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RESEARCH ARTICLE

INCREASED SERUM LEVELS OF TUMOR NECROSIS FACTOR-A AND INTERLUEKIN-6 IN ADULT BAHRAINI SICKLE CELL DISEASE PATIENTS WITH HYPOVITAMINOSIS D AND LOW BONE MINERAL DENSITY

[1,*]Adla B. Hassan, Taysir Garadah, Diab E. Diab, Reginald P. Sequeira, Ahmed Jaradat, Azzeghaibi Saleh Nasser and Fathia Al Qurashi

[1]Department of Internal Medicine, Arabian Gulf University Kingdom of Bahrain, Manama
[2]Al Jawhra Centre, Arabian Gulf University Kingdom of Bahrain, Manama
[3]Departmentof Pharmacology and Therapeutics, Arabian Gulf University Kingdom of Bahrain, Manama
[4]Departmentof Family and Community Medicine, Arabian Gulf University Kingdom of Bahrain, Manama
[5]Alfarabi Private College, Arabian Gulf University Kingdom of Bahrain, Manama
[6]Departmentof Hematology, Salmaniya Medical Complex, Arabian Gulf University Kingdom of Bahrain, Manama

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ABSTRACT

Objective: To investigate serum levels and relationship between tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), C-reactive protein (CRP), bone mineral density (BMD) and vitamin D in Bahraini sickle cell disease (SCD) patients compared to healthy controls.

Methods: Seventy patients with SCD followed at department of hematology at Salmaniya Medical Complex and 70 sex-matched healthy controls were included prospectively in this study. Serum levels were measured using ELISA kits for cytokines and UPLC-MS for vitamin D. BMD was estimated using Dual Energy X-ray Absorptiometry (DEXA). Osteopenia (T score < -1>-2.5) and osteoporosis (≤ -2.5) were diagnosed as per WHO criteria.

Results: SCD patients had increased serum levels of TNF-α (56.72 pg/ml; range 27.43-98.93) compared to controls (39.49 pg/ml; range 27.56-119.95; p<0.0001). Our patients had increased serum levels of IL-6 (49.5 pg/ml; range 12.27-101.81) compared to controls (30.08 pg/ml; range 4-275; p=0.002). While, 94% of patients had low vitamin D, only 6% had optimal levels. However, 58% of patiens had abnormal BMD and 42% had normal BMD. A positive correlation between vitamin D and TNF-α (r=0.333, p=0.005) and vitamin D and CRP (r=0.265, p=0.027) were found, but not between vitamin D and BMD.

Conclusions: The presence of low-grade inflammation in Bahraini SCD patients in steady-state is suggested by increased levels of proinflammatory cytokines and CRP. The cytokines (TNF alpha and IL-6) serum levels were associated with hypovitaminosis D. Vitamin D and BMD tests should be offered to all SCD patients for early intervention, if indicated.

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INTRODUCTION

Several studies have reported a high prevalence of vitamin D deficiency (VDD) among patients with sickle cell disease (SCD) (Adewoye *et al.,* 2008, Adla H Bakri, 2013, Buison *et al.,* 2004, Rovner and O'Brien, 2008). Moreover, there appears to be a substantial overlap between symptoms of chronic pain in SCD and VDD, (Lotfi *et al.,* 2007, Straube *et al.,* 2009) and both conditions are associated with increased risk of low bone mineral density (Osunkwo, Osunkwo *et al*.). Vitamin D deficiency as a contributor to multiple forms of chronic pain has been well recognized (Gloth and Greenough, 2004, Holick *et al.,* 2007), although there was no consistent pattern that vitamin D treatment was associated with greater efficacy than placebo in any painful condition (Straube *et al.,* 2009). Vitamin D deficiency in SCD remains both under-recognized and under-treated. In adolescents with SCD, a low-grade inflammation due to increased oxidative stress in relation to elevated resting energy expenditure has been reported (Akohoue *et al.,* 2007). In children with SCD it has been postulated that at steady-state the inflammation was associated

Corresponding author: Adla B. Hassan,
Departmentof Internal Medicine, Arabian Gulf University Kingdom of Bahrain, Manama

with hypermetabolism and was mainly related to C-reactive protein (CRP), but not cytokines (Hibbert *et al.,* 2005). The active form of vitamin D modulates the inflammatory response by inhibiting tumor necrosis factor-α (TNF-α)-induced NF-κB activation, as well as TNF-α-induced VCAM-1 expression (Kudo *et al*.). Furthermore, a novel pathway by which vitamin D inhibits monocyte/macrophage proinflammatory cytokine (IL-6 and TNF-α) production by targeting MAPK phosphatase-1 (MKP-1) has been described (Kudo *et al*.). There is a strong association between vitamin D deficiency and increased serum levels of TNF-α in patients with chronic heart failure secondary to coronary disease (Milovanovic *et al*.). Moreover, it has been shown that vitamin D was required to control TNFα-induced synovial inflammation (van Hamburg *et al*.). On the other hand, the development of senile osteoporosis arises mainly through the effects of estrogen deficiency and secondary hyperparathyroidism (Lane, 2006). However, osteoporosis that arises in young adults, either healthy or with certain diseases such as SCD, is poorly understood. The complexity of how vitamin D modulates the immune system in the pathophysiology of osteoporosis, specifically the role of cytokines, has not been well clarified (Clowes *et al.,* 2005).

We have tested the hypothesis that in acute or chronic imbalance of the immune system in SCD patients due to low vitamin D levels could contribute to bone loss. There are no previous reports testing this hypothesis in Bahraini SCD patients; also the hypothesis whether such an inflammatory state augments the chronic musculoskeletal pain in these patients. In the present study we have evaluated the indicators of inflammatory process and correlated these with bone metabolism markers, as well as, clinical condition of bone damage, in steady-state, in adult Bahraini SCD patients; healthy Bahraini subjects carrying normal hemoglobin genotype (HbAA) were used as control. Pro-inflammatory cytokines (TNF-α and IL-6) and CRP were measured as markers for inflammation, vitamin D as marker for bone metabolism, and BMD as marker for clinical bone damage (osteopenia or osteoporosis).

MATERIALS AND METHODS

Seventy Bahraini patients (42 females) with sickle cell disease (SCD) were included prospectively in this study. Patient's medical records were reviewed to ascertain the HPLC pattern showing presence of HbS, F and A2 and a diagnosis of SCD. All patients were followed at department of oncology at Salmaniya Medical Complex (SMC). The mean age of all patients was 32.27 years (range 18 – 57 years); for females the mean age was 32.45 years (range 18 – 57 years) and males 32 years (range 18 – 50 years). The patients were enrolled over the period between April and October 2012. Patient selection was consecutive from those attending the hematology out-patient clinic in Salmaniya Medical Complex (SMC) for follow up. SMC is the main governmental referral hospital in the Kingdom of Bahrain. Seventy sex-matched and age-matched (1-5 years) healthy Bahraini subjects (42 females) were also included prospectively in this study as the control group. The mean age of the control group was 47.71 years (range 31–70 years). Whereas the mean age of females 46.32 years (range 31– 66 years), the mean age of males was 49.57 years (range 40–70 years).

Except BMD, all other parameters such as vitamin D, TNF-α, and IL-6 were measured in SCD patients (n = 70) in steady state, which defined as a period when the patient is free of infection, pain or other active disease process (Juwah *et al.,* 2004). All parameters were compared with non-anemic, sex-matched normal Bahraini subjects (n = 70). Although BMD service was offered to all SCD patients only 50/70 patients (30 females) underwent the Dual Energy X-Ray Absorptiometry (DEXA) test for BMD determination. The mean age of patients was 31.9 years (range 18 – 50).

The following inclusion criteria were used for enrolling SCD patients: Patients with positive genotypes for sickle cell disease (HbS, F and A2), Bahrainis, adult male or female, age equal to or greater than 18 years old, last blood transfusion equal to or greater than 30 days prior, patients on hydroxyurea treatment (if it cannot be withheld). Exclusion criteria: any chronic co-morbidity (renal, liver diseases, or hypercalcemia), recent hospitalization (in less than 6 weeks), recent blood transfusion (in less than 30 days), high dose current vitamin D therapy, or treatment with other drugs which are known to influence serum vitamin D levels. Serum levels of vitamin D were measured by using Ultra Performance Liquid Chromatography-interfaced with tandem Mass Spectrometry at Al Jawhara Center for Molecular Medicine as described before. (Adla H Bakri, 2013, Diab Diab, 2014) The following published reference range for vitamin D levels were used: normal/optimal (≥50 nmol/l), insufficient (30-49 nmol/l) and deficient (< 30 nmol/l). (Golbahar *et al*., ?). For quantification of serum levels of TNF-α and IL-6, ELISA kits (Creative Diagnostic, USA) were used. BMD measurements were performed at SMC using DEXA at upper/neck femur, forearm and lumbar spine.(Garadah *et al.*, 2015). The diagnosis of osteopenia (T score ≤ -1 and > -2.5) and osteoporosis (T score ≤ -2.5) were made as per WHO criteria (Leslie *et al.,* 2006, Woodson, 2000).

Statistical software, SPSS (Statistical Package of Social Sciences) version 19.0, was used for data analysis. Spearman correlation and Chi-square tests were used to analyze the data. Data are presented as mean ± SD. Two-tailed P value was calculated and a value of ≤ 0.05 was considered significant. The study was approved by the Research and Ethics Committee of the College of Medicine and Medical Sciences, Arabian Gulf University, and the Ethics Committee of the Salmaniya Medical Center, Ministry of Health, Kingdom of Bahrain. All subjects signed an informed consent form (Arabic or English language version) prior to entering the study and a copy was given to patients. This study is part of an ongoing clinical trial registered at the Australia-New Zealand Clinical Trials Registry (No. ACTRN12612000560897), and supported by grant #95/2013-15 from Arabian Gulf University.

RESULTS

Patients with SCD had increased mean serum levels of TNF-α (56.72 pg/ml; range 27.43-98.93) compared to the controls (39.49pg/ml; range 27.56-119.95; p-value<0.005). Furthermore, the patients had increased serum levels of IL-6 (49.05 pg/ml; range 12.27-101.81) compared to the controls (30.08; range 4-275) (p-value=0.002) (Table1 and Table 2).

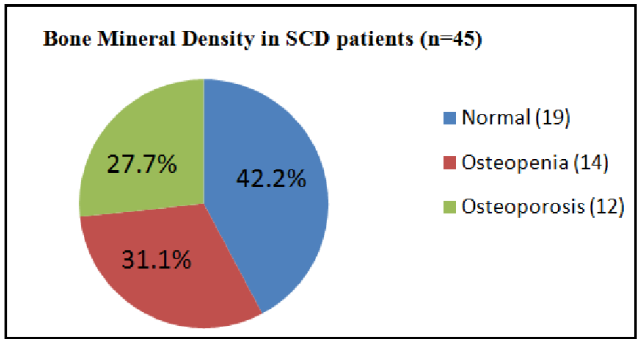

Figure 1. Bone mineral density in sickle cell disease patients

Table 1. Serum levels of vitamin D, TNF-α, IL-6 and CRP in patients with sickle cell disease compared to the controls

Factor	Group	Min.	Max.	Mean ± SD	95% C.I.	P-Value
TNF-α	Patient	27.43	98.93	56.72±22.28	(10.81,	< 0.005
	Control	27.56	119.95	39.49±15.52	23.65)	
IL-6	Patient	12.27	101.81	49.05±16.56	(6.92,	0.002
	Control	4.00	275.00	30.08±48.20	31.01)	
VD	Patient	0.00	68.40	26.08±14.42	(-14.57, -	< 0.005
	Control	7.00	85.20	35.56±16.03	4.38)	
CRP	Patient	0.00	103.00	15.45±21.31	-	-
	Control	-	-	-		
Hb	Patient	7.10	13.20	9.85±1.57	-	-
	Control	-	-	-		
Hct	Patient	0.20	0.43	0.30±0.05	-	-
	Control	-	-	-		

TNF-α=Tumor necrosis factor-alpha, IL-6=Interleukin-6 , CRP= C-reactive protein, VD = 25 hydroxycholecalciferol, Hb = Hemoglobin, Hct = Hematocrit

Table 2. Comparison between vitamin D status and bone mineral density in patients with sickles cell disease

Vitamin D	BMD (n=45)			ND (n=20)
	Osteoporosis	Osteopenia	Normal	
Deficient (n=46)	4	12	10	17
Insufficient n=(20)	5	2	8	3
Optimal (n=4)	3	-	1	-
Total (n=70)	12 (27.7%)	14 (31.1%)	19 (42.2%)	20

BMD = Bone mineral density, ND = Not determined,
VD = 25 hydroxycholecalciferol

Table 3. Pearson Correlations between CRP, vitamin D, TNF-α, and IL-6 in patients with sickle cell disease

	IL-6	CRP	VD
TNF-α	-0.050	-0.041	0.333**
IL-6		-.034	0.007
CRP			0.265*

** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)
TNF-α = tumor necrosis factor-alpha, IL-6=Interleukin-6, CRP = C-reactive protein, VD = 25 hydroxycholecalciferol

Osteoporosis was detected in 26.7% (12/45) of the patients, osteopenia in 31.1% (14/45), while 19/45 (42.2%) patients had normal BMD. The majority of patients (94.3%) had low vitamin D whereas only 5.7% had an optimal. Only, 57.8% of patients had abnormal BMD, and 42.2% had normal BMD (Table 3 and Figure 1). Out of the 26 (57.8%) SCD patients who had abnormal BMD 23 (88.5%) had low vitamin D levels and only 3 (11.5%) patients had optimal vitamin D levels (Figure1). There was a correlation between BMD and serum vitamin D levels, but that was statistically not significant. There was no statistical difference in abnormal BMD between males and females; it was present in approximately 60% of SCD patients in each group (data not shown). A positive correlation was found between vitamin D and TNF-α (r=0.333, p-value=0.005), but also between vitamin D and CRP (r=0.265, p-value=0.027) (Table 4). There is a positive association between BMD and CRP (p-value=0.01; Table 4). However, as mentioned above, no correlation was found between vitamin D and BMD, or between IL-6 and CRP.

DISCUSSION

Vitamin D has effective anti-inflammatory properties (Zhang *et al.*). Up-regulation of vitamin D occurs at later stages of immune activation, thus, providing a late negative feedback loop and down-regulating immune responses (i.e. anti-inflammatory) (Fritsche *et al.,* 2003). Therefore, we investigated the impact of vitamin D on pro-inflammatory markers, namely TNF-α and IL-6 cytokines, which have been implicated in SCD patients, as well as CRP. Furthermore, we also assessed their impact on clinical bone damage (osteopenia/osteoporosis) by BMD measurements. We have reported previously low vitamin D level and low BMD in SCD patients compared with age and sex-matched subjects (either healthy or with other types of hemoglobinopathies). (Adla H Bakri, 2013, Garadah *et al.,* Taysir Garadah, 2014a, Taysir Garadah, 2014b, Taysir S. Garadah, 2014). What is new in our current study is the inflammatory markers (TNF-α, IL-6 and CRP), but also our controls (who are healthy Bahraini subjects), carefully selected for the genetic study (Diab Diab, 2014). It is noteworthy that we matched our patients with sex, but with age (1-5 years). Our rationale for using older subjects as controls is based on a previous study that reported vitamin D deficiency is associated with inflammation-linked vascular endothelial dysfunction in healthy middle-aged and older adults (Jablonski *et al.*). Interestingly, despite the fact that our healthy controls had low vitamin D levels as expected, yet the difference was statistically significant in favor of the patients.

Table 4. Associations between bone mineral density (BMD), TNF-α, IL-6, CRP and vitamin D in Bahraini patients with sickle cell disease (n=45)

		BMD				
		Normal Count (%)	Osteopenia Count (%)	Osteoporosis Count (%)	Total	P-value
TNF	Low	1 (33.3)	1 (33.3)	1 (33.3)	3	0.942
	High	18 (42.9)	13 (31.0)	11 (26.2)	42	
IL 6	Low	4 (66.7)	1 (16.7)	1 (16.7)	6	0.427
	High	15 (38.5)	13 (33.3)	11 (28.2)	39	
CRP	Normal	3 (16.7)	9 (50.0)	6 (33.3)	18	0.014
	Abnormal	16 (59.3)	5 (18.5)	6 (22.2)	27	
VD	Deficient	10 (40.0)	9 (36.0)	6 (24.0)	25	0.205
	Insufficient	8 (50.0)	5 (31.3)	3 (18.8)	16	
	Optimal	1 (25.0)	0 (0.0)	3 (75.0)	4	
Total		19 (42.2)	14 (31.1)	12 (26.7)	45	

TNF-α=Tumor necrosis factor-alpha, IL-6=Interleukin-6, CRP= C-reactive protein, VD = 25 hydroxycholecalciferol

Furthermore, in the present study we found increased serum levels of pro-inflammatory cytokines- TNF-α and IL-6, but also CRP in our Bahraini SCD patients. Our results about cytokines and CRP are consistent with the results of other studies that reported elevated levels of TNF-α, IL-6 and CRP in steady state, in Omani SCD patients (Pathare *et al.,* 2004, Pathare *et al.,* 2003). Raised inflammatory markers found in our study could be related to frequent crises resulting in severe or chronic pain. Vitamin D deficiency in patients with SCD may further augment chronic musculoskeletal pain leading to more frequent painful crisis (Osunkwo *et al*., ?). Vitamin D is considered a hormone; it acts locally as a cytokine, defending the body against microbial invaders (Adams and Hewison, Adams and Hewison, 2008). Thus, our finding of low vitamin D is compatible with observed increase in both TNF-α and IL-6. Although we report strong correlation between bone metabolism (assessed by vitamin D status) and inflammatory condition (assessed by TNF-α and CRP), but not with IL-6. Interestingly, high CRP levels observed in SCD patients did not correlate with IL-6 or with TNF-α. Perhaps our findings may imply that SCD patients share some distinct immunological features with inflammatory diseases. The inverse relation between vitamin D and TNF-α (low vitamin D and high TNF-α) observed in our study suggests that hypovitaminosis D in SCD may be associated with endothelial dysfunction at steady-state; and activation of monocytes that may play a role in pathophysiology of vaso-occlusive crisis in SCD (Zhang *et al*.).

In SCD patients, BMD was found to be low compared to (elderly healthy) controls suggesting bone damage. Low BMD correlated with raised CRP, but not with other pro-inflammatory cytokines. This finding is consistent with a previous report in children with SCD, which revealed that in stable state the inflammation was mainly correlated with CRP but did not correlate with cytokine levels (Hibbert *et al.,* 2005). In contrast, two recent studies from Kingdom of Saudi Arabia found a significant positive correlation between low vitamin D level and reduced BMD among both healthy individuals and patients with SCD (Sadat-Ali *et al.,*?*;* Sadat-Ali *et al*., ?). We did not find this correlation. While vitamin D and CRP, as well as BMD and CRP had good correlation, vitamin D and BMD had poor correlation. Although the BMD results from our analysis should be considered as preliminary based on the limited number of patients who underwent BMD measurement, yet it reflects indirectly the effects of vitamin D on clinical bone damage (osteoporosis) and indicate that vitamin D status could be considered as a biomarker for bone damage in Bahraini SCD patients.

Study Limitations

Only 50/70 SCD patients underwent BMD assessment of whom five patients were excluded due to additional abnormal findings. We also did not evaluate the CRP and BMD for our healthy subjects as we relied upon the standard reference range values for CRP and the WHO criteria for BMD. Moreover, we did not investigate the pro-inflammatory markers (TNF-α, IL-6 and CRP) in sex and age-matched healthy cohort for comparison with current controls. The results of our study should be interpreted with caution because of the age mismatch between study group and controls. It would have been useful to consider kidney function status while interpreting the levels of vitamin D. There are considerable differences in operational definition of the 'steady-state' in SCD (Akinola *et al.,* 1992b, Juwah *et al.,* 2004, Ohene-Frempong *et al.,* 1998). This methodological issue is relevant while comparing the results from different investigators. A contrarian view is that the term 'steady-state' is a misnomer, being characterized by biochemical and rheological fluctuations consistent with minor episodes of micro-vascular occlusion that are insufficient to cause the overt tissue infarction of painful crisis (Akinola *et al.,* 1992a).

Conclusion

Low vitamin D status is associated with an imbalance in cytokine production that results in raised serum levels of TNF-α and IL-6, and C-reactive protein. The presence of low-grade inflammation in Bahraini SCD patients, at steady-state, is characterized by pro-inflammatory cytokines and CRP elevation. Vitamin D, BMD and pro-inflammatory cytokines interact differently with each other. Vitamin D and BMD tests should be offered to all Bahraini SCD patients for early intervention and maintenance of vitamin D levels mainly via supplementation. It would be interesting to further characterize serological phenotypes based on cytokine profile of Bahraini SCD patients in whom musculoskeletal pain is a predominant clinical feature.

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