were homozygous for the wild-type VDR rs4516035, a VDR germline variation associated with development of DLBCL (24). One case-control study found that VDR SNP rs1544410 is at a higher frequency in AA patients compared to healthy controls (33). Furthermore, VDR SNPs were associated with different severities of AA, with rs1544410 and rs7975232 correlating with nonsevere AA and rs2228570 coinciding with severe AA (33). Given the multitude of studies, VDR SNPs may serve as predictive indicator of disease prognosis and risk of haematological disease development. Additional studies are required to better understand the biological consequences of these SNPs and correlate the various VDR SNPs with more haematological diseases.

IV. Effects on Healthy Immune Cells

Since many haematological disorders affect peripheral blood mononucleocytes (PBMCs), which include T-cells, B-cells, natural killer (NK) cells, monocytes, and dendritic cells, many studies have investigated the effects of vitamin D on these healthy cells as well as those involved in cancers and autoimmune diseases. For example, vitamin D and its analogs inhibited proliferation and TNF-α production in PMBC from healthy donors and Crohn's patients (34, 35) as well as blocked NF-κB activation in PBMCs from Crohn's patients (35). Vitamin D exerts a

variety effects on specific PBMC subtypes as outlined below.

T-cells

T-cells are direct targets of vitamin D in addition to being indirectly modulated by vitamin D through dendritic cells and B-cells as reviewed in later sections (36). Activated T-cells have high levels of VDR while naïve T-cells have very low or undetectable levels of VDR (36-38). T-cell activation increases levels of VDR and CYP27B1, peaking at 48 hours post-activation, contrary to CYP24A1 which is not detectable at the time of isolation or post-activation (36). VDR levels further increase with calcitriol (36) or $25(OH)D_3$ treatment (38). Interestingly, when treated with calcitriol repeatedly for a longer time course, T-cells experience a modest increase in CYP24A1 (36). As CYP24A1 is responsible for the catabolism of calcitriol to $1,24,25(OH)_3D_3$, which has a significantly lower binding affinity for VDR (6), T-cells are able to convert 25(OH)D3 to its active form, calcitriol, and are also able to then break down calcitriol into a less active form.

Various subsets of T-cells respond differently to calcitriol treatment. One study examined the response of CD45RO+ and CD45RA+ T-cells to calcitriol. Proliferation of CD45RO+ T-cells was reduced by calcitriol treatment, while CD45RA+ T-cell proliferation was unchanged (39). In both subsets of T-cells, IFN-γ and IL-2 production was decreased (39), matching several accounts of cytokine reduction in the literature. Additional studies found that IFN-γ and IL-10 production in T-cells were significantly inhibited when treated with calcitriol at the time of activation or when VDR levels were maximal two days after activation (36). However, in long term culture, calcitriol was required every two days to substantially maintain reduced IFN-γ and IL-10 production (36). Calcitriol inhibited IL-2 induced IFN-γ production in human T-cells (40), with IL-2 required for inhibition of IFN-γ and IL-10 production in mice (41). VDR itself has been implicated in controlling proliferation and IFN-γ and IL-17 production in CD8+ T-cells (42). When VDR is knocked out, CD8+ T-cells exhibit increased proliferation and production of IFN-γ and

IL-17 compared to wildtype CD8+T-cells (42).

The direct and indirect effects of calcitriol on CD4+ T-cells have been extensively studied. Activated, but not naïve, CD4+ T-cells are able to convert $25(OH)D_3$ to calcitriol (38). Furthermore, calcitriol was found to stabilize VDR, prevent its degradation and extend its half-life in CD4+ T-cell (38). When CD4+ CD25- T-cells were stimulated and treated with calcitriol, IFN-γ, IL-17, and IL-21 pro-inflammatory cytokine production decreased while expression of CTLA-4 and FoxP3 and production of IL-10 increased (43). Proliferation of CD4+ T-cells was not significantly altered by calcitriol treatment (43). However, when these CD4+ T-cells were treated with both calcitriol and IL-2, the cells mimicked adaptive regulatory T-cells in their ability to suppress the proliferation of resting CD4+ T cells (43).

B-cells

VDR is found in activated B-cells but not detectable in naïve B-cells (37), which appears to be a common theme among multiple cell types. Calcitriol treatment inhibits proliferation (29, 44) and promotes apoptosis of activated B-cells (29). For example, calcitriol and vitamin D analogs inhibited the proliferation of IL-2/*Staphylococcus aureus* stimulated B-cells (45). Furthermore, calcitriol inhibits the production of plasma cells and postswitch memory B-cells (44) as well as the production of immunoglobulin, potentially by reducing IL-2 receptor levels (46). Although VDR and CYP24A1 are at low levels and undetectable, respectively, in freshly isolated B-cells, calcitriol increases both VDR and CY24A1 expression in B-cells regardless of their activation state (44). Defined intracellular changes have been documented when B-cells are treated with calcitriol, which include a decrease in NF-κB activation in naïve B-cells through impaired NF-κB p65 binding to the p105 promoter (47). While calcitriol can act on individual immune cells, it is evident that this can also modify how cells interact with each other. For example, calcitriol treated B-cells exhibited reduced co-stimulatory molecule expression, leading to a decrease in ability to activate T-cells as evidenced by a decrease in T-cell cytokine

production and proliferation (48).

Dendritic cells

Murine and human dendritic cells (DCs) have measurable levels of CYP27A1, CYP2R1, and CYP27B1, indicating that these cells can metabolize vitamin D (5). In addition, transcriptionally active VDR has been detected in these cells (5). Previous work has shown that calcitriol treatment of human and mouse DCs decreases T-cell proliferation, possibly by decreasing IFN-γ output by DCs (5). Calcitriol was also found to decrease maturation, activation, differentiation, and survival of DC cells, leading to a suppression of T-cell activation and proliferation (49). Taken together, calcitriol dampens the immune response through inhibition of the ability of DC to activate T-cells, preventing autoimmunity and hyper responsive T-cells.

NK cells

The effects of calcitriol treatment on NK cells, a type of cytotoxic lymphocyte, are currently unclear as contradictory findings have been reported. One study found that calcitriol acts on early NK progenitors to decrease NK cell development, proliferation, differentiation, and IFN-γ production of stem cell-derived NK cells, which created a smaller NK cell population that was largely immature with reduced function (50). Interestingly, mature NK cells were unaffected by these parameters (50). Cytotoxic capabilities were inhibited at the level of NK cell activation, while these cytotoxic functions were unaffected in NK cells that were in the effector phase, suggesting that calcitriol inhibits cytotoxic functions of NK cells during the activation stage only (51). IL-2 and IFN-γ production were deceased upon calcitriol treatment, while supplementation of exogenous IL-2 reversed this inhibition (51). On the other hand, another study found that calcitriol increases the cytotoxicity of NK cells through IFN-γ production, which enhanced killing capabilities of primary NK cells against leukemia and solid tumor cell lines (52). It was

determined that the mechanism for susceptibility to NK cell-mediated killing was due to downregulation of the microRNAs miR-302c and miR-520c within tumor cells, which increased the transcription of the NKG2D ligands, MICA, MICB, and ULBP2 (52). Upregulation of these NKG2D ligands serves as a danger signal to the immune system, targeting these tumor cells for destruction (52).

Macrophages

The primary role of macrophages in cancer progression is less clear as macrophages have the ability to be pro-tumorigenic and anti-tumorigenic, depending on their exerted functions (28). Despite their ability to promote metastasis, macrophages are able to exhibit antibody-dependent cellular cytotoxicity and participate in phagocytosis (28). Some tumor-associated macrophages (TAMs) are unable to execute these cytotoxic functions, leading to escape of cancerous cells. This process may be a result of vitamin D deficiency, which has been demonstrated in Burkitt's lymphoma (BL). Inflammatory M1 macrophages release cathelicidin, an antimicrobial peptide, which kills the B-cell lymphoma cells through targeting the BL cell mitochondria (28). However, this process is vitamin-D dependent and, interestingly, BL patients have fewer M1 and more M2 macrophages (28). These M2 macrophages are anti-inflammatory, exhibit reduced production of cathelicidin, and cannot kill BL cells (28). Although M2 macrophages exhibit altered vitamin D metabolism, when these macrophages were treated with calcitriol, cathelicidin production was induced and rituximab-mediated cytotoxicity was restored (28). This held true for treating M2 macrophages from patients *in vitro* and for treating 25(OH)D_3 deficient individuals with vitamin D (28).

IV. Vitamin D effects on malignant haematological cells

Vitamin D exerts numerous effects on malignant haematological cells (Figure 2). It has been well established that vitamin D induces differentiation in AML cells as well as apoptosis in

other leukemic and lymphoma cell lines. However, the supplementation dose required to achieve this unfortunately often induces hypercalcemia *in vivo* (53). Thus, significant efforts have been made to develop vitamin D analogs with increased specificity and potency; investigate combinatorial treatments that utilize vitamin D to enhance sensitivity to chemotherapy treatments; and utilize alternative vitamin D delivery methods that would circumvent the negative side effect yet inhibit proliferation and promote differentiation or apoptosis of cancerous cells.

Calcitriol & Analogs

Calcitriol and its noncalcemic analogue MC903 induce differentiation and inhibit proliferation in SU-DHL4 and SU-BUL5 B-cell lines representative of follicular NHL possessing the common 14;18 translocation (54). The concentrations required to demonstrate these effects *in vitro* were higher than the physiological range (54). Interestingly, there was still an observed clinical response to calcitriol in patients with follicular NHL, even at lower calcitriol doses than required for effects in the cell lines (54). This suggests that calcitriol acts on the CD4+ T-cells that are implicated in the induction of lymphoma cell proliferation rather than the tumor cells themselves (54). In another type of NHL, DLBCL, treatment of the cell line models with calcitriol and vitamin D analogs induced necrosis (45). Calcitriol also inhibits growth of malignant B-cell progenitors from acute lymphoblastic leukemia (ALL), while leaving viability and differentiation state unchanged (29).

EB1089, a calcitriol analog, has been well studied in solid tumor models and has been shown to induce apoptosis in a cell cycle independent mechanism in B-cell chronic lymphocytic leukemia (B-CLL) patient cells (26). Bcl-2 and Mcl-1 protein levels as well as extracellular signal-regulated kinases (ERK) activity were decreased in B-CLL apoptotic cells while p38 mitogen-activated protein (MAP) kinase and caspase-3 were activated in these apoptotic cells (26). B-CLL patient cells exhibited similar responses to EB1089 regardless of their current

treatment regime (26). This apoptosis was unique to the malignant cells, as they were significantly more sensitive to EB1089 treatment compared to B-cells from healthy donors (26). This finding demonstrates that the calcitriol-induced apoptosis is selective for the malignant cells, leaving the normal cells unharmed.

EB1089 negatively affects NCI-H929 myeloma cells cell growth and viability (55). Growth arrest was achieved through induction of G_1 phase cell cycle arrest, while apoptosis involved increased activity of caspase 3 protease and p38 MAP kinase (55). PARP protein degradation, p44 ERK activity inhibition, and decreased Bcl-2 levels were observed during apoptosis (55).

Calcitriol analogs with side chain modifications, PRI-1906 and PRI-1907, increased differentiation of blast cells from patients with AML compared to calcitriol (56). CD14 protein levels were increased upon analog or calcitriol treatment (56). Interestingly, these analogs had increased effectiveness in blasts from patients with mutated NPM1 or a normal karyotype, compared to patients with mutated FLT3 receptor, whose cells exhibited the least blast differentiation (56).

Vitamin D_2

Although many studies utilize calcitriol and its analogs, vitamin D_2, also known as ergocalciferol, is being studied in animal and cell models since ergocalciferol has less toxicity compared to vitamin D_3. Ergocalciferol was investigated in the HL-60 AML cell line (57). This vitamin D compound induced apoptosis through a reactive oxygen species (ROS) mechanism leading to a loss of mitochondrial membrane potential, production of ROS compounds, depletion of glutathione (GSH), release of cytochrome c, increase in pro-apoptotic proteins, decrease in anti-apoptotic proteins, and Fas receptor induction (57).

Synergism with anti-cancer therapies

Synergism of drug combinations is a principle that could be exploited in regards to vitamin D combinatorial treatment. Many studies have shown that combining a vitamin D analog with a cancer drug produces a powerful effect in vitro. The combination of calcitriol with azacitidine was tested in the AML cell lines HL-60 and MOLM13 (16). The combination significantly inhibited proliferation more than either drug alone (16). Another study combined glycogen synthase kinase 3 (GSK3) inhibitors with a low concentration of calcitriol and demonstrated that multiple AML cell lines, including HL-60, OCI-AML3 and Mono-mac3, were able to differentiate (58). This promotion of differentiation was greater than calcitriol alone or GSK3 inhibitors combined with an additional FDA-approved GSK3 inhibitor in the HL-60 cell line (58). When treated with GSK3 inhibitor, the majority of primary AML patient cells tested experience a sensitization of the cells to calcitriol-induced differentiation (58). In the AML cell lines, an increase in irreversible growth arrest in G_0-G_1 phase of the cell cycle and decreased expression of cyclin A were observed with the GSK3 inhibitor and calcitriol treatment (58). The role of GSK3 in this sensitization to calcitriol-induced differentiation was confirmed by utilizing GSK3B knockdown cells, which exhibited increased differentiation and VDR transcriptional activity compared to GSK3B wild-type cells (58). GSK inhibition in combination with calcitriol led to an induction of VDR serine-208 phosphorylation, a site that affects VDR transcriptional activity, and increased JNK activation, potentially elucidating the mechanism of action of these combined treatments (58). Furthermore, treatment of a mouse model of human AML with the combination of the GSK3 inhibitor and calcitriol prolonged animal survival (58). In addition to the GSK3 inhibitor and calcitriol synergistic effects, the effects of vitamin D_2 analogs in combination with plant polyphenol carnosic acid have been investigated. This combination inhibited G_1-S cell cycle transition and induced cell differentiation in multiple AML cell lines (59).

Combinational treatments have been explored for CTCL. Treatment of the MyLa cell line, a model of CTCL, with 25(OH)D_3 or calcitriol alone did not affect proliferation. However, a

combination treatment of 25(OH)D_3 or calcitriol and bexarotene, an RXR agonist, inhibited proliferation (22). The role of vitamin D sufficient and insufficient levels was demonstrated in this experiment, as 33 nM of 25(OH)D_3, representative of insufficient vitamin D, did not significantly increase apoptosis of the MyLa cell line and CTCL patient samples (22). A dose of 100 nM of 25(OH)D_3 was necessary to see effects, with greater apoptosis observed when bexarotene was added (22).

Alternative vitamin D delivery methods

An additional method to circumvent hypercalcemia and create a more specific cancer target is to alter the calcitriol delivery method. Calcitriol was packaged into a liposome that would deliver it to the HL-60 AML cell line or primary peripheral blood cancer cells AML patients (60). Proliferation was inhibited by 65% when treated with 48 nM of calcitriol using liposomal delivery (60). Liposomal delivery of the vitamin D stereoisomer analog, MC1288, achieved this inhibition at an one log lower dosage of 4.8 nM (60). Additionally, the effects were specific to AML cells as proliferation was not significantly altered in normal T-cell lines (60). Differentiation of HL-60 cells was induced as evidenced by increased CD14 expression and decreased CD33 expression (60)

Resistance to calcitriol treatment

Although calcitriol treatment shows promise in many leukemia and lymphoma cell lines, mouse models, and primary patient cells, there are cell lines that are resistant to the effects of calcitriol. For these malignancies, the mechanism behind this resistance has been studied in an effort to perturb this pathway and restore calcitriol sensitivity. KG1, an AML cell line, is resistant to the differentiating effects of calcitriol contrary to numerous observations of calcitriol sensitivity in other AML cell lines (61, 62). This cell line exhibited very low VDR gene expression due to degradation of VDR mRNA (61). Additionally, KG1 had constitutively active STAT1 and

increased levels of interferon stimulated genes (ISGs) resulting from a constitutively active fusion protein FOP2-FGR1 (61). KG1 exhibited restored sensitivity to calcitriol when FOP2-FGR1 was disrupted (61). VDR expression increased after FOP2-FGR1 was disrupted (61).

Calcitriol and the JAK-STAT pathway

Dysregulation of the Janus kinase (JAK)-signal transducers and activators of transcription (STAT) pathway is common in leukemias and lymphomas including large granular lymphocytic leukemia (LGLL) (63, 64), T-cell lymphomas (65), AML (61, 66), B-CLL (67), and T-cell acute lymphoblastic leukemia (T-ALL) (68). This aberrant activation can be due to an increase in JAK-STAT promoting cytokines, repression of JAK-STAT inhibitors, activating mutations in the JAKs or STATs themselves (69), or fusion proteins as observed in KG1 (61).

Inhibition of the JAK-STAT signaling pathway leads to apoptosis of several cancer cells, including LGLL (64). Thus, investigations into potential JAK-STAT pharmacological inhibitors are underway. Unfortunately, limited potency of STAT inhibitors and negative side effects currently prevent the clinical use of STAT inhibitors (70). Vitamin D may serve as a novel JAK-STAT inhibitor as vitamin D has been found to reduce STAT3 activation in the mouse model of experimental diabetes (71) and experimental allergic encephalomyelitis (EAE) (72) as well as human esophageal squamous cell carcinoma (SCC) cell lines (73). Furthermore, vitamin D decreases STAT1 activation and pro-inflammatory cytokine output in the mouse model of EAE, correlating with a lesser disease state and improved symptomology (72). In hepatocellular carcinoma this reduction in pro-inflammatory cytokines is hypothesized to occur in a p27(kip1) gene dependent manner (74).

Recently the effects of vitamin D on the JAK-STAT pathway in LGLL have been investigated. Although 30-40% of LGLL patients exhibit somatic activating mutations in STAT3 (63, 75), constitutive activation of STAT3 and STAT1 is also observed in patients without STAT3 mutation (64) and to date no activating mutations in STAT1 have been found (27). The reason

for the increased activation in non-mutated individuals may be due to aberrant cytokine signaling, particularly IL-6, and repression of JAK-STAT inhibitors, specifically suppressor of cytokine signaling 3 (SOCS3) (76). This suppression of SOCS and increase in IL-6 production is found in other cancers (77-79). We recently found that calcitriol increases VDR protein levels, reduces STAT1 and STAT3 activation, and decreases pro-inflammatory cytokine production, specifically IFN-γ production (27). Viability remained unaltered in both the T-LGLL cell line and primary patient cells, supporting a potential shift away from pro-inflammatory cells to a more anti-inflammatory phenotype (27).

Similarly, calcitriol alters pro-inflammatory cytokine production in PBMCs from AA patients. One study found that IFN-γ, TNF-α, IL-10, IL-17A, and TGF-β1 levels were elevated in AA PBMCs, but calcitriol treatment significantly decreased the production of IFN-γ, TNF-α, and IL-17A while increasing TGF-β1 (31). Calcitriol also inhibited the differentiation of Th17 and Th1 cells while increasing Th2 cells (31). Calcitriol decreased T-bet mRNA levels in AA patient cells (31), supporting a shift from Th1 phenotype. Several autoimmune and haematological disorders share a similar cytokine profile of higher IFN-γ, IL-10, IL-6, and IL-17, as observed across ITP, AIHA, Evan's syndrome, and CIN (23). Although this study did not measure changes in cytokine profile after vitamin D treatment, anti-erythrocyte autoantibody production was decreased following vitamin D treatment in patients with AIHA (23), suggesting that vitamin D may decrease the autoimmune nature of autoimmune cytopenias.

Given that vitamin D can decreases the JAK-STAT pathway activation and inflammatory cytokine output, further studies are warranted in cancers with this pathway dysregulation. Future work could determine whether vitamin D treatment of JAK-STAT activated cancers works via the same mechanism to cause inhibition, or whether unique mechanisms are at play.

V. CONCLUSION

With the discovery of vitamin D deficiency in many haematological disorders, understanding the role of vitamin D in the progression and treatment of such disorders is critical. Treatment with vitamin D or its analogs has shown promise in primary patient cells and cell lines in numerous haematological disorders and malignancies. Interestingly, the exact effects of calcitriol vary for different cell types. In general, calcitriol suppresses pro-inflammatory cytokine production, curtails proliferation, and inhibits antibody production in normal lymphocytes. In malignant cells, calcitriol inhibits proliferation, induces apoptosis, promotes differentiation, sensitizes malignant cells to anti-cancer therapies, and enhances cell cycle arrest. Interestingly, the exact mechanism(s) responsible for these anti-cancer effects is not yet known and should be determined in an effort to better understand how calcitriol exerts its effects.

Furthermore, vitamin D and its analogs have been well tolerated in the clinic (7, 8) and show promise in clinical trials for MDS (8). These studies have shown the potential of vitamin D as a combinatorial treatment. However, the adequate serum vitamin D level in humans and which form of vitamin D is the most appropriate to use for everyday supplementation versus cancer treatment needs to be determined. Regardless, further investigations are warranted to better elucidate the effects of calcitriol on abnormal cells and to determine its potential utility in the clinical management and treatment of haematological diseases.

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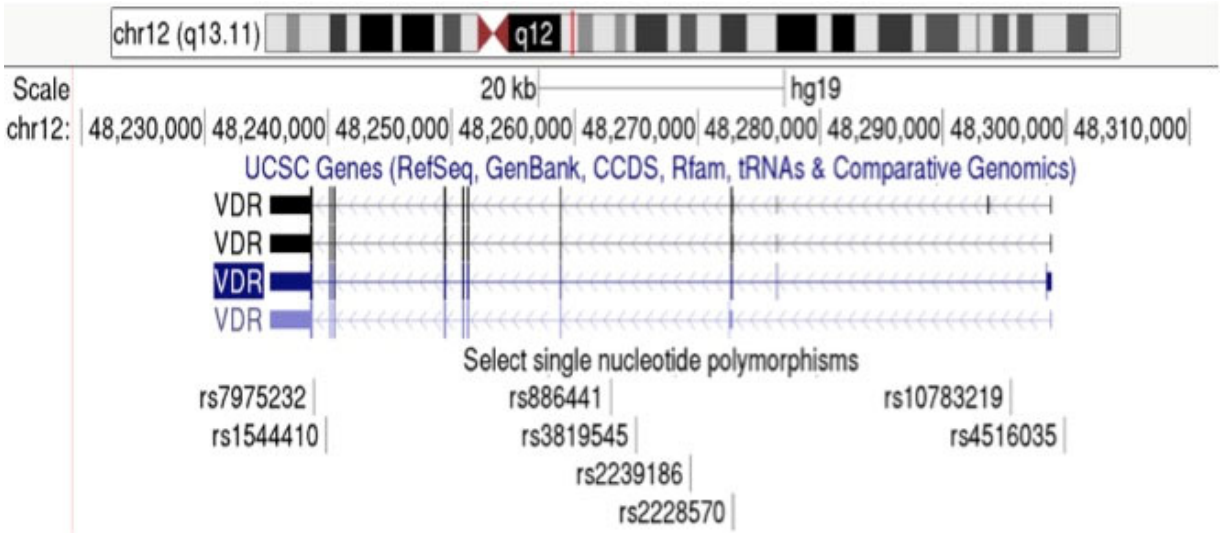

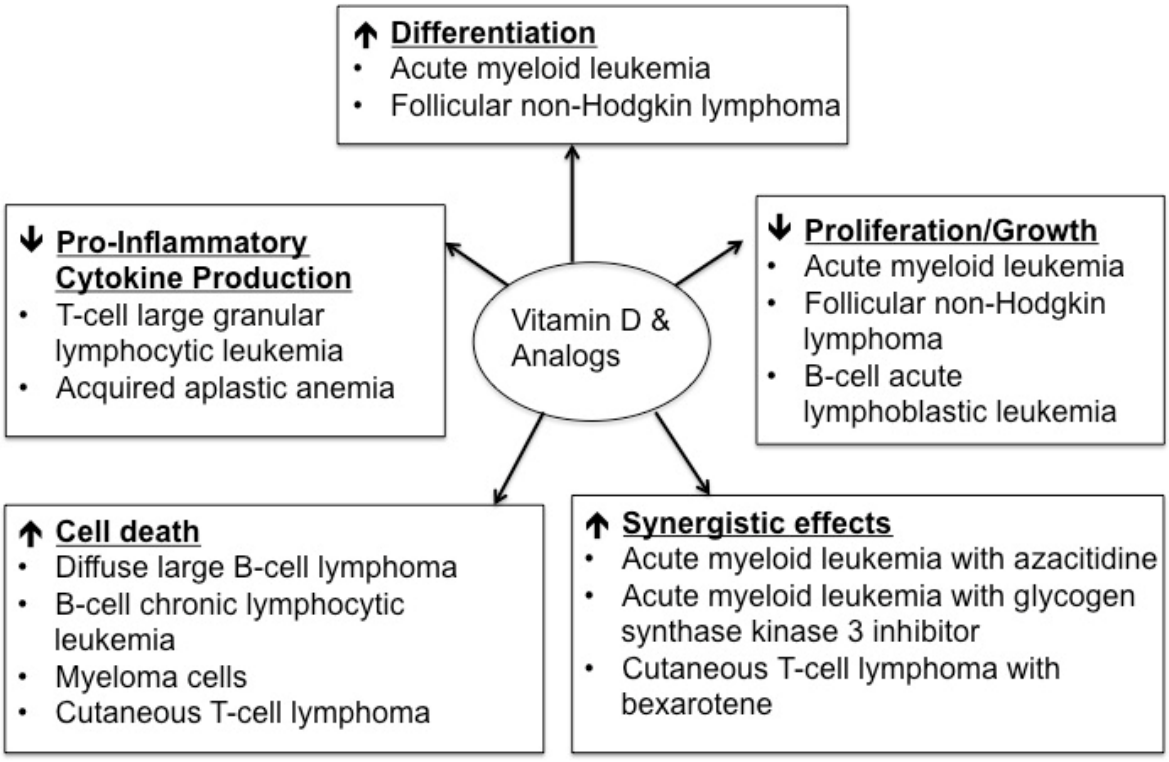

Figure 1. Visual representation of polymorphisms discussed in this review. Figure was captured from the UCSC genome browser (https://genome.ucsc.edu) utilizing the GRCh37/hg19 human genome assembly. Chromosome cytoband, chromosome coordinates and relative position of exons (wide bars), introns (line with arrow) and untranslated regions (narrow bars) of four isoforms of the VDR are shown. Eight germline SNPs are noted.

Figure 2. Summary of anti-cancer actions of vitamin D in haematological disorders and malignancies as described in the review. See text for references.