A Case-control Study of Urinary Tract Infection, 25-Hydroxyvitamin D Status and Associated Inflammatory and Regulatory Responses

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A B S T R A C T

Background: Vitamin D plays a key role in the modulation of numerous immune functions against infectious agents. We aimed to explore the association between serum 25-hydroxyvitamin D (25[OH] D) levels and cytokine responses, along with hematological changes, in patients with urinary tract infection (UTI).

Materials and Methods: Vitamin D level, cytokines (interferon [IFN]−γ, interleukin [IL]−4, IL−6, IL–10, IL−17A, tumor necrosis factor [TNF]−α, and transforming growth factor [TGF]−β), hematological indices (neutrophil-to-lymphocyte ratio [NLR], monocyte-to-lymphocyte ratio [MLR], neutrophil-to-monocyte ratio [NMR], platelet-to-lymphocyte ratio [PLR], and mean platelet volume [MPV]), C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR) were evaluated in a case-control human study included 65 patients and 45 controls.

Results: Among the enhanced cytokine levels in patients, the cytokines IFN-γ, IL-17A, and IL-10 had a significant association with 25(OH)D, but not IL-6, TNF-α, and TGF-β. The IL-4 levels remained unchanged. By comparing hematological indices, we found the association of increased NLR and MLR with 25(OH)D and the cytokines IFN-γ and IL-17A, along with a decrease in the PLR without showing such an association. The NMR did not show any significant difference. The platelet count showed an association with IL-6, IL-17A, and TGF-β, but the association of MPV with 25(OH)D was significant. The ESR results exhibited statistically non-significant differences. CRP elevation was directly associated with IL-6 and IL-17A, but not with 25(OH)D.

Conclusion: 25(OH)D-mediated inflammatory cytokine milieu might alter the proportion and function of peripheral blood cells in a regulated manner to support bacterial clearance which needs further studies to be validated.

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1. Introduction

Vitamin D affects both innate and adaptive immunity. Immune cells express the vitamin D receptor (VDR) and can metabolize 25(OH)D to the active form 1,25(OH)2D3, suggesting its importance in the immune response to infection [1, 2]. Urinary tract infection (UTI), primarily caused by uropathogenic *Escherichia coli* (UPEC), is a common bacterial infection that include cystitis (bladder infection) and pyelonephritis (kidneys infection) [3].

The augmentation of host defense relies on the infiltration of phagocytes and CD4+ T helper (Th) cells involved in controlling infection, in part, via distinct cytokines response [4]. The polarized T helper type 1 (Th1) and Th17 responses with associated release of interferon (IFN)-γ and interleukin (IL)-17A increase bacterial clearance [5, 6]. Similarly, the cytokines IL-6 and tumor necrosis factor (TNF)-α promote the establishment and development of immune inflammatory responses [7]. On the other hand, the anti-inflammatory/immunosuppressive cytokines, such as IL-4, IL-10 and transforming growth factor (TGF)-β, is essential to attenuate infection- and inflammation-related injury of the host tissue. Alternatively, the TGF-β induces the Th17 phenotype, in a synergistic action with IL-6, and has a pro-fibrotic function [8, 9]. Infection and inflammatory state is thought to be reflected by alterations in the circulating quantity and function of various leukocytes, as well as platelets that may occur partly following the resultant cytokine milieu [10-12].

With this background, we aimed to evaluate the association between 25-hydroxyvitamin D [25(OH)D] levels and several variables, including serum concentrations of the signature cytokines of Th1 (IFN-γ), Th2 (IL-4), Th17 (IL-17A), Treg (IL-10 and TGF-β) responses, as well as innate inflammatory cytokines TNF-α and IL-6, and associated hematological markers of systemic inflammation between UTI patients and controls.

2. Materials and Methods

Study subjects

Ninety women 20-60 years old with urine culture proven UTI who attended outpatients clinics of Ardabil University of Medical Sciences, Ardabil, Iran and 45 age-matched healthy women as the control group were included in the present study. Sixty-five of these patients had *E.coli* isolated from their urine samples and they were selected for further analyses. The sample size was calculated using the software OpenEpi (http://www.openepi.com) with α level of 0.05 and a power of 80%. A diagnosis of lower urinary tract infection (cystitis) in those patients with clinical symptoms of dysuria, the urgency and frequency of urination, and or suprapubic pain, was confirmed by urine analysis and a positive urine culture (growth of >105 CFU/mL of a single pathogen in a midstream urine sample). Those with diabetes mellitus, renal failure, liver disease, gastrointestinal disease, asymptomatic bacteriuria, kidney stones, gynecological problems, immunosuppressive diseases, anemia, chronic inflammatory disease, glucocorticoids therapy, and antibiotic therapy were excluded from the study. The study was approved by the institutional local Ethics Committee (IR.IAU.TABRIZ.REC.1396.83). Informed consent was obtained from all the study subjects.

Laboratory analysis

Blood samples were taken from the study subjects at the end of winter and during spring 2019, and all the sera were obtained from blood samples and stored at −80°C for further analysis. Hemograms were performed with an automated hematology analyzer (Sysmex Corporation, Kobe, Japan). Serum 25(OH)D levels were quantified by using the EUROIMMUN kit (Mediziische Labordiagnostika AG, Lübeck, Germany) according to the manufacturer's instructions. The levels of IFN-γ, IL-4, IL-17A, IL-10, IL-6, TNF-α and TGF-β cytokines were measured using commercially available ELISA Kit (U-CYTech Biosciences, Utrecht, the Netherlands) according to the manufacturer's instructions. Serum levels of cytokines were expressed in pg/mL, and 25(OH)D was expressed in ng/mL. The CRP (mg/dL) and ESR (mm/h) measurement was carried out using immunoturbidimetric assay and the Westergren method, respectively. Complete blood count (CBC)-derived parameters, such as NLR, NMR, MLR, and PLR, were calculated as the ratio of neutrophil to lymphocyte counts, the ratio of neutrophil to monocyte counts, the ratio of monocyte to lymphocyte counts, and the ratio of platelet to lymphocyte counts, respectively.

Statistical analysis

The Kolmogorov-Smirnov test was used to determine whether the data were normally distributed. For comparing variables between the patient and control groups, independent t-test for normally distributed continuous variables, and the Mann–Whitney U test for non-normally distributed continuous variables were used. The rela-

tionship between the continuous variables and 25(OH)D levels was determined using the Pearson's correlation analyses. Subsequently, variables that were significantly associated with 25(OH)D were analyzed by logistic regression to predict serum 25(OH)D status (insufficient [<30 ng/mL] and sufficient [>30 ng/mL]). The obtained data were expressed as mean or median (inter-quartile range). A P value of less than 0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism 5.04 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

Study population characteristics

The study included 65 female patients diagnosed with UPEC cystitis and 45 sex- and -age matched healthy controls. The patients had the typical symptoms (dysuria, urgent/frequent urination, and or suprapubic pain), pyuria (≥10 leukocytes per high power field [HPF]), and bacteriuria (≥105 CFU/mL) of uropathogenic *E. coli* (UPEC). The healthy controls had negative urine culture and no evidence of UTI symptoms.

There was no significant difference between the two groups with Mean±SD age of 36.21±8.13 vs 34.86±6.09 years, P=0.214. Compared to controls, patients showed a statistically insignificant difference when serum 25(OH)D levels were compared between these groups with Mean±SD of 28.09±10.63 vs 31.54±12.75 ng/mL, P=0.309. According to 25(OH) D levels, 19 patients were 25(OH)D deficient, i.e., 25(OH)D concentration <20 ng/mL, 16 patients had insufficient levels of 25(OH)D concentration of between 20 and 30 ng/mL, and 30 patients had 25(OH)D sufficient concentration of >30 ng/mL.

Comparison of cytokines response and hematological parameters between the patient and control groups

A specific polarization cytokine milieu mediates a proinflammatory and/or anti-inflammatory response following infection with a pathogen. The assessment of serum cytokine concentrations indicated an elevation of IFN-γ, IL-17A, IL-10, IL-6, TNF-α, and TGF-β in patients with no statistically significant difference regarding the IL-4 levels, as compared to the controls (Table 1).

With regard to hemogram values, the White Blood Cell (WBC), Red Blood Cell (RBC) and platelet (PLAT) indices were compared between these two groups. Among the studied variables, the counts of WBC, neutrophil,

monocyte and the values of mean platelet value (MPV), NLR, MLR and MPV/PLAT were significantly higher in the patient group than in controls while the platelet count and PLR value decreased significantly in comparison between the two study groups. Also, we did not observe significant differences between the two groups with respect to red cell indices and the values of platelet distribution weight (PDW) and NMR. The comparison of the CRP and ESR results showed that although the ESR values were not different between these two groups, the patients had an increase in the CRP levels as compared to controls (Table 2). In the analysis of serum CRP concentration, CRP values < 10 mg/dL were found in 29.2% of patients and 62.2 % of controls. The patients with CRP values between 10-30 mg/dL were 55.4% compared to 22.2% in the controls. The CRP values >30 mg/dL were observed in 15.4% and 15.6% of both patients and controls, respectively. Approximately 63% and 67% of women under 50 years old in both patients and controls had ESR levels of less than 20 mm/h. The ESR was less than 30 mm/h in 37% and 33% of women over 50 years old in the patient and control groups, respectively.

These findings might provide evidence of conditions associated with Th1 and Th17 polarization cytokine responses, partly contributing to the increased numbers of neutrophils and monocytes in the peripheral blood as reflected by an elevation of NLR and MLR values. A decrease in the PLR resulted from the decrease in platelet count seems to be due to platelet consumption during inflammation.

Serum 25(OH)D levels, associated cytokines response and hematological indices

We conducted correlation analysis to investigate further the association between 25(OH)D and cytokine levels. The following cytokines showed significant correlations with 25(OH)D: IFN-γ (r=0.50, P=0.000), IL-17A (r=0.34, P=0.006), and IL-10 (r=0.33, P=0.007). Such correlations were not found for other cytokines in patients. The patient group was compared according to insufficient (<30 ng/mL) and sufficient (>30 ng/mL) 25(OH)D status. Serum levels of the IFN-γ, IL-17A, and IL-10 cytokines differed significantly between patients with serum concentration of 25(OH)D <30 ng/mL and >30 ng/mL, while such significant differences were not observed in terms of IL-4, IL-6, TNF-α, and TGF-β (Figure 1). Also, logistic regression analysis showed that individuals with sufficient 25(OH)D had a greater probability of changes in serum levels of IFN-γ (odds ratio [OR]: 1.26, 95% confidence interval [CI]: 1.18-2.02), IL-17A (OR: 1.67, 95% CI: 1.15-3.42), and IL-10 (OR:

Table 1. Serum levels of cytokines (Mean±SD) in UTI Patients (n=65) and Controls (n=45)

Variables	Mean±SD		P
	Patient Groups	Control Groups	
IFN-γ (pg/mL)	393.26±31.09	249.35±15.15	<0.0001
IL-4 (pg/mL)	46.81±10.59	49.21±8.23	0.836
IL-6 (pg/mL)	29.39±10.96	20.64±9.60	0.0008
IL-10 (pg/mL)	347.28±32.21	288.90±26.79	0.0014
IL-17A (pg/mL)	57.10±5.56	36.09±5.08	<0.0001
TNF-α (pg/mL)	44.01±4.95	29.20±4.93	0.0005
TGF-β (pg/mL)	38.73±9.87	29.90±9.04	0.0002

IL: Interleukin; TGF: Transforming Growth Factor; IFN: Interferon; TNF: Tumor Necrosis Factor; UTI: Urinary Tract Infection.

1.09, 95% CI: 1.02-1.38) compared to 25(OH)D insufficiency. It seems likely that sufficient levels of vitamin D mediate VDR-regulated target gene expression since it modulates induction of both Th1 and Th17 polarizing cytokines milieu following the activation of toll-like receptor (TLR) signaling through pathogen recognition.

Considering peripheral blood-derived hematological indices, there was significant correlations between NLR (r=0.70, P=0.000) and MLR (r=0.64, P=0.000) with 25(OH)D, while both PLR and NMR were not significantly correlated with 25(OH)D. Despite insignificant correlations between 25(OH)D and the platelet count together with its related indices such as PLR and PDW, MPV showed a significant correlation with 25(OH)D (r=0.28, P=0.021) (Table 3). This finding may suggest the production of the large platelets and their activity as well. Similarly, there were no statistically significant differences in terms of CRP and ESR levels (r=0.22, P=0.284; r=−0.15, P=0.157, respectively).

An elevation in the levels of the above-mentioned variables may propose the role of serum 25(OH)D levels, as an environmental modulator agent, in supporting the preparation of an inflammatory milieu involved in host defense of bacterial infection. However, increased synthesis of IL-10 with concomitant production of TGF-β1 might reflect the need for the regulation of induced immune response.

The association of immune/inflammatory cytokines and hematological indices

Meanwhile, the possible correlations between serum cytokine levels of patients and hematological indices were examined (Table 3). We found a significant correlation between the MLR and IL-17A. With regard to the NLR, it was correlated not only with IL-17A but also with IFN-γ. The IFN-γ and IL-17A cytokines appear to be involved in the recruitment of circulating monocyte and neutrophil into the site of inflammation and their activation to produce these cytokines which may subsequently create a distinct response by migrated T lymphocyte. For both PLR and NMR, we could find insignificant correlations. Moreover, although there was an inverse correlation between the platelet count and serum levels of IL-17A and TGF-β, it was found to have a positive correlation with the IL-6 levels. Similarly, the PDW also showed statistically significant correlations with the IL-17A, IL-6, and TGF-β. The MPV was positively correlated with 25(OH)D despite insignificant differences between the above cytokines. We might speculate that IL-6, in cooperation with 25(OH)D, helps to compensate the consumption of platelets due to their migration to the site of inflammation, thereby triggering thrombocytosis characterized by larger and more activated platelets. Of note, the TGF-β has a possible regulatory effect on platelets under inflammatory conditions. The assessment of correlation between CRP and the IL17A and IL-6 cytokine levels, the representative cytokines of the Th17 cell commitment, indicated that they are correlated positively (r=0.40, P=0.001; r=0.27, P=0.030), whereas such a significant correlation was not found between CRP and 25(OH)D (r=0.08, P=0.509), as well as the other cytokines mentioned in this study (P> 0.05). Thus, the prominent Th17 response may be responsible for regulating infection-related platelet and acute phase responses.
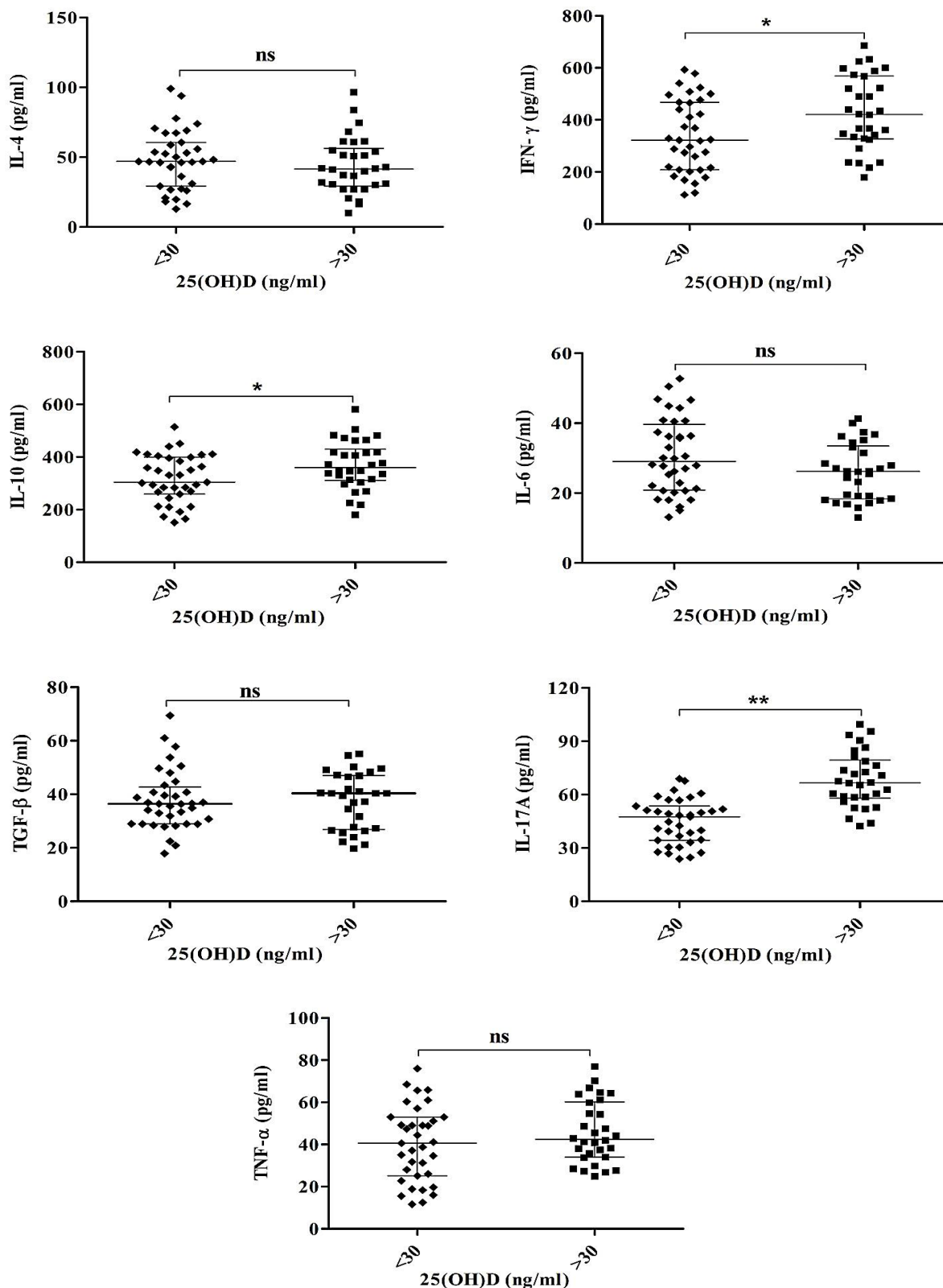
Figure 1. Comparison between serum levels of pro-inflammatory and regulatory cytokines and serum 25(OH)D

Status of patients with <30 ng/mL and >30 ng/mL; the data displayed as median with interquartile range; *P<0.05; **P<0.01 and NS: Not Significant.

Table 2. Comparison of Laboratory Parameters (mean±SD) between UTI Patients (n=65) and Controls

Variables		Mean±SD		P
		Patient Group	Control Group	
	Vitamin D (ng/ml)	28.09±10.63	31.54±12.75	0.309
White blood cell indices	WBC (103/mm³)	7.76±2.76	6.27±1.89	0.040
	Neutrophil (×10⁹/L)	4.81±1.54	3.45±1.15	0.012
	Lymphocyte(×10⁹/L)	2.25±0.63	2.73±0.56	0.247
	Monocyte (×10⁹/L)	0.70±0.15	0.47±0.09	0.023
Red blood cell indices	RBC (×10¹²/L)	4.52±0.40	4.50±0.30	0.180
	Hb (g/dL)	13.08±0.65	13.11±0.88	0.135
	MCV (fL)	86.78±4.50	87.78±3.89	0.083
	RDW (%)	12.63±0.86	12.90±1.44	0.128
Platelet indices	Platelet (10³/mm³)	240.55±50.17	315.40±46.40	0.034
	MPV (fL)	10.20±1.87	9.18±2.63	0.040
	PDW (%)	10.99±1.67	11.10±2.10	0.220
	MPV/PLAT	0.042±0.002	0.029±0.003	<0.001
Inflammatory indices	NLR	2.14±1.01	1.26±0.48	0.023
	PLR	106.91±40.35	115.53±32.90	0.042
	MLR	0.31±0.44	0.17±0.20	0.030
	NMR	6.87±6.40	7.34±4.81	0.487
	CRP (mg/dL)	20.10±7.43	10.49±6.31	<0.001
	ESR (mm/h)	22.84±11.40	20.43±9.76	0.112

Hb: Hemoglobin; WBC: White Blood Cell; RBC: Red Blood Cell; MCV: Mean Corpuscular Volume; RDW: Red Distribution Width; PLAT: Platelet; MPV: Mean Platelet Volume; PDW: Platelet Distribution Width; NLR: Neutrophil-to-Lymphocyte Ratio; PLR: Platelet-to-Lymphocyte Ratio; MLR: Monocyte-to-Lymphocyte Ratio; NMR: Neutrophil-to-Monocyte Ratio; CRP: C-reactive Protein; ESR: Erythrocyte Sedimentation Rate; UTI: Urinary Tract Infection.

4. Discussion

Vitamin D is one of the potential contributors to modulate immune-inflammatory responses [13]. A consideration of alterations in humoral and cellular factors of both innate and adaptive immunity appears to characterize significantly the type of immune response [14]. Thus, we were interested in evaluating whether serum 25(OH)D status may be associated with serum levels of representative cytokines of the Th17 (IL-17A), Th1 (IFN-γ), Th2 (IL-4), and Treg (IL-10 and TGF-β) cells commitment, as well as innate inflammatory cytokines TNF-α and IL-6, and peripheral blood-derived hematological

indices in women with bacterial cystitis. Both CRP and ESR were also analyzed as markers of inflammation associated wiyh 25(OH)D levels. Probably, these results may provide the view of a differential regulatory role of vitamin D in inflammatory and immune responses in response to UPEC-mediated UTI.

Significantly higher levels of pro-inflammatory cytokines of IFN-γ, IL-17A, IL-6, and TNF-α with anti-inflammatory cytokines of IL-10 and TGF-β were observed in the patients than those in the controls. However, the production of IL-4 known as the Th2-polarizing cytokine showed an insignificant difference. Among pro-

Table 3. Correlation analysis of hematological indices with 25(OH)D and cytokines in UTI patients

Variables		25(OH)D	IFN-γ	IL-4	IL-10	IL-17A	IL-6	TNF-α	TGF-β
WBC	r	0.498	0.343**	−0.111	0.01	0.194	−0.137	−0.182	0.076
	P	0.308	0.05	0.379	0.995	0.121	0.275	0.146	0.545
NLR	r	0.705**	0.471**	0.076	0.382	0.246**	−0.156	0.115	−0.156
	P	0.0	0.0	0.550	0.079	0.040	0.215	0.361	0.214
MLR	r	0.640**	0.225	0.019-	0.187	0.397**	−0.159	−0.037	−0.045
	P	0.0	0.072	0.883	0.135	0.01	0.206	0.769	0.723
NMR	r	0.020	0.094	0.035	0.022	0.02	-0.136	0.201	0.04
	P	0.871	0.456	0.780	0.863	0.990	0.281	0.109	0.977
PLR	r	−0.225	−0.074	0.145	−0.179	−0.177	0.054	0.194	−0.072
	P	0.072	0.557	0.250	0.154	0.158	0.672	0.122	0.568
PLAT	r	−0.178	−0.168	−0.057	0.033	−0.296*	0.350**	−0.208	−0.258*
	P	0.156	0.182	0.651	0.796	0.017	0.04	0.096	0.038
MPV	r	0.285*	−0.152	−0.150	−0.20	−0.029	0.171	0.074	0.138
	P	0.021	0.228	0.234	0.110	0.819	0.174	0.557	0.272
PDW	r	−0.178	−0.168	−0.057	0.033	−0.296*	0.350**	−0.208	−0.258*
	P	0.156	0.182	0.651	0.796	0.017	0.04	0.096	0.038

IL: Interleukin; TGF: Transforming Growth Factor; IFN: Interferon; TNF: Tumor Necrosis Factor; NA: Non-applicable; WBC: White Blood Cell; PLAT: Platelet; MPV: Mean Platelet Volume; PDW: Platelet Distribution Width; NLR: Neutrophil-to-Lymphocyte Ratio; PLR: Platelet-to-Lymphocyte Ratio; MLR: Monocyte-to-Lymphocyte Ratio; NMR: neutrophil-to-Monocyte Ratio; UTI: Urinary Tract Infection; *P<0.05; **P<0.01.

inflammatory cytokines, the production of IFN-γ (Th-1cytokine signature), IL-17A (Th17 cytokine signature), and IL-10 had an association with sufficient 25(OH)D status. For other cytokines, such significant associations were not observed. Induction of the IFN-γ and IL-17A cytokines has been reported to generate a protective immune response against infection with UPEC [15]. The observation that NLR and MLR have increased may reflect elevated IFN-γ and IL-17A production by Th1 and Th17 cells associated with the infiltration of neutrophils and monocytes to the infected bladder. It commonly happens during the early stages of infection because of their crucial roles in UPEC clearance [16-18]. Alternatively, neutrophils and macrophages themselves may be essential sources of IFN-γ and IL-17A [19, 20].

Furthermore, an IL-6 increase is proposed to promote the Th1 and Th17 polarization in a synergistic action with TNF-α and TGF-β, respectively. This cytokine up-

regulates the induction of an antibody response as well [8, 21]. In UPEC infection, the protective role of IL-6 together with TNF-α has been demonstrated to enhance bacterial clearance by regulating TGF-β and endogenous anti-microbial peptides expression [22]. Substantially, the IL-6 and IL-17A have synergistic effects on the promotion of serum CRP levels which may explain the observed correlation between CRP and these cytokines [23, 24]. CRP itself is believed to increase the activation of NF-κB- and AP1-mediated expression of inflammatory genes [25]. Based on the results, the platelet count had a significant direct association with the IL-6 levels, suggesting its regulatory role in increased platelet count and function [26, 27]. On the other hand, the levels of IL-17A and TGF-β showed an inverse relationship with the platelet count. Recent studies have reported that platelets have the regulatory effects on Th17 cell differentiation and related functions, in part through inhibition of TGF-β signaling [28, 29]. Moreover, the changes in the value of MPV

that had a direct association with 25(OH)D may suggest the platelet VDR signaling required to release larger and more reactive platelets than smaller ones as a reaction to infection-related platelet consumption [30, 31].

Apart from the role of TGF-β in the differentiation of Th17 cells, this cytokine, along with IL-10, is known as an immunosuppressive cytokine produced by Tregs. Recently, TGF-β-mediated up-regulation of the expression of VDR has been suggested to indirectly induce the expression of IL-10 [32, 33]. An elevation of IL-10 production seems to reveal its involvement in the innate response to control UTI [34]. Thus, the simultaneous existence of these pro- and anti-inflammatory cytokines would be necessary for the regulation of immune and inflammatory responses involved in pathogen clearance to attenuate the possible detrimental effects on the host tissue by keeping homeostasis.

A single gender-matched case-control study with a small sample size may limit the generalizability of our findings. The parameters were evaluated based on a single measurement. Therefore, it remains to be seen whether they will change or not over time. Also, a possible impact of any unmeasured confounding variable should be considered on the observed associations. The findings revealed the direction and strength of the association between 25(OH)D and cytokines responses. On the other hand, interventional and or mechanistic studies exploring a potential direct effect of vitamin D on immune/inflammatory response in UTI are needed to highlight further the associations found in the present study.

5. Conclusion

The findings provide evidence that a sufficient amount of vitamin D may contribute to a linkage between innate and adaptive immunity in a regulated manner as reflected by the Th1 and Th17 polarization cytokine responses. This event is partly responsible for a significant increase in the number of phagocytes (neutrophils and monocytes) and thrombocytes reactivity in the peripheral blood. Accordingly, vitamin D is suggested to influence an inflammatory milieu to promote the host defense functions. The maintenance of immune homeostasis might be mediated by direct and or indirect effects of vitamin D on regulatory cytokines production to attenuate an infection-associated inflammation-related tissue injury. However, there is a complexity in the initiation or resolution of innate and adaptive immune responses in response to infectious stimuli that should be further evaluated.

Ethical Considerations

Compliance with ethical guidelines

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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