A Study of Vitamin D Status and Cathelicidine Plasma Levels in Pediatric Population with Sepsis

Ahmed M. Ismail[1], Sherief H. Abdelrahman[1], Ahmad H. Elsayed[1], Gamal A. Alkheshen[2] and Mohamed A. EL Sadaney[1]

Pediatrics[1] and clinical pathology[2], Al-azhar University, Cairo, Egypt

Abstract: Background Sepsis is a major cause of morbidity and mortality in the paediatric population, despite progresses encountered in the last decades. Antimicrobial peptides have been shown to have an important role in the first line of mucosal immunity. Two main families of antimicrobial peptides, the defensins and the cathelicidine (LL-37), are expressed in immune cells and at epithelial surfaces. Deficiency in these peptides results in increased susceptibility to infection. Recent evidence suggests that vitamin D may enhance the innate immune response by induction of cathelicidin (LL-37). Thus, the relationship between vitamin D status and cathelicidine (LL-37) production may be of importance for host immunity, but little data is available on this subject, especially in the setting of neonatal sepsis syndrome and other critical illness. **Objective:** The aim of the study is to assess and correlate vitamin D status and cathelicidine serum levels in infants and children with sepsis and compare it to levels in healthy controls. **Subjects/Methods:** This prospective case control study was conducted on 30 full term neonates (20 of them with proved late onset sepsis and 10 apparently healthy neonates of matched gender and age as control) and 30 children (20 with sepsis and 10 apparently healthy children as control).Blood culture, complete blood count, CRP quantitative assay ,erythrocyte sedimentation rate was carried out for patients. VIT D and Cathelicidine serum level by enzyme linked immunosorbent assay (ELISA) was done for both patients and control group.**Results:** **Results** showed statistically significant differences between patients and controls regarding plasma Vit D, and cathelicidine in both groups of study. Mean plasma 25(OH)D concentrations, and mean plasma LL-37 levels were significantly lower in patients with sepsis compared to healthy controls .In group of neonates mean Vit D was(26.922±11.27 in patients and 50.060 ±15.463 in control with p<0.001*0), while mean cathelicidine was (24.285±8.832 in patients and 35.800±14.639 in control with p0.012*). In the group of children mean vit D level was (24.525±6.561 in patients and 54.328 ± 24.738 in control P<0.001*) , while mean cathelicidine value was(29.850±15.851 in patients and 32.790±8.020 in control with p 0.587). There was a significant positive association between circulating 25(OH)D and LL-37 levels. **Conclusion**: This study demonstrates an association between critical illness(sepsis) and lower 25(OH)D ,and plasma LL-37 in critically ill patients as compared to healthy controls. It also establishes a positive association between vitamin D status and plasma LL-37, which suggests that systemic LL-37 levels may be regulated by vitamin D status.

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1. Introduction:

Vitamin D is a steroid hormone that has long been known for its important role in regulating body levels of calcium and phosphorus, and in mineralization of bone. It is essential for promoting calcium absorption in the gut and maintaining adequate serum calcium and phosphate concentrations to enable normal mineralization of bone and prevent hypocalcemic tetany. It is also needed for bone growth and bone remodeling by osteoblasts and osteoclasts ***(Cranney et al, 2007).*** Vitamin D has other roles in human health, including modulation of neuromuscular and immune function and reduction of inflammation. Many genes encoding proteins that regulate cell proliferation, differentiation, and apoptosis are modulated in part by vitamin D ***(Hayes et al, 2003).*** More recently, it has become clear that receptors for vitamin D are present in a wide variety of cells, and that this hormone has biologic effects which extend far beyond control of mineral metabolism **(HoIick , 2004)**.

Cells of the innate and adaptive immune system including macrophages, lymphocytes and dendritic cells express the vitamin D receptor and respond to stimulation by $1,25(OH)_2D$ (**Adams & Hewison ,2008 , Liu *et al, 2006*)** . Cathelicidin (known as LL-37); is an endogenous antimicrobial peptide active against a broad spectrum of infectious agents including gram negative and positive bacteria, fungi and mycobacteria **(Dürr et al., 2006)**. Cathelicidin is highly expressed at barrier sites including respiratory and colonic epithelium, saliva, and skin and thus provides an important first line defense mechanism for the innate immune system to respond to infectious insults. Cathelicidin are small peptides with

amphipathic structures that allow them to disrupt the integrity of the pathogen cell membrane, resulting in its death. Patients with severe infections as in sepsis have a high prevalence of vitamin D deficiency **(Nierman & Mechanick ,1998.,and Berghe ,2003)**, and high mortality rates **(Angus et al. 2001)** Furthermore, epidemiologic findings have implicated vitamin D insufficiency as a risk factor for sepsis **(Grant , 2009)** . The role of vitamin D treatment in sepsis syndrome has been evaluated in animal models of sepsis where 1, $25(OH)_2D_3$ administration was associated with improved blood coagulation parameters in sepsis associated disseminated intravascular coagulation (DIC) , **(Asakura et al. ,2001)** . Vitamin D treatment in vitro has also been demonstrated to modulate levels of systemic inflammatory cytokines such as TNF-α and IL-6 **(Equils et al. 2006, and Sadeghi et al. 2006)**. These effector functions of vitamin D may be of importance in the pathogenesis of sepsis and sepsis-related DIC, especially when considered together with the potential for vitamin D to enhance anti-microbial peptide production.

The role of vitamin D in sepsis syndrome has not been fully evaluated in humans. Therefore, we performed this cross-sectional study of vitamin D status including plasma levels of 25(OH) D and its relationship to systemic LL-37 levels in a group of critically ill patients with sepsis and normal controls without sepsis.

2. Subjects and methods:

This prospective case control study was conducted on 30 neonates and 30 children recruited from Shobra general hospital and Sayed Galal university hospital.

The group of neonates consisted of 20 cases incubated at NICU with sepsis ,diagnosed by clinical score for predicting neonatal sepsis (Griffin et al.,2007), and proved by culture, 12 male and 8 females ,weighing 1.4 kg to 3.8 kg., and their age ranged between 4 days and 26 days. The control group involved 10 apparently healthy neonates of matched gender and age.

The pediatrics group included 20 children admitted with sepsis, 10 males and 10 females, their ages ranged between 2 and 12 ys, beside a group of 10 healthy children of matched gender and age as controls .

Exclusion criteria:

Cases with affected vitamin level or immunity) by congenital disorders e.g. renal or cardiac……etc, chronic diseases e.g. renal, cardiac, endocrinal ……etc, or medications e.g. steroids, cytotoxic drugs.

All cases were subjected to:

Complete History taking, thorough clinical examination, and laboratory Investigations, including; Complete blood count with differential leucocytic count, C-reactive protein (CRP) done by slide assay using the BIOTEC kit .Erythrocyte sedimentation rate (ESR) and blood culture, 25-dihydroxyvitamin D and Cathelicidine serum level by enzyme linked immunosorbent assay (ELISA). The cathelicidine concentration in each plasma sample was determined using the Human cathelicidine ELISA Test Kit (Hycult Biotechnology, HK321).

Blood samples were taken from patients within 2 days of severe sepsis onset, about 5 mls of venous blood, withdrawn into plain tubes, left to clot for 30 minutes and separated serum was divided into two portions, one for direct assay of serum cathelicidine level, and the other portion was stored at -20°C until assay of 25(OH)D..Vitamin D Insufficiency was diagnosed at vitamin d level < 30 ng/ml.

3. Results:

The results of this work are summarized in the following tables and figures

Table &Figure (1): Plasma 25-dihydroxyvitamin D distribution in studied groups

Table (1)

Neonate Group	Vit D (ng/ml)		T-test	
	Range	Mean±SD	t	P-value
Control	29.3-88	50.060±15.463	7.006	<0.001*
Patients	6.1-50.6	26.922±11.273		

Pediatric Group	Vit D (ng/ml)		T-test	
	Range	Mean±SD	t	P-value
Control	27.8-100.1	54.328±24.738	5.120	<0.001*
Patients	12.4-46.5	24.525±6.561		

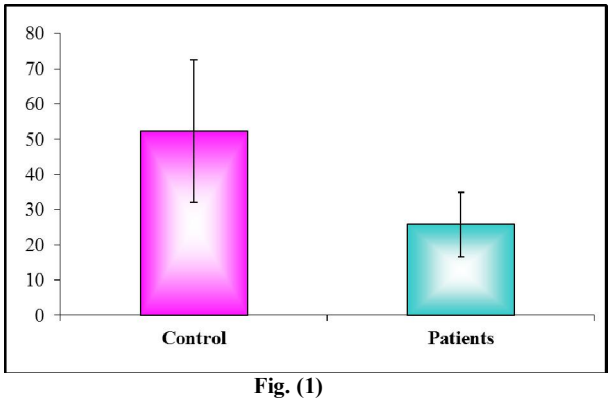

Fig. (1)

Table &fig. (1), show statistically significant lower level of vitamin D p< 0.001 in both neonatal and pediatric patients with sepsis.

Table & Fig. (2):- Cathelicidine distribution in studied groups

Table & Fig. (2) show statistically significant lower level of cathelicidine in patients with sepsis in the group of neonates $p < 0.012$.

Table (2)

Neonatal Group	Cathlecidin (ng/ml)		T-test	
	Range	Mean±SD	t	P-value
Control	19.1-66.5	35.800±14.639	2.694	0.012*
Patients	10.0-42.5	24.285±8.832		

Pediatric Group	**Cathlecidin (ng/ml)**		**T-test**	
	Range	Mean±SD	t	P-value
Control	25.0-48.2	32.790±8.020	0.549	0.587
Patients	10.5-87.5	29.850±15.851		

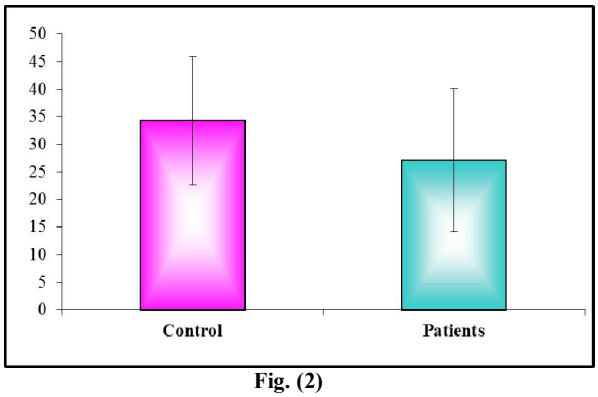

Fig. (2)

Table (3):- Statistical comparison of laboratory data between patients and controls in the group of neonates .

	Neonates							
	Control			**Patients**			**T-test**	
	Mean	±	SD	Mean	±	SD	t	P-value
Age by days	10.900	±	4.954	16.550	±	7.640	-2.117	0.043*
Weight	3.415	±	0.464	2.390	±	0.751	3.936	<0.001*
WBCs	8.900	±	1.370	19.540	±	2.733	-11.536	<0.001*
staff	8.300	±	2.312	22.900	±	3.905	-10.852	<0.001*
segmented	55.700	±	4.855	57.050	±	3.900	-0.824	0.417
i/t ratio	0.134	±	0.018	0.281	±	0.036	-11.976	<0.001*
RBCs	4.800	±	0.397	3.995	±	0.820	2.919	0.007*
HGB	12.620	±	1.168	12.400	±	2.709	0.244	0.809
HCT	37.350	±	2.861	36.965	±	7.974	0.147	0.884
MCV	81.280	±	12.641	92.470	±	8.984	-2.805	0.009*
MCH	28.270	±	4.855	31.065	±	3.657	-1.768	0.088
MCHC	32.240	±	1.427	33.370	±	0.978	-2.556	0.016*
PLT	344.000	±	49.710	145.150	±	85.276	6.783	<0.001*
CRP	4.5	±	1.269	174	±	100.517	-54.895	<0.001*
Vit D	50.060	±	15.463	26.922	±	11.273	4.678	<0.001*
Cathlecidin	35.800	±	14.639	24.285	±	8.832	2.694	0.012*

This table shows statistically significant differences between patients and controls regarding (laboratory signs of sepsis as CRP , WBCs, I/T ratio, staff and platlet count) and also statistically significant differences between patients and controls regarding (Vit D and cathelicidine) in the group of neonates

Table (4):- Statistical comparison of laboratory data between patients and controls in pediatrics group.

	Pediatrics							
	Control			**Patients**			**T-test**	
	Mean	±	SD	Mean	±	SD	t	P-value
Age by years	11.100	±	6.790	8.050	±	7.516	1.080	0.289
Weight	15.050	±	5.505	11.875	±	7.248	1.217	0.234
WBCs	9.016	±	1.267	18.500	±	1.850	-14.538	<0.001*
staff	8.900	±	2.183	23.550	±	4.925	-8.917	<0.001*
segmented	55.900	±	4.557	57.450	±	5.404	-0.778	0.443
i/t ratio	0.132	±	0.032	0.285	±	0.037	-11.192	<0.001*
RBCs	4.980	±	0.123	4.627	±	0.628	1.746	0.092
HGB	12.150	±	0.502	12.180	±	2.128	-0.044	0.966
HCT	36.928	±	1.419	35.615	±	6.143	0.662	0.514
MCV	77.130	±	1.653	78.400	±	4.492	-0.859	0.398
MCH	25.801	±	1.384	26.675	±	1.622	-1.456	0.156
MCHC	31.921	±	1.037	33.700	±	0.906	-4.835	<0.001*
PLT	406.020	±	16.429	270.600	±	115.628	3.654	<0.001*
CRP	6.500	±	1.269	143	±	100.524	-65.349	<0.001*
Vit D	54.328	±	24.738	24.525	±	6.561	5.120	<0.001*
Cathlecidine	32.790	±	8.020	29.850	±	15.851	0.549	0.587

This table shows significant differences between patients and controls regarding (laboratory signs of sepsis as CRP-,WBCs, I/T ratio, staff and platlet count), and also statistically significant differences between patients and controls regarding Vit D p < 0.001.

Table (5):- Correlation between vitamin D, Cathelicidine in both neonatal and pediatric group

Neonates	Vit D	
	r	P-value
Cathlecidine	0.714	<0.001*

Pediatrics	Vit D	
	r	P-value
Cathlecidine	-0.001	0.997

This table shows significant positive correlation between Vit D and Cathilicidine

4. Discussion:

Recent studies suggest that vitamin D may have other actions outside of its classic functions related to bone and calcium homeostasis *(Holick MF 2007*).

Cathelicidine is an endogenous antimicrobial peptide active against a broad spectrum of infectious agents including gram negative and positive bacteria, fungi and mycobacteria *(Dürr UH,et al 2006*). Cathelicidine has been demonstrated to possess multiple other immunoregulatory functions, from chemoattraction of inflammatory cells, to promotion of wound healing, and regulation of angiogenesis (Ramanathan *B et al, 2002*). Deficiency in these peptides results in increased susceptibility to infection.

The present study aimed to assess vitamin D status and cathelicidine in infants and children with sepsis and was conducted on 30 full term neonates (20 of them with proved late onset sepsis and 10 apparently healthy neonates of matched gender and age as control) and 30 children (20 with sepsis and 10 apparently healthy children as control).

All cases included in the study were subjected to full medical history with special emphasis on symptoms and signs of sepsis and thorough clinical examination. Also laboratory investigations including:Complete blood count with differential leucocytic count,CRP quantitative assay,Erythrocyte sedimentation rate (E.S.R),Blood culture, VIT D and Cathelicidine plasma level by enzyme linked immunosorbent assay (ELISA).

The results obtained from this study showed statistically significant differences between patients and controls regarding Vit D and cathelicidine in both the neonatal group and the pediatric group. Mean plasma 25(OH)D concentrations, and mean plasma LL-37 levels were significantly lower in patients with sepsis compared to healthy controls .In group of neonates mean Vit D was(26.922±11.27 in patients and 50.060 ±15.463 in control with p<0.001*0), while mean cathelicidine was (24.285±8.832 in patients and 35.800±14.639 in control with p0.012*). In the group of children mean vit D level was (24.525±6.561 in patients and 54.328 ± 24.738 in control P<0.001*) , while mean cathelicidine value was(29.850±15.851 in patients and 32.790±8.020 in control with P0.587). There was a significant positive association between circulating 25(OH) D and LL-37 levels.

Vitamin D insufficiency is a common condition in patients admitted to the intensive care unit, (Berghe et al. 2001,) . In the present study we found that; 75% of patients with sepsis had vitamin D deficiency, while in control subjects 10% only had vitamin D deficiency.

Leo Jeng et al (2009), in a similar study in adults found that > 95% of their critically ill patients had vitamin D insufficiency.

When they examined plasma levels of the endogenous antimicrobial peptide LL-37 in relationship to 25(OH) D, they found that lower levels of 25(OH)D were also associated with lower systemic levels of LL-37.

This association supports recent *in vivo* data that vitamin D plays some roles in regulating the production of antimicrobial peptides such as LL-37 in cultured macrophages (Liu et al. 2006). Since many cells of the immune system possess the vitamin D receptor, vitamin D status may prove to be an important factor in management of sepsis syndrome and other critical illness.

Recent evidence suggests that vitamin D may also play an important role in enhancing innate immunity against infection. Liu et al(2006) demonstrated that 1,25(OH)2D3 treatment of macrophages infected with *Mycobacterium tuberculosis* in vitro resulted in enhanced production of an endogenous anti-microbial peptide, cathelicidin or LL-37, and in improved killing of the microorganisms . Administration of LL-37 has been demonstrated to be protective in rodent models of sepsis,(Kirikae et al.1998 ,and Cirioni et al., 2006) . A recent randomized, placebo controlled trial of vitamin D supplementation in patients with pulmonary tuberculosis in Indonesia demonstrated significantly higher sputum conversion rates at earlier time points in the group randomized to receive vitamin D compared to the group assigned placebo,(Nursyam et al. , 2006) . A smaller study of post-menopausal women also suggested that vitamin D may have activity against influenza, (Aloia & Li-Ng, 2007) . No prospective clinical study has confirmed that intervention with vitamin D would raise LL-37 concentrations and improve activity against infection.

Wayse et al., 2004 studied the association of subclinical vitamin D deficiency with severe acute lower respiratory infection in Indian children under 5 years and cocluded that subclinical vitamin D deficiency is a significant risk factor for severe infections in Indian children.

On the other hand, Roth et al.(2009) who studied the association between vitamin D status and susceptibility to infection in young Canadian children found serum 25(OH)D concentrations were similar among cases and controls, and concluded That vitamin D status was not associated with the risk of hospitalization for sepsis.

Similar findings were recorded by McN ally et al.(2009) they found no difference between patients with infection and control group.

Roth et al. (2009) explained the divergent findings between their results and the Indian study may be due to different infection epidemiology as bacterial infection is common in developing countries, whereas viral infection predominates in developed countries. The active vitamin D metabolite may exert antimicrobial actions that reduce susceptibility to bacterial infection but one of its dominant immunomodulatory effects is a shift towards T helper cell (TH)-2, which may not be beneficial in the response to viral infections, the severity of which is inversely proportional to the (Th)-1 response.

Our cross-sectional study design does not allow us to determine whether restoring vitamin D status to optimal levels would increase LL-37 levels systemically or result in improved immunity against infection. It is unknown at this time whether circulating levels of LL-37 translate directly into antimicrobial activity.

In conclusion, we have determined that nearly all critically ill patients we studied had sub-optimal vitamin D status and a higher rate of vitamin D insufficiency compared to healthy subjects. This finding is associated with lower systemic levels of LL-37, a vitamin D dependent antimicrobial peptide which appears to have multiple effector roles within the immune system.

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