ANTICANCER RESEARCH *42*: 5043-5048 (2022)
doi:10.21873/anticanres.16013

A Novel Immunomodulatory Mechanism by Which Vitamin D Influences Folate Receptor 3 Expression to Reduce COVID-19 Severity

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Abstract. *Background/Aim: Identify potential mechanisms involving gene expression changes through which vitamin D supplementation could be beneficial in preventing adverse COVID-19 outcomes. Materials and Methods: We performed a literature review to identify differentially expressed genes (DEGs) in the blood between severe and mild COVID-19 patients. We compared these with the top DEGs induced by 6 months of 10,000 IU/day vitamin D supplementation in healthy adults who were vitamin D deficient/insufficient. We used bioinformatic tools to look for a vitamin D response element (VDRE) in DEGs. Results: FOLR3, RGS1, GPR84, and LRRN3 were the most significantly altered genes by 6 months of 10,000 IU/day vitamin D supplementation whose expression levels were also involved in COVID-19 severity. FOLR3 and GPR84 were found to be consistently up-regulated and RGS1 and LRRN3 consistently down-regulated in severe COVID-19 infection. FOLR3 and LRRN3 were down-regulated and RGS1 and GPR84 were up-regulated by 10,000 IU/day vitamin D supplementation. Conclusion: FOLR3 and RGS1 are expressed in neutrophils and lymphocytes, respectively. Vitamin D supplementation may decrease the neutrophil-lymphocyte ratio as has been reported in patients admitted with severe symptoms. There is evidence that vitamin D directly influences the expression of the RGS1 gene through vitamin D receptor binding. A potential negative VDRE (nVDRE) in an intron of the FOLR3 gene was found, which was homologous with two known nVDREs. Combined with other transcription factor elements near the newly identified nVDRE, these observations may explain the mechanism by which vitamin D regulates these genes, thus influencing COVID-19 outcomes.*

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Key Words: COVID-19, SARS-CoV-2, vitamin D, 25(OH)D, gene expression, blood, neutrophil-lymphocyte ratio, 1,25(OH)2D, vitamin D response element, VDRE, review.

Vitamin D deficiency has also been associated with a variety of medical conditions, including cardiovascular disease, autoimmune diseases, type 2 diabetes, and cancers (1). Vitamin D sufficiency is defined as having a serum concentration of 25-hydroxyvitamin D (25(OH)D), the major circulating form of vitamin D, greater than 30 ng/ml (1). The COVID-19 pandemic has been a cause of devastating mortality worldwide. However, a previous study has found that vitamin D sufficient individuals had a 54% decrease in COVID-19 infection compared to vitamin D deficient individuals (2). Previous studies have also suggested that vitamin D sufficiency correlates with a reduced risk of COVID-19 severity and mortality (3, 4). Nevertheless, approximately 70% of the United States population has been found to be either vitamin D deficient or insufficient (5). Therefore, vitamin D supplementation could potentially be a simple and inexpensive way to reduce the risk of the healthy population developing adverse COVID-19 outcomes.

While there have been many clinical trials investigating the effect of vitamin D supplementation on COVID-19 prognosis, there have been few studies examining the molecular pathways through which vitamin D supplementation may reduce risk of COVID-19 severity, particularly through gene expression. A previous study has found that 6 months of 10,000 IU/day vitamin D supplementation for deficient and insufficient individuals induced gene expression changes in peripheral white blood cells (6). The study observed 1,289 differentially expressed genes, including 800 up-regulated and 489 down-regulated genes (6).

This analysis aims to compare transcriptional changes resulting from severe COVID-19 and vitamin D supplementation in order to identify potential mechanisms involving changes in gene expression through which vitamin D supplementation could be beneficial in preventing adverse COVID-19 outcomes.

Materials and Methods

We used the dataset of differentially expressed genes (DEGs) in the white blood cells of initially vitamin D deficient or insufficient healthy adults [serum 25(OH)D concentration < 30 ng/ml] who received 6 months of 600, 4,000, or 10,000 IU/day vitamin D supplementation compiled by Shirvani *et al*. (6). The top 10 up-regulated and down-regulated DEGs (all have *p*-value<0.05) in adults receiving 6 months of 10,000 IU/day vitamin D supplementation were the focus of our literature search (Table I and Table II). We searched for studies in the PubMed database through July 2022 identifying differentially expressed genes (DEGs) in white blood cells involved in COVID-19 severity, and compared them to the top 10 up-regulated and down-regulated DEGs by 10,000 IU/day vitamin D supplementation. We compared a sequence of candidate VDREs with known VDREs to identify a VDRE in the *FOLR3* gene. We also searched for other transcription factor elements to confirm VDRE functionality.

Results

Studies on blood cell gene expression profile influencing COVID-19 severity. We identified in the literature search that *FOLR3, RGS1, GPR84*, and *LRRN3* were genes whose expression was significantly altered (|fold change| >2, *p*-value <0.05) between severe and mild COVID-19 patients (7-9). *TNFAIP3, SLED1, HILPDA, JUN, IGIP, WLS,* and *ZNF594*, which were also part of the top 10 up-regulated and top 10 down-regulated genes by 6 months of 10,000 IU/day vitamin D supplementation, were found by some studies to be differentially expressed in blood cells between severe and mild COVID-19 patients, although not by others (9, 10).

Differential expression of FOLR3. *FOLR3* (folate receptor 3) expression was consistently found to be up-regulated (fold change >2, *p*-value <0.05) in blood cells of severe COVID-19 patients compared to mild COVID-19 patients (7-9). A study also found *FOLR3* expression to be up-regulated (fold change >2, *p*-value <0.05) in the whole blood transcriptomes of clinical COVID-19 patients compared to individuals with entirely asymptomatic COVID-19 (11). In contrast, Vitamin D supplementation down-regulated *FOLR3* expression in a dose-dependent manner (fold change=–1.0, –1.7, and –2.7 for the 600, 4,000, and 10,000 IU/day supplementation groups, respectively; *p*-value <0.05 for the 4,000 and 10,000 IU/day groups) (6). We also found a potential negative VDRE (nVDRE) in an intron of the *FOLR3* gene that was homologous with two known nVDREs. There were also other transcription factor elements near the newly identified nVDRE. All of these may indicate a causal relationship between vitamin D supplementation and *FOLR3* expression. Therefore, *FOLR3* could be a gene of interest involving vitamin D's effect on COVID-19 outcomes, as its dose-dependent down-regulation by vitamin D supplementation in white blood cells could counter its up-regulation of expression with increasing COVID-19 severity (Figure 1). Long-term vitamin D supplementation for deficient and insufficient adults could potentially reduce the risk of developing severe COVID-19 through a mechanism associated with the down-regulation of *FOLR3* gene expression in the white blood cells.

Other genes. *RGS1* (regulator of G-protein signaling 1) was consistently found to be down-regulated (fold change <-2, *p*-value <0.05) in the blood cells of severe COVID-19 patients compared to mild COVID-19 patients (8, 9). Conversely, 6 months of 10,000 IU/day vitamin D supplementation significantly up-regulated RGS1 expression (fold change=4.2, *p*-value <0.05). Interestingly, the up-regulation of RGS1 by vitamin D supplementation was not dose-dependent; 6 months of 600 and 4,000 IU/day vitamin D supplementation was found to down-regulate *RGS1* (fold change=-2.1 and -1.6, respectively, *p*-value <0.05). There is evidence to show that vitamin D directly influences expression of the RGS1 gene through vitamin D receptor binding (12).

Table I. *Top 10 up-regulated differentially expressed genes in adults receiving six months of 10,000 IU/day vitamin D supplementation.*

1.	*C15orf48*	Chromosome 15 open reading frame 48
2.	*NR4A2*	Nuclear receptor subfamily 4, group A, member 2
3.	*TNFAIP3*	Tumor necrosis factor, alpha-induced protein 3
4.	*SLED1*	Proteoglycan 3 pseudogene
5.	*GPR84*	G protein-coupled receptor 84
6.	*CXCL2*	Chemokine (C-X-C motif) ligand 2
7.	*HILPDA*	Hypoxia inducible lipid droplet-associated
8.	*MIR221*	microRNA 221
9.	*JUN*	Jun proto-oncogene
10.	*RGS1*	Regulator of G-protein signaling 1

Table II. *Top 10 down-regulated differentially expressed genes in adults receiving six months of 10,000 IU/day vitamin D supplementation.*

1.	*ZNF594*	Zinc finger protein 594
2.	*ZNRD1-AS1*	ZNRD1 antisense RNA 1
3.	*SLC35E2*	solute carrier family 35, member E2
4.	*FOLR3*	Folate receptor 3 (gamma)
5.	*FPR2*	Formyl peptide receptor 2
6.	*MPZL1*	Myelin protein zero-like 1
7.	*CD180*	CD180 molecule
8.	*WLS*	Wntless homolog (Drosophila)
9.	*LRRN3*	Leucine rich repeat neuronal 3
10.	*IGIP*	IgA-inducing protein homolog (Bos taurus)

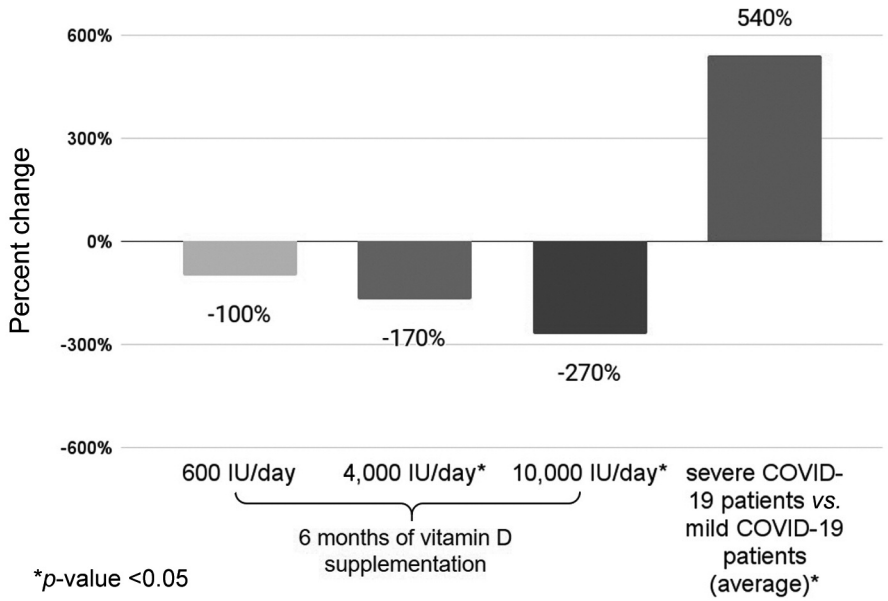

Figure 1. *FOLR3 expression was down-regulated with vitamin D supplementation in a dose-dependent manner (fold change=–1.0, –1.7, and –2.7 for the 600, 4,000, and 10,000 IU/day supplementation groups, respectively). FOLR3 expression was up-regulated in severe COVID-19 patients compared to mild COVID-19 patients (average fold change=5.4). *p-Value <0.05.*

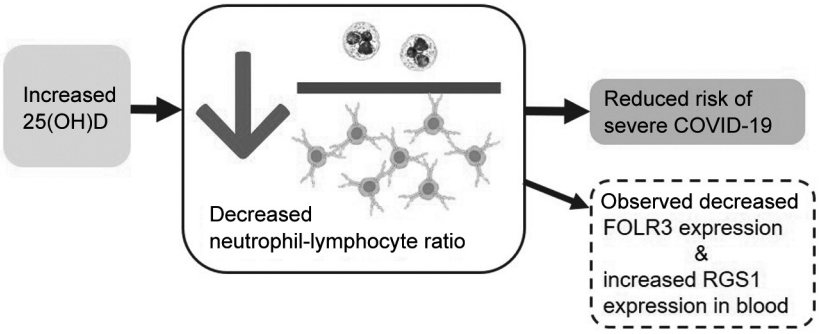

Figure 2. *Changes in the immune system associated with vitamin D supplementation. Vitamin D sufficiency has been found to decrease the ratio of neutrophils compared to lymphocytes, which would lead to improved COVID-19 outcomes, as well as explain the observed decrease in FOLR3 mRNA transcript levels and increase in RGS1 transcript levels in the blood.*

GPR84 (G protein-coupled receptor 84) was consistently found to be up-regulated (fold change >2, *p*-value <0.05) in blood cells of severe COVID-19 patients compared to mild COVID-19 patients (8, 9). *GPR84* had been also found to be up-regulated (fold change >2, *p*-value <0.05) by 6 months of 10,000 IU/day vitamin D supplementation (6). *LRRN3*, a gene down-regulated by 6 months of 10,000 IU/day vitamin D supplementation (6), was also consistently found to be down-regulated (fold change <-2, *p*-value <0.05) in blood cells in severe COVID-19 compared to mild COVID-19 (8, 9).

Discussion

Vitamin D supplementation & decreased neutrophil-lymphocyte ratio. The *FOLR3* gene encodes the folate receptor gamma protein, a soluble secretory protein released from neutrophil granulocytes as part of the innate immune response (13). The *RGS1* gene encodes the regulator of G-protein signaling 1 protein, which is not expressed in neutrophils, but highly expressed in various types of lymphocytes, including natural killer cells and T cells (14). Hence, a decreased FOLR3 and increased RGS1 expression

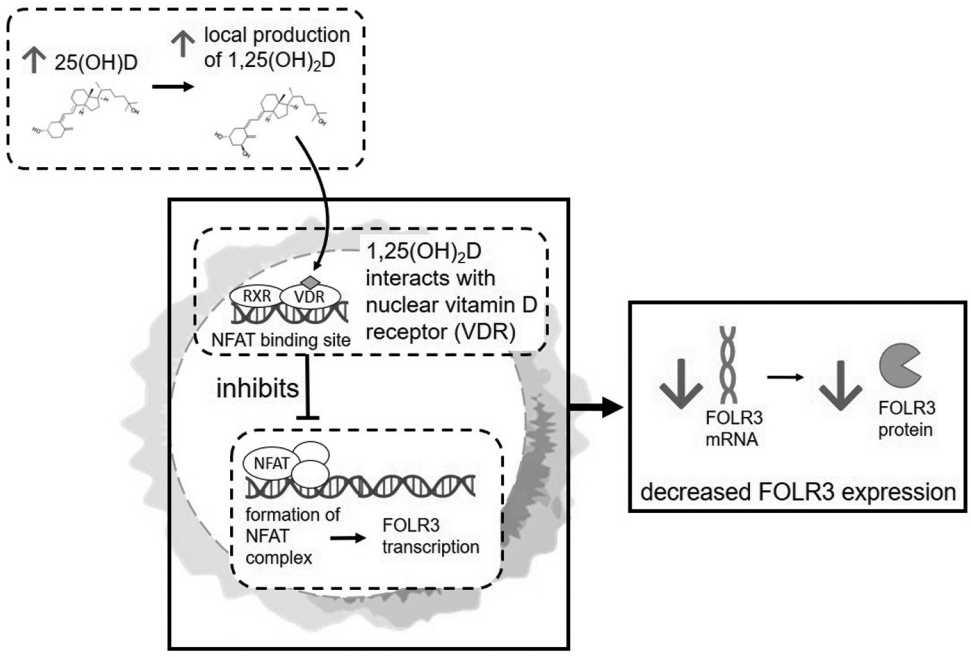

Figure 3. *Possible direct repression of FOLR3 expression by the 1,25(OH)$_2$D-bound vitamin D receptor (VDR). We hypothesize that increased 25(OH)D levels increase immune cells' local production of the active form of vitamin D [1,25(OH)$_2$D], which may bind to its nuclear receptor on an NFAT transcription factor binding site in the FOLR3 promoter, thus inhibiting the formation of the NFAT complex necessary for FOLR3 transcription.*

level observed in the group that received 6 months of 10,000 IU/day vitamin D supplementation could modulate the immune system by decreasing neutrophil count and increasing lymphocyte count. A high neutrophil-lymphocyte ratio on patient admission has been found to predict COVID-19 severity and mortality (15). Therefore, decreasing the neutrophil-lymphocyte ratio could be a mechanism through which vitamin D supplementation reduces the risk of adverse COVID-19 outcomes (Figure 2). This is consistent with clinical findings that oral 25(OH)D_3 treatment correcting vitamin D deficiency/insufficiency was associated with a significant decrease in neutrophil-lymphocyte ratio in COVID-19 patients (16).

Direct effect of active form of vitamin D on FOLR3 expression. Vitamin D supplementation could also have an effect on COVID-19 outcomes by directly suppressing *FOLR3* expression. The active form of vitamin D, 1,25-dihydroxyvitamin D [1,25(OH)$_2$D], has been found to directly affect the expression of over 1000 genes in most tissues and cell types via activating the nuclear transcription factor vitamin D receptor (VDR) (1, 17). The VDR and the enzyme 25-hydroxyvitamin D-1α-hydroxylase (*CYP27B1*), which metabolizes 25(OH)D to 1,25(OH)$_2$D, are expressed in several immune cell types, including macrophages, activated monocytes, neutrophils and T lymphocytes (1, 18). Therefore, increased levels of circulating 25(OH)D could lead to increased local production of its active metabolite 1,25(OH)$_2$D in white blood cells. The active form of vitamin D [1,25(OH)$_2$D] could then potentially bind with its nuclear vitamin D receptor (VDR), creating a complex that would bind to a vitamin D response element (VDRE) on the target genes. The potential vitamin D response element on the *FOLR3* gene may explain the mechanism by which vitamin D regulates *FOLR3* in immune cells. The potential *FOLR3* VDRE is homologous to known negative VDRE sequences in other genes. This could be a mechanism through which 1,25(OH)$_2$D directly represses *FOLR3* expression. The vitamin D receptor (VDR) has been shown to compete with *NFAT* for DNA binding to regulate *NFAT's* target genes (19, 20). Therefore, 1,25(OH)$_2$D may indirectly down-regulate expression of *FOLR3* through *VDR* competing with *NFAT* for DNA binding sites in the *FOLR3* gene (Figure 3).

It has been hypothesized that upon secretion, the *FOLR3* protein binds to 5- methyltetrahydrofolate (5-methylTHF), which deprives cells of this natural folate (13). 5-methylTHF is required for the methyl group transfer in converting homocysteine to methionine (21). A lack of 5-methylTHF would lead to an excess of homocysteine, which has been shown to induce neutrophil extracellular trap (NET) formation (22). NETs have been found to be a major cause of the immunothrombosis, organ damage, and acute respiratory

distress syndrome (ARDS) associated with COVID-19 morbidity and mortality (23). Therefore, direct inhibition of *FOLR3* expression by vitamin D supplementation could play a role in reducing severe COVID-19 by reducing NET formation.

COVID-19 is not the only disease associated with increased expression of the *FOLR3* gene. *FOLR3* has been found to be over-expressed in autoimmune diseases, such as rheumatoid arthritis and psoriasis (24), cardiovascular diseases, such as coronary artery disease (25), various cancers (26), and Alzheimer's disease and mild cognitive impairment (27). Therefore, decreasing *FOLR3* expression could be a way through which being vitamin D sufficient with a 25(OH)D of at least 30 ng/ml has a protective effect against other diseases as well. Indeed, considering how 10,000 IU/day of vitamin D supplementation decreased *FOLR3* expression nearly trifold but severe COVID-19 raised it five-fold, further studies should be conducted to determine the effects of higher than 10,000 IU/day of vitamin D supplementation on *FOLR3* expression to potentially reduce severity of COVID-19 in at-risk patients.

Conflicts of Interest

Michael F. Holick has served as a consultant for Biogena Inc., Ontometrics Inc. and Solius Inc, and has grants from Carbogen Amcis BV and Solius Inc. The Authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Authors' Contributions

M.F. Holick designed the project. R.J. Lu performed the literature search and analyzed the data. P. Shirvani completed the vitamin D response element (VDRE) evaluation. R.J. Lu, P. Shirvani, and M.F. Holick drafted the manuscript. M.F. Holick provided final approval.

Acknowledgements

The Authors are grateful to Boston University's RISE Internship program for offering the opportunity and connecting RJL to complete a research project in the Vitamin D, Skin and Bone Research Laboratory. RJL is also thankful for the support of her fellow RISE interns Ramneek Kaur and Aashi Mittal, and everyone who has made this an enjoyable & insightful experience.

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Received July 30, 2022
Revised August 25, 2022
Accepted August 26, 2022