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ORIGINAL ARTICLE

Vitamin D Deficiency and Its Association with Inflammatory Markers, Lipid Profile and Regulatory T-cells in Pediatric Sickle Cell Disease Patients

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Abstract We investigated vitamin D deficiency in pediatric sickle cell disease patients and its association with selected bone, lipid and inflammatory parameters. The study included 64 patients (33 SS and 31 SB) and 21 carriers (AS). Blood was obtained to assess levels of vitamin D, WBC, CRP, Ca, P, ALP, PTH, triglyceride, total cholesterol, LDL, VLDL, HDL, IL-2, IL-12, TNF-α, IL-4, IL-6, IL-10 and regulatory T cells. The patients were grouped according to their genotype (SS, SB) and vitamin D status (low or normal). Carriers were also grouped as low or normal vitamin D. Laboratory findings were similar between low and normal Vit D groups in SS, SB and AS genotypes except a lower IL-12 in SB-low vitamin D compared SB-normal vitamin D group. Acute chest syndrome was more frequent in SS-low Vit D (63%) compared to SS-normal Vit D (25%), SB-low Vit D (21%) and SB-normal Vit D (33%) ($P = 0.045$). Both SS and SB with low vitamin D had higher VLDL ($P = 0.006$ and $P = 0.022$), TNF-α ($P = 0.001$) and regulatory T cells ($P = 0.000$) compared to AS-low vitamin D. Both SS and SB with normal vitamin D had higher levels of regulatory T cells ($P = 0.000$) compared to AS-normal vitamin D. Vit D was not a modifier of selected inflammation, bone and lipid parameters in sickle cell disease. Acute chest syndrome was comparably more frequent in SS-low vitamin D. Increase of regulatory T cells in the patients was a result of chronic inflammation in sickle cell disease.

Keywords Sickle cell disease · Genotype · Vitamin D · Cytokine · TNF-α · Regulatory T cell · Lipid · VLDL

Introduction

Sickle cell disease (SCD) and its variants are inherited disorders resulting from the presence of a mutated form of hemoglobin (Hb), named HbS. Polymerization of mutant hemoglobin under hypoxia, acidosis and dehydration results with sickling of the erythrocyte that causes vaso-occlusion and endothelial injury with concurrent inflammation in the circulation [1]. This inflammation has been reflected by a relative increase in the levels of systemic inflammation markers such as C reactive protein (CRP) and cytokines both in stable and crisis patients compared to controls [2–4]. Increased IL-1, IL-6 and IFN-γ in the serum of stable patients and increased TNF-α and IL-6 in crisis patients were also reported [5]. There are two separate groups of cytokines: IL-2, IL-12 and TNF-α are proinflammatory and mainly secreted by helper T lymphocytes type 1 (T_{H1}) which provide immunity against intracellular pathogens where IL-4, IL-6 and IL-10 are anti-inflammatory and secreted by T_{H2} cells which provide immunity against extracellular pathogens [6]. Taylor et al. [7] reported increased IL-6 levels in stable pediatric SCD patients compared to controls and also suggested an altered balance between proinflammatory and anti-inflammatory cytokines.

Part of the findings of this study was presented in 56th ASH Annual Meeting in 2014 [27].

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Vitamin D (Vit D) deficiency is a frequent laboratory finding in SCD according to various reports [8–10]. Children with SCD have five times more Vit D deficiency compared to controls, after correcting for season and age [10]. Besides having a key role in bone metabolism, Vit D has been reported to have receptors on almost all cells of the immune system and been suggested to have a role in mediating inflammation [11]. Vit D regulates innate immunity by stimulating macrophages and monocytes and adaptive immunity (T_{H2} response) by increasing levels of IL-4, IL-10 and number of regulatory T cells (Treg) [12]. Deficiency of Vit D in SCD may be associated with the uncontrolled, chronic inflammation and concurrent vaso-occlusions seen in the course of the disease. The association of Vitamin D status with inflammation parameters and proinflammatory and anti-inflammatory cytokines in SCD has been investigated in a recent article [13].

Hypocholesterolemia and relative hypertriglyceridemia have been frequently detected in SCD patients compared to the controls [14]. Relative hypertriglyceridemia has been previously linked to chronic inflammation in SCD [15]. Low Vit D status is associated with high Tg levels in healthy children [16]. However, the relation between Vit D status and levels of serum lipids, particularly Tg, has not been investigated in SCD patients.

In this respect we conducted a study exploring the association between Vit D status, inflammation and lipids in SCD.

Materials and Methods

Patients and Study Design

Sixty-four SCD patients who have received routine follow up were enrolled in the study. Their ages were between 2 and 18 years old. Blood samples were obtained from 37 stable and 27 crisis patients. Stable patients had no transfusion or crisis for the last 3 months. Crisis patients were hospitalized patients for vaso-occlusive crisis. Genotypes of the patients were either SS or S-beta thalassemia (SB) determined by HPLC and genetic analysis. Vitamin D deficiency was defined as a serum 25(OH) D_3 level below 20 ng/mL [17]. The patients were grouped into four as SS-low Vit D, SS-normal Vit D, SB-low Vit D, and SB-normal Vit D.

Number of blood transfusions, vaso-occlusive and sequestration crises, hospitalization; history of hydroxyurea, chelation and penicillin therapies, acute chest syndrome, stroke, and splenectomy were collected from medical records for each patient. The patients didn't have renal or hepatic diseases, use Vit D or calcium supplement and get blood transfusion in the last 3 months.

Twenty-one carriers who were chosen from relatives of the patients were also enrolled in the study as controls. They were also grouped as AS-low Vit D and AS-normal Vit D.

Ethics Committee approved the study and informed consent was obtained.

Laboratory Analysis

Vit D Status and Bone Metabolism Vit D status was assessed with serum levels of 25-OH-vitamin D_3 measured by HPLC (Chromosystems, GmbH Germany). Levels of total calcium, phosphorous and alkaline phosphatase (ALP) in serum were determined by using commercial kits in Cobas Integra 800 (Roche Diagnostics, Germany). Parathormone (PTH) levels were determined by immunoassay with Immulite 2000 XPI (Siemens, United Kingdom).

Systemic Inflammation White blood cell (WBC) count was provided by Sysmex XT 2000İ (Roche Diagnostics, Germany). CRP levels were determined by Architect c8000 (Abbott, Germany).

Percentage of Treg cells was determined using flow cytometry by using anti-Foxp3 antibody (BD Biosciences). For this purpose blood samples were collected into BD Vacutainer CPT^{TM} (4 mL) tubes containing heparin. Mononuclear cells were separated using Ficoll gradient. Surface stain for CD4 FITC and CD25 and intracytoplasmic stain with FoxP3 PE were performed. After staining cells were analysed by Facs Calibur flow cytometry instrument. After determining the gating for lymphocytes according to size and granular content, 10,000 cells were counted. Treg count was evaluated according to positivity for CD4, CD25 and FoxP3 and expressed as percentage of total number of lymphocytes.

Levels of Proinflammatory Cytokines (*IL-2, IL-12, and TNF-α*) *and Anti-inflammatory Cytokines* (*IL-4, IL-6 and IL-10*) were determined by ELISA system (Dynex Technologies, USA).

Lipid Metabolism Triglyceride (Tg), total cholesterol (TChol), very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), high-density lipoprotein (HDL) and ferritin in serum were determined by using commercial kits in Cobas Integra 800 (Roche Diagnostics, Germany).

Statistics Statistical analysis was carried out using IBM SPSS Statistics 22. Data are reported as mean ± standard deviation. Statistical significance was set at $P < 0.05$. Kruskal–Wallis, test was used to evaluate the differences among groups. Mann–Whitney *U* test was used to compare two groups. Bonferonni correction was done where needed. Chi square test was used to assess the difference between descriptive characteristics among groups. Association between quantitative variables was assessed using

Spearman's correlation coefficient. A cut off value of + 0.7 to + 1.0 was taken to imply a strong positive association.

Results

The study included 64 SCD patients of whom 33 were SS and 31 were SB genotype and 21 SCD carriers with AS genotype. Selected bone, lipid and inflammation parameters for each genotype with either low or normal Vit D were shown on Table 1.

1. SS genotype; there was no significant difference among all the measured laboratory parameters when low and normal Vit D groups were compared.
2. SB genotype, IL-12 levels were significantly lower in low Vit D group compared to normal Vit D ($P = 0.045$).
3. AS genotype, there was no significant difference among all the measured laboratory parameters, when low and normal Vit D groups were compared.

Low Vit D Groups When we compared the low Vit D groups including SS, SB and AS genotypes with each other, VLDL ($P = 0.006$), HDL ($P = 0.007$), IL-2 ($P = 0.002$), TNF-α ($P = 0.003$) and T reg ($P = 0.000$) levels were different among groups. IL-2 levels were lower in SS-low Vit D patients compared to SB-low Vit D patients ($P = 0.000$). SS-low Vit D patients had higher VLDL ($P = 0.006$), lower HDL ($P = 0.007$), higher TNF-α ($P = 0.001$) and higher T reg ($P = 0.000$) compared to

Table 1 Laboratory parameters of the patient and carrier groups

Laboratory parameters	SS (N = 33)		SB-thalassemia (N = 31)		AS (carrier) (N = 21)	
	Low Vit D (N = 19)	Normal Vit D (N = 14)	Low Vit D (N = 16)	Normal Vit D (N = 15)	Low Vit D (N = 7)	Normal Vit D (N = 14)
Vitamin D	11.3 ± 4.2	26.6 ± 4.2	13.1 ± 3.5	26.6 ± 4.8	17.0 ± 1.6	25.1 ± 4.0
Bone parameters						
Calcium (mg/dL)	9.6 ± 0.6	9.3 ± 1.0	9.6 ± 0.7	10.0 ± 0.6	10.1 ± 0.4	10.2 ± 0.4
Phosphate (mg/dL)	4.7 ± 0.8	4.7 ± 0.6	4.9 ± 0.4	5.1 ± 0.8	4.6 ± 0.6	4.7 ± 0.5
ALP (IU/L)	135.0 ± 44.5	127.4 ± 20.5	143.5 ± 46.6	152.7 ± 46.1	125.4 ± 81.4	163.5 ± 66.8
PTH (pg/mL)	65.7 ± 33.5	53.4 ± 18.2	73.5 ± 40.0	76.1 ± 36.6	48.8 ± 26.7	59.7 ± 31.8
Lipid profile						
Tg (mg/dL)	121.2 ± 52.4	131.5 ± 96.8	127.0 ± 95.0	112.5 ± 33.4	73.4 ± 49.2	84.8 ± 5.6
TChol (mg/dL)	114.1 ± 20.3	103.2 ± 17.1	123.5 ± 30.0	116.0 ± 23.6	118.3 ± 36.9	133.9 ± 36.1
VLDL (mg/dL)	26.4 ± 12.4	32.8 ± 25.0	26.6 ± 18.2	22.5 ± 6.7	14.7 ± 9.8**,b	17.0 ± 11.1
LDL (mg/dL)	54.7 ± 23.1	46.9 ± 7.8	62.6 ± 26.5	49.3 ± 22.9	68.6 ± 17.7	76.1 ± 27.8
HDL (mg/dL)	31.3 ± 7.0	28.9 ± 6.7	34.8 ± 10.6	40.6 ± 15.6	39.2 ± 6.6	42.3 ± 9.2
Inflammatory markers						
WBC ($\times 10^3/mm^3$)	13.7 ± 4.4	13.2 ± 3.0	11.5 ± 3.1	12.2 ± 5.1		
CRP (mg/L)	4.6 ± 3.3	7.1 ± 4.1	4.7 ± 4.2	5.4 ± 3.4		
Proinflammatory cytokines						
IL-2 (IU/mL)	0.3 ± 0.1	0.6 ± 0.6	0.6 ± 0.2*,a	0.6 ± 0.7	0.3 ± 0.2	0.5 ± 0.2
IL-12 (pg/mL)	203.7 ± 120.0	293.1 ± 140.4	214.0 ± 122.2	325.6 ± 214.5	175.0 ± 91.9	228.6 ± 160.3
TNF-α (pg/mL)	9.7 ± 4.3	12.0 ± 5.3	9.1 ± 3.2	8.9 ± 3.3	5.6 ± 1.1#,b	6.6 ± 2.8
Antinflammatory cytokines						
IL-4 (pg/mL)	9.6 ± 6.1	15.1 ± 19.6	11.1 ± 5.1	14.9 ± 18.2	8.4 ± 2.7	10.0 ± 4.1
IL-6 (pg/mL)	79.8 ± 30.4	108.7 ± 138.1	96.6 ± 54.8	148.9 ± 183.6	78.4 ± 26.3	135.6 ± 153.2
IL-10 (pg/mL)	3.9 ± 0.5	3.6 ± 0.4	4.3 ± 1.6	3.7 ± 0.5	4.3 ± 2.0	**3.9** ± 1.1
Treg (%)	2.3 ± 1.2	1.6 ± 0.8	2.4 ± 1.6	2.2 ± 1.0	0.4 ± 0.2*,b	0.3 ± 0.2*,c

* $P = 0.000$; ** $P = 0.022$; # $P = 0.001$

[a]SS-low Vit D versus SB-low Vit D

[b]SA-low Vit D versus SS-low Vit D and SB-low Vit D

[c]SA-normal Vit D versus SS-normal Vit D and SB-normal Vit D

Table 2 Clinical parameters of the patients

Clinical condition (frequency)	SS patients		SB patients	
	Low Vit D (N = 19)	Normal Vit D (N = 14)	Low Vit D (N = 16)	Normal Vit D (N = 15)
Transfusion				
< 2	14	10	13	14
≥ 2	5	4	3	1
Vaso-occlusive crisis				
< 2	9	8	11	10
≥ 2	10	6	5	5
Sequestration crisis	6	4	2	0
Acute chest syndrome	12*	3	4	5
Splenectomy	3	2	3	2
Stroke	1	2	3	1
Hydroxyurea	14	13	8	12
Chelation	2	2	1	1

* $P = 0.045$

AS-low Vit D carriers. SB-low Vit D patients had lower VLDL ($P = 0.022$), higher IL-2 ($P = 0.022$), higher TNF-α ($P = 0.001$) and higher T reg ($P = 0.000$) compared to AS-low Vit D carriers. As a conclusion both SS and SB patient groups with low Vit D had higher VLDL, TNF-α and Treg compared to carriers with low Vit D.

Normal Vit D Groups When the normal Vit D groups with SS, SB and AS genotypes were compared, TNF-α ($P = 0.002$) and T reg levels ($P = 0.000$) were found significantly different. TNF-α and T reg levels were higher in SS patients-normal Vit D compared to AS carriers-normal Vit D ($P = 0.000$). T reg levels were higher in SB patients-normal Vit D compared to AS carriers-normal Vit D ($P = 0.000$). As a conclusion patients with normal Vit D had higher levels of Treg compared to carriers with normal Vit D.

Acute chest syndrome was the clinical conditions that was more frequent in SS patients with low Vit D (63%) compared to SS patients with normal Vit D (25%), SB patients with low Vit D (21%) and SB patients with normal Vit D (33%) ($P = 0.045$) (Table 2).

Discussion

We hypothesized a possible link between low Vit D status and chronic inflammation in SCD patients and conducted this study. We measured selected inflammation, bone, lipid and immune system parameters in our SS and SB patients as well as in AS carriers. We found that Vit D status was not a modifier of selected laboratory parameters in our SCD patients and carriers except a lower mean IL-12 in SB-low Vit D patients compared SB-normal Vit D. Interestingly frequency of acute chest syndrome in the last year was highest in SS patients with low Vit D compared to normal Vit D group.

A previous study reported Vit D deficiency in 76% of SCD children and secondary hyperparathyroidism in 38% of them [20]. Our patients had a similar incidence of Vit D deficiency and hyperparathyroidism that stands at 60.9 and 40% respectively. We couldn't find any association of Vit D with phosphate, PTH and ALP neither in SS nor in SB patients in this study.

Serum lipids were not different between our low and normal Vit D groups of SCD patients. Low Vit D and high serum Tg levels were found to be associated in atherosclerosis patients [18]. We expected to find such a link in SCD with a vascular pathology characterized by endothelial inflammation similarly. Previously it was reported that Vit D levels were negatively correlated to Tg and positively correlated to HDL in serum [19]. We think further studies with a larger number of patients may help to clarify the possible metabolic link between Vit D and serum lipids. It might be better to investigate this relation in a subset of SCD patients having vascular inflammation as the prominent phenotype.

Both SS and SB patients with low Vit D levels had significantly higher VLDL levels compared to carriers with low Vit D. VLDL particles are synthesized in liver and carry almost all the Tg in the circulation. The relative hypertriglyceridemia in SCD was linked to chronic inflammation [15]. When we compared Tg levels of our patients with the carriers irrespective of their Vit D status, we also found significantly higher Tg in patients than carriers.

Table 3 Correlation between triglyceride (Tg) and VLDL in each group

Tg-VLDL	SS (N = 33)		SB-thalassemia (N = 31)		AS (carrier) (N = 21)	
	Low Vit D (N = 19)	Normal Vit D (N = 14)	Low Vit D (N = 16)	Normal Vit D (N = 15)	Low Vit D (N = 7)	Normal Vit D (N = 14)
R values	0.581	0.758	0.768	0.989	1.000	1.000
	$P = 0.009$	$P = 0.002$	$P = 0.001$	$P = 0.000$	$P = 0.000$	$P = 0.000$

We also analyzed the correlation between Tg and VLDL levels in each group and found significant correlations (Table 3). Interestingly the correlation coefficient (R) decreased relatively as clinical condition and Vit D status got worse. R was highest (1.000) in carriers whereas it was the lowest (R = 0.581) in SS patients with low vitamin D. This suggests a disturbance in the composition of VLDL particle as well as its quantity as a possible consequence of the metabolic alterations in SCD. As we didn't investigate the composition of the VLDL particle in this study, we can only suggest that the oxidative medium in SCD resulted with some kind of a structural alteration that the strength of the correlation between Tg and VLDL was altered as shown in Table 3.

Vit D was shown to induce differentiation and functioning of T reg cells that suppress exaggerated or autoimmune responses of other immune cells [20]. However we didn't found any effect of Vit D status on the number of T reg cells in the patient and carrier groups.

On the other hand, T reg counts, as marker of systemic inflammation, were significantly increased in both SS and SB patients either with low or normal Vit D compared to AS carriers with low or normal Vit D. This finding is a result of chronic inflammation observed in SCD [21]. There is only one study in the literature about increased T reg cells in SCD patients with leg ulcers [22].

A recent study reported increased proinflammatory cytokines in Vit D deficient stable pediatric patients with SCD and 3 months of daily vitamin D supplementation reversed the proinflammatory state [13]. They suggested Vit D supplementation to serve as an anti-inflammatory treatment in the management of SCD. However, we could not show a proinflammatory state related to Vit D status in our patients that were grouped according to genotype.

Acute chest syndrome in the last year was significantly more frequent in SS-low Vit D patients compared to all other patient groups. This finding may suggest a link between the occurrence of acute chest syndrome and low Vit D levels with SS patients. SB patients usually have a better clinical course than SS patients. There was no previous report about Vit D status and history of acute chest syndrome in the literature, but there is a recent study reporting a potential association between vaso-occlusive crisis and low Vit D levels in pediatric SCD patients [23]. Acute chest syndrome is a result of vaso-occlusion within the pulmonary vasculature of patients with SCD who are hospitalized and mostly experienced a prior vaso-occlusive crisis [24]. We suggest that larger number of SCD patients should be investigated for a link between acute chest syndrome and Vit D deficiency.

Carriers with AS genotype are not usually regarded as patients for their complications being either uncommon or mild. A study investigated inflammation in exercise in sickle cell trait and concluded that some sickle cell trait patients may present with an exercise induced inflammation [25]. CRP levels in AS carriers were found significantly lower than SS patients and similar to AA controls in a study investigating markers of coagulation activation and inflammation in SCD [26]. But more studies are needed to comment that heterozygous states in SCD have any role in altering chronic inflammation and Vit D status.

The lacuna of this study is the limited number of patients in each group. Further studies with larger number of patients and novel markers of inflammation may help to clarify any possible link between Vit D and particularly endothelial inflammation in SCD. Additionally measuring 1,25-OH-Vit D that is the metabolically active form of Vit D may help to better evaluate the Vit D status of SCD patients.

As a conclusion we did not found a significant effect of Vit D status on selected bone, lipid and inflammation markers measured in the blood of SCD patients. However we found significantly increased frequency of acute chest syndrome in our SS-low Vit D SCD patients. It is possible that many other factors may modify the measured laboratory parameters; but this clinical condition associated with Vit D deficiency may imply a role for Vit D in the clinical course of the disease.

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Compliance with Ethical Standards

Conflict of interest Authors YO, SU, GE, LT and NO declare that they have no conflict of interest.

Research Involving Human Participants and/or Animals All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual children and their parents.

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