Ministry of higher education and scientific research

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"The Immunomodulatory Markers in Patient with Chronic Urinary Tract Infection and Brucellosis "

A graduation project submitted to the college of pharmacy /Babylon University as partial fulfillment of the requirement of the BSc degree in pharmacy

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بسِـــــمِ اللـَّهِ الرَّحْمَـٰنِ الرَّحِيمِ

يرْفعِ اللَّه اللذِينَ آمَنوُا مِنكُمْ وَاللذِينَ أوُتوُا الْعِلْمَ دَرَجَاتٍ واللَّه بِمَا تعْمَلوُنَ خَبيِر

صدق الله العلي العظيم

الاية ﴿11﴾ سورةالمجادلة

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TABLE OF CONTENTS

List of figure & tables

figure: -

tables: -

علامات تعديل الجهاز المناعي لدى مرضى التهاب المسالك البولية المزمنة وداء البروسيلات

الخلاصة:

الخلفية والأهداف:

الحمى المالطية هو مرض حيواني المنشأ تسببه أنواع البروسيلا. هذا المرض منتشر في العراق وانتشاره في ازدياد. تقيس هذه الدراسة مستويات فيتامين د في الدم ، و IL-6 و TNF-α في المرضى الذين يعانون من عدوى المسالك البولية المزمنة وداء البروسيلات.

المواد والأساليب:

تم إجراء هذا البحث بمدينة الحلة العراقية. شمل حجم العينة المحسوب 92 مريضا يعانون من التهاب المسالك البولية المزمنة وداء البروسيلات. تم إجراء قياس مستويات فيتامين د ، IL-6 و TNF-α بواسطة ELISA.

النتائج:

تم تصنيف متوسط مستوى فيتامين د في الدم إلى ثلاث فئات (عادي = 32.81 نانوغرام / مل ± 7.61 ، ضعيف كاف = 18.22 نانوغرام / مل ± 6.89 وغير كاف = 7.56 نانوغرام / مل ± 4.18). (يُقبل مستوى فيتامين د في الدم <10 نانوغرام / مل على أنه نقص ، 10-30 نانوغرام / مل على أنه قصور ، 30-100 نانوغرام / مل كفاية ، و> 100 نانوغرام / مل كسمية). كان مدى مستويات IL-6 **هو 133.94-438.90 بيكوغرام / مل بمتوسط مستوى 389.45 ± 70.88 ومرتبط بنسبة 66٪ من المشاركين أعلى من النطاق الطبيعي. كان مدى مستويات** TNF-α 78.51-162.31 **بيكوغرام / مل بمتوسط مستوى 118.78 ± 44.11 ومرتبط بنسبة 43٪ من المشاركين أعلى من النطاق الطبيعي**

استنتاج:

ارتفاع معدل الإصابة بداء البروسيلات المصحوب بالتهاب المسالك البولية المرتبط بانخفاض مستوى فيتامين (د) المصحوب بارتفاع مستويات المصل من IL-6. **قد تترافق هذه النتيجة السلبية للعدوى مع شكل من أشكال أمراض المناعة الذاتية.**

الكلمات الدالة:

داء البروسيلات ، عدوى المسالك البولية ، فيتامين د ، IL-6 و TNF-α

1. Abstract

Background and Objectives:

Brucellosis is a zoonotic disease that is caused by the *Brucella* species. This disease is common in Iraq and its incidence is increasing. This study measures serum vitamin D levels, IL-6 and TNF-α in patients with chronic urinary tract infection and brucellosis.

Materials and Methods:

This research was conducted Hilla city, Iraq. The calculated sample size included 92 patients with chronic urinary tract infection and brucellosis. The measurement of vitamin D levels, IL-6 and TNF-α were performed by ELISA.

Results:

The mean serum vitamin D level was categorized into three categories (normal= 32.81 ng/ml ±7.61, poor sufficient= 18.22 ng/ml ±6.89 and insufficient= 7.56 ng/ml ±4.18). (Serum vitamin D level <10 ng/mL is accepted as deficiency, 10–30 ng/mL as insufficiency, 30–100 ng/mL as sufficiency, and >100 ng/mL as toxicity). The rang of IL-6 levels was 133.94-438.90 pg/ml with mean level of 389.45±70.88 and associated with 66% of participant higher than normal rang. The rang of TNF-α levels was 78.51-162.31 pg/ml with mean level of 118.78±44.11 and associated with 43% of participant higher than normal rang

Conclusion:

High incidence of brucellosis with UTI associated with low level of Vitamin D accompanied with high serum levels of IL-6. This adverse outcome of infection may be associated with some form of autoimmune diseases.

Keywords:

Brucellosis, urinary tract infection, vitamin D, IL-6, TNF-α

2. Introduction

Brucellosis is an infectious disease caused by *Brucella* species. It is known by many other names, including remitting fever, undulant fever, Mediterranean fever, Maltese fever, Gibraltar fever, Crimean fever, goat fever, and Bang disease [1].

The disease was described by George Cleghorn, a British army surgeon stationed in Menorca, in his 1751 work Observations on the Epidemical Diseases in Minorca from the Year 1744 to 1749.

Brucellosis again came to the attention of medical officers of the British army on the island of Malta following the Crimean War. The genus Brucella is named for Major-General Sir David Bruce, who in 1886 led the Malta Fever Commission that identified *Brucella melitensis* as the organism responsible for the disease. In 1905, Sir Themistocles Zammit identified that infected goats transmitted brucellosis in their milk [2].

Bacteria of the genus *Brucella* cause disease primarily in domestic, feral and some wild animals and most are also pathogenic for humans. In animals, brucellae typically affect the reproductive organs, and abortion is often the only sign of the disorder. Human brucellosis is either an acute febrile disease or a persistent disease with a wide variety of symptoms. It is a true zoonosis in that virtually all human infections are acquired from animals [3].

Etiology

Brucellosis is a zoonotic disease that can be caused by four different Brucella species in humans: *B. suis, B. melitensis, B. abortus,* and *B. canis*. Among these, *B. melitensis* is the most virulent, followed by *B. suis*. As few as 10 to 100 organisms can cause the disease in humans.

All Brucella species are gram-negative, nonmotile, facultative intracellular coccobacilli. Brucella species do not form spores or toxins. The animal host of *B. suis* is swine; the hosts of *B. melitensis* are sheep and goats; the host of *B. abortus* is cattle; and the hosts of *B. canis* are dogs [3].

Epidemiology

The disease is transmitted from animals to humans by consumption of unpasteurized milk and dairy products, consumption of undercooked meat, or skin penetration of those in contact with livestock. It also has been shown to be transmitted by inhalation of contaminated aerosols, conjunctival inoculation, blood transfusions, transplacentally from mother to fetus, and rarely from person to person [4].

Pathogenesis

Brucellae are facultative intracellular parasites, multiplying mainly in monocyte-macrophage cells. This characteristic dominates the pathology, clinical manifestations and therapy of the disease. The organisms may gain entry into the body through a variety of portals. Because the infection is systemic it is often not possible to determine which portal was involved in a particular case. Oral entry, by ingestion of contaminated animal products (often raw milk or its derivatives) or by contact with contaminated fingers, probably represents the most common route of infection even though this portal may not be the most vulnerable one. Inhalation of aerosols containing the bacteria, or aerosol contamination of the conjunctiva, is another route. Inhalation probably underlies some industrial outbreaks. Percutaneous infection through skin abrasions or by accidental inoculation has frequently been demonstrated [5].

Animal studies suggest that invading brucellae are rapidly phagocytosed by polymorphonuclear leukocytes. Brucellae are frequently able to survive and multiply in these cells because they inhibit the bactericidal myeloperoxidase-peroxide-halide system by releasing 5′-guanine and adenine. Early in infection, macrophages are also relatively ineffectual in killing the intracellular brucellae. In systemic spread, it is not clear whether the bacteria are transported within neutrophils and macrophages or in the blood stream outside cells but organisms may disseminate widely from regional lymphoid tissue appropriate to the portal of entry and may localize in certain target organs such as lymph nodes, spleen, liver, bone marrow, and (especially in animals) the reproductive organs. In humans, the tissue lesions produced by Brucella species consist of minute granulomas that are composed of epithelioid cells, polymorphonuclear leukocytes, lymphocytes and some giant cells. In cases of infection with B melitensis these granulomas are particularly small [6] .

Clinical Manifestation

The presentation of brucellosis is characteristically variable. The incubation period is often difficult to determine but is usually from 2 to 4 weeks. The onset may be insidious or abrupt. Subclinical infection is common.

In the simplest case, the onset is influenza like with fever reaching 38 to 40°C. Limb and back pains are unusually severe, however, and sweating and fatigue are marked. The leukocyte count tends to be normal or reduced, with a relative lymphocytosis. On physical examination, splenomegaly may be the only finding. If the disease is not treated, the symptoms may continue for 2 to 4 weeks. Many patients will then recover spontaneously but others may suffer a series of exacerbations. These may produce an undulant fever in which the intensity of fever and symptoms recur and recede at about 10 day intervals. Anemia is often a feature. True relapses may occur months after the initial episode, even after apparently successful treatment [7].

Most affected persons recover entirely within 3 to 12 months but some will develop complications marked by involvement of various organs, and a few may enter an ill-defined chronic syndrome. Complications include arthritis, often sacroiliitis, and spondylitis (in about 10 percent of cases), central nervous system effects including meningitis (in about 5%), uveitis and, occasionally, epididymo orchitis. In contrast to animals, abortion is not a feature of brucellosis in pregnant women [8].

Based on brucellosis literature a comprehensive list of clinical manifestations :associated with brucellosis cases was developed

General: documented fever, sweats, chills, fatigue, headache, malaise, weight loss, nausea/vomiting

Abdominal: abdominal pain, splenomegaly, hepatomegaly, hepatitis

Musculoskeletal: arthralgia, arthritis, myalgia, back pain, spondylitis, sacroiliitis

Specific organ involvement: epididymo-orchitis, abortion, endocarditis, respiratory and neurological signs, cutaneous changes

Death occurs in 2% of cases. Endocarditis rarely occurs; however, it is the most common cause of death from brucellosis. Aortic fistulas have also been documented [9].

Diagnosis

Brucellosis may be diagnosed by blood cultures in tryptose medium; however, because of the slow-growing nature of Brucella, the cultures may take a week or more to become positive. Bone marrow culture has a higher yield than blood cultures. Standard agglutination testing is the most common method of diagnosis in endemic areas. Indirect enzyme-linked immunosorbent assay (ELISA) and Rose Bengal testing also may be used. Laboratory testing on the patient may show anemia, leukopenia, or pancytopenia as well as elevated C Reactive protein, erythrocyte sedimentation rate, serum lactate dehydrogenase, alkaline phosphatase, and transaminases. Pedro Pons sign, erosion of the anterior superior aspect of lumbar vertebrae with osteophytosis, is associated with

spondylodiscitis caused by Brucella. Disc space narrowing, bone destruction, and sclerosis may be seen on imaging in patients with spondylitis. Nonspecific hepatitis and granulomas may be observed on liver biopsy [10].

Treatment / Management

Tetracycline 500 mg in every six hours orally) or Doxycycline (100 mg in every hours orally, a long acting tetracycline analogue for at least six weeks are now 12 the preferred drug for the treatment of unsophisticated brucellosis in adults and children eight years old and more seasoned. But when tetracycline or Doxycycline is given alone, the rate of relapse stays between 10 to 20 %. Thus most specialists suggest an amino-glycoside (Streptomycin as a dose of 1 g/day intramuscularly) managed for 2-3 weeks in addition to the tetracycline or Doxycycline for the first 2-3 weeks of treatment. As per recommendation by the WHO Expert Committee in 1986, the combination of Doxycycline (200 mg/day orally) plus rifampicin)600–900 mg/day orally), with the 2 medications controlled for about a month and a half was advised to give a completely oral regimen to treat brucellosis. For secondary alternative therapy drugs like quinolones in mix with different other drugs, for example, doxycycline or rifampicin; trimethoprim or sulfamethoxazole are advised. For children less than eight years old, trimethoprim/ sulfamethoxazole (8/40 mg/kg/day twice daily orally) for about a month and a half in addition to streptomycin (30 mg/kg/day once daily intramuscularly) for three weeks or gentamicin (5 mg/kg/day once daily intravenously or intramuscularly) for 7 to 10 days as directed. Alternatives such as trimethoprim/ sulfamethoxazole in addition to rifampicin (15 mg/kg/day orally) each for about a month and a half, or rifampicin in addition to an aminoglycoside are likewise suggested. Until additional experience is obtained with these medicines, it is preposterous to expect to characterize the cure of decision. There is no persuading proof regarding the advantage of controlling Brucella vaccines or antigen arrangements, nor for the utilization of invulnerable framework modulators. Alert ought to be practiced in the utilization of mitigating specialists to manage local troubles; where conceivable, special advice to be required [11].

Vitamin D and Immunomodulatory Markers

Vitamin D has a potential role in immune regulation and it prevents this infections especially urinary tract infections UTI. Therefore it has a positive regulatory role in both acute and recurrent infections especially in women of reproductive.

The role of vitamin D is to block the effects of adaptive immune system leading to prevent the autoimmunity that proved by animal studies which showed with absent of calcitriol, the immune cells are attacked the healthy cells of the body

leading to autoimmunity. Moreover, vitamin D can be blocking the production of pro-inflammatory cytokines like interleukin-6 (IL-6), Tumor Necrotizing Factor-α (TNF- α) and Interferon-γ (IFN- γ) and that may also have associated with stimulating the production of anti-inflammatory cytokines and decreasing in killer T cells production. On other hand, the deficiency of vitamin D can inhibit the production of B lymphocytes leading to blocks the production of immunoglobulins [12].

In normal immune response, T helper-1 cells produce INF-γ, IL-2, and TNF-α, and this response is strongly addressed to intracellular pathogens as viruses while T helper-2 lymphocytes produce mainly Tissue Growth Factor (TGF)-β1and IL-4 and IL-5, and these mediators are linked with extracellular pathogens as bacteria and parasites that proved the same role of VD decreases the production of T helper-1 producers like INF-γ, IL-2, and TNF-α and increases the production of T helper-2 producers like IL-4 and IL-5.

Furthermore, the immunomodulating effects of VD and its analogs have been well-characterized in dendritic cells (DCs), which are as antigen presenting cells (APCs). In response to inflammatory signals, VD strongly impairs the migration and maturation of DCs, which reduces antigen presentation capacity and activation of T lymphocytes with marked reduction in pro-inflammatory ILs that contribute as an induction of tolerogenic state [13].

The aim of study

The aim was to investigate the levels of VD and some immunomodulatory markers (IL-6, TNF-α and INF-γ) in pregnant women with TA and their effects on their obstetric health.

Subjects and Methods

Patients: The number of patients was 92, study was carried out at General hospitals in Babel province, Iraq. Sample size was taken by consecutive manner.

Exclusion criteria: Eligible patients should not document with known chronic liver, kidney, thyroid or gastrointestinal diseases, hypertension, diabetes, or using any medications that affect vitamin D level (e.g. anticonvulsants, antituberculosis drugs, etc)

Confirmation of Brucellosis: detection of IgM and IgG against *Brucella* by ELISA.

Ethics statement: The study was carried out in compliance with the Declaration of Helsinki principles and was approved by the University of Babylon/College of Pharmacy's Institutional Review Committee. All-important study material was communicated to the patient group, and they were told that their rejection or withdrawal from the trial would have no impact on the medical care they

received. After gaining their verbal consent, the data was collected by a well-trained researcher using a standardized questionnaire.

Demographic and clinical variables: Age, residency, socioeconomic states, educational and occupation states, state of anemia and body mass index (BMI) were collected by questionnaire to all participants.
The effects of age were investigated using continuous and categorical approaches (coded as age < 25, 25-30, 30-35, and 35-40 and > 40 years).
Medical records were abstracted to a certain their anthropomorphic characteristics as well as their medical status throughout gestation.

Laboratory Investigation:

During admission period, 5ml venous blood sample was collected to measure vitamin D, IL-6 and TNF-α.

Level of Serum vitamin D3 (25-hydroxyvitamin-D): Estimation of vitamin D level from blood samples were determined by using a commercial enzyme-linked immunosorbent assay (ELISA) (Demediteq: DE1971, Germany). According to the manufacturer's protocol, each assay was run with known standards (provided with the kit) that were used to determine the quantity of vitamin D in each sample in ng/ml. The range of detection was 3–300 ng/mL. Patients' vitamin D status was evaluated according to vitamin D concentrations into deficient Levels below 25 ng/ml, insufficient between 25–75 ng/ml and normal level ≥75 ng/ml [(8)].

Immunomodulatory markers:

IL-6 and TNF-α levels

Inflammatory cytokines indices were measured by ELISA technique (elabscience company, USA). IL-6 is by E-EL-H0192 kit with normal values ranging from (104.35-238.90 pg/ml) and TNF-α by E-EL-H 0109 kit with normal values ranging from (24.10-86.50 pg/ml). According to the manufacturer's protocol; each assay was run with known standards (provided with the kit) that were used to determine the quantity of cytokines in each sample.

Statistical analysis

To explore relationships and differences in Demographic, clinical factors, VD, and immunomodulatory markers, descriptive measures, one way and two way ANOVA analysis were utilized. For all analyses, the significance level was fixed at a probability (P) of less than or equal to 0.05. GraphPad Prism 5.3 on Windows was used to conduct all analyses (GraphPad Software, San Diego, CA, USA).

Results

Serum VD levels ranged from 2.8 to 68.5 ng/ml with mean level was 19.83±43.59 ng/ml. The distribution of VD among participant population was 84% (mean: 16.2±10.6 ng/ml) with deficient vitamin D (< 25 ng/ml), 11% (mean: 32.8±17.6)

with insufficient vitamin D (25-75 ng/ml) and only 5% (mean: 76.5±3.6 ng/ml) with normal vitamin D level (>75 ng/ml) (Table 1). The rang of IL-6 levels was 133.94-438.90 pg/ml with mean level of 389.45±70.88 and associated with 66% of participant higher than normal rang. The rang of TNF-α levels was 78.51-162.31 pg/ml with mean level of 118.78±44.11 and associated with 43% of participant higher than normal rang (Table 1).

Table 1. Serum Levels of Vitamin D, Interleukin-6 (IL-6) and Tumor Necrotizing Factor-α (TNF- α) in patient with Brucellosis and UTI (n=92).

Immunomodulatory Markers	Means	Std. Deviation	Std. Error	Lower 95% CI of mean	Upper 95% CI of mean
Vitamin D	19.83 ng/ml	43.59	3.794	12.32	27.34
IL-6	389.5 pg/ml	70.88	6.169	377.2	401.7
TNF-α	118.8 pg/ml	44.11	3.839	111.2	126.4

The patients in the study ranged in age from 18 to 71 years old, with 70% of them being between the ages of 25 and 55. The majority of the participants were from a middle socioeconomic status (40%) with a secondary education (54%) and were primary education (77%). BMI was 56% of the women were between 25-30 kg/m^2, while 31 % for participant more than 30 kg/m^2 and only 12 % was below 25 kg/m^2. Regarding the anemia, most of participant was anemic (88%) (Table 2).

Moreover, there was a significant relation (P> 0.001) between the levels of cytokines and the levels of VD (Figure 1) that increase in the significances between the levels of cytokines in the deficient status of vitamin D (P<0.001).

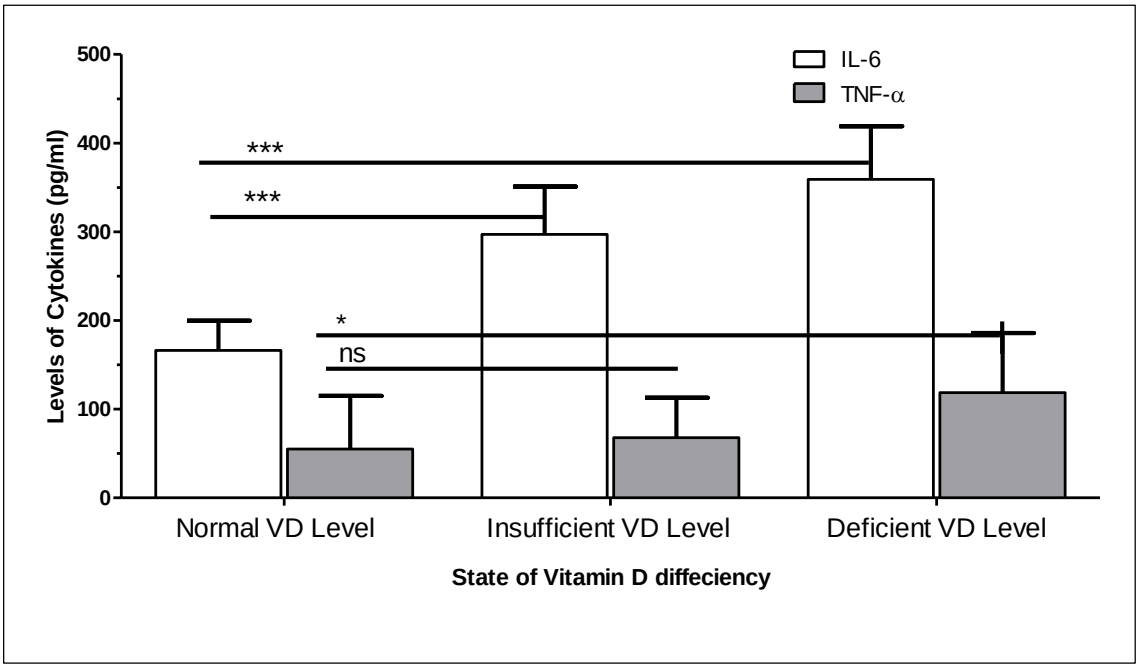

Figure 1. Relation between the levels of vitamin D and the levels of cytokines (Interleukin-6 (IL-6) and Tumor Necrotizing Factor-α (TNF- α) in study participant (n=92). The number of asterisks (*) corresponds to the level of the statistical significance (**P<0.01, ***P<0.001).

Moreover, there was also a relation between the levels of VD and the levels of IL-6 and TNF-α (Figure 2 and Figure 3), these relations varied according the in significances ranging from non-significant to P<0.001).

Table 2. sociodemographic data, previous medical information in the studied population in relation to the serum level of vitamin D.

Parameters	**Sub-parameters**	**Normal vitamin D N = 7**	**Insufficient vitamin D N = 15**	**Deficient vitamin D N = 69**	***P value***
Age	< 25 yrs	2 (2%)	2 (3%)	10 (15%)	*P<0.001*
	25-30 yrs	3 (2%)	5 (6%)	27 (45%)	
	31-35 yrs	1 (1%)	3 (2%)	17 (13%)	
	36-40 yrs	0 (0%)	2 (3%)	8 (6%)	
	> 40 yrs	1 (0%)	3 (0%)	7 (1%)	
Socioeconomic state	Low	0 (0%)	4 (3%)	17 (34%)	*P<0.001*
	Medium	2 (2%)	5 (4%)	32 (39%)	
	High	3 (3%)	4 (4%)	14 (11%)	
Educational Level	Elementary	0 (0%)	4 (3%)	29 (22%)	*P<0.001*
	Secondary	3 (2%)	8 (6%)	39 (48%)	
	Bachelor or >	4 (3%)	3 (2%)	12 (14%)	
Occupation	Free job	4 (3%)	11 (8%)	62 (68%)	*P<0.001*
	Employee	3 (2%)	4 (3%)	21 (16%)	
BMI (kg/m^2)	<25	0 (0%)	3 (2%)	13 (10%)	*P<0.001*
	25-30	3 (2%)	3 (2%)	68 (52%)	
	> 30	4 (3%)	10 (8%)	28 (22%)	
	Good	5 (4%)	8 (6%)	39 (29%)	
Anemia	> 11 g/L	3 (2%)	3 (2%)	8 (12%)	*P<0.001*
	< 11 g/L	3 (3%)	7 (9%)	50 (72%)	

BMI: body mass index

Conclusion:

In summary, High incidence of brucellosis with UTI associated with low level of Vitamin D accompanied with high serum levels of IL-6. This adverse outcome of infection may be associated with some form of autoimmune diseases. Hypovitaminosis D was correlated with high proinflammatory markers (IL-6 and

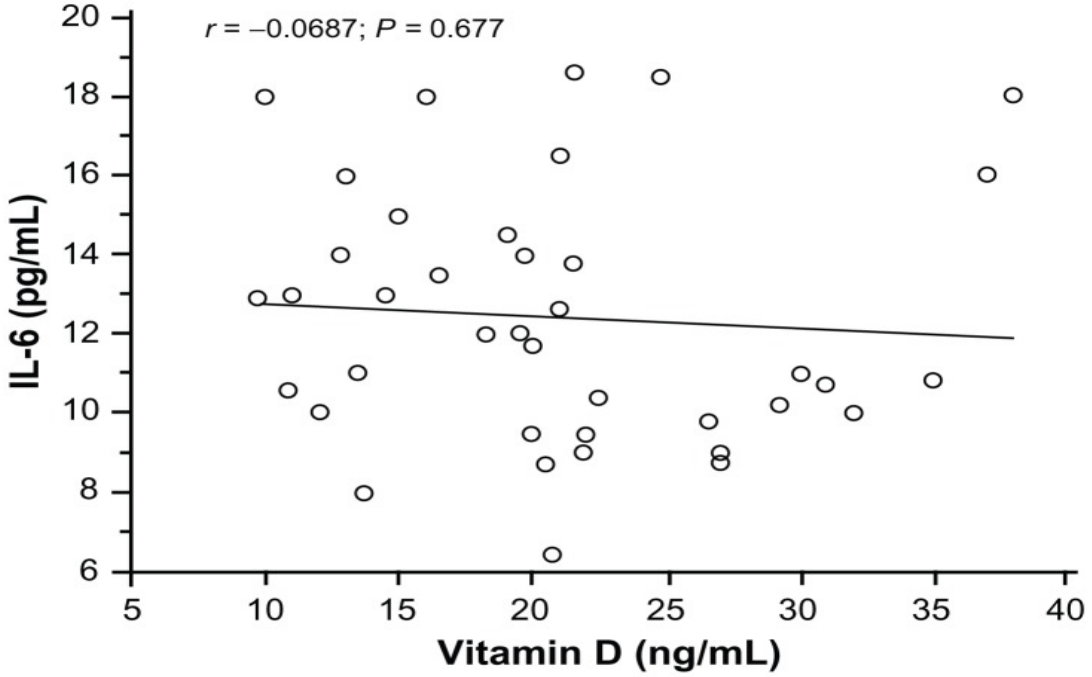

Figure 2. Correlation between the levels of vitamin D levels and Interleukin-6 (IL-6) in relation to participant (n=92)

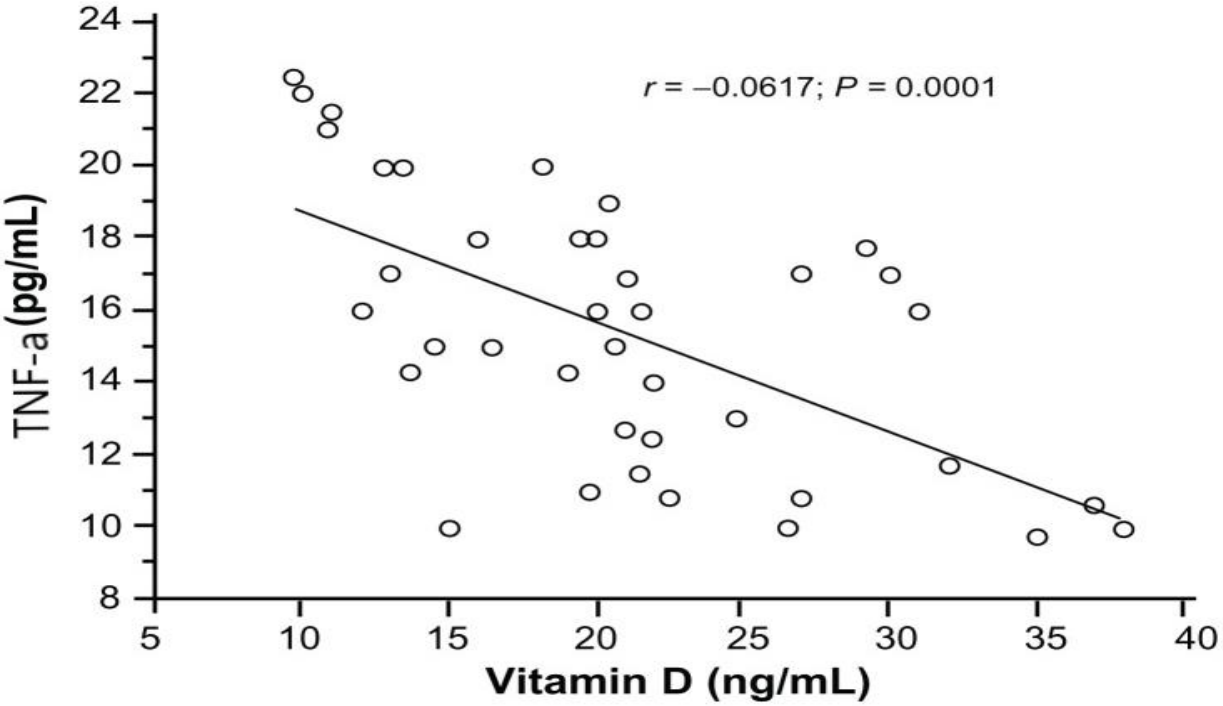

Figure 3. Correlation between the levels of vitamin D levels and Tumor Necrotizing Factor-α (TNF- α) in relation to participant (n=92).

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