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Immunological Biomarker of Leishmania Donovani

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Abstract: Leishmania donovani are major disease causes and humans are the main reservoir. In addition to Leishmania infantum, Leishmania archibaladi, and Leishmania chgasi. infection, such as cytokines IL-2, IL-10, IL-12, and TGF-β, was detected using ELISA technology. The results showed a significant increase in the concentration of these cytokines in the test group (131.58 ± 15.92, 335.68 ± 15.57, 823.62 ± 98.75, 341.16 ± 15.19), respectively, for patients infected with visceral leishmaniasis compared to the healthy control group

Keywords: ELISA, IL-2, IL-10, IL-12, TGF-β

1. Introduction

Leishmaniasis is a neglected tropical disease NTD. More than one billion people are at risk of contracting this disease in all continents. This disease may debilitate many people and prevent them from living a decent life and preventing productive work properly **[1].**The physicians Leishman and Donovan were the first to describe the visceral leishmaniasis pathogen at 1903, it is found in stained smears from the spleen of infected patients who showed symptoms of a disease similar to malaria, and later they called it Leishmania donovani **[2].**
Visceral leishmaniasis (VL) is the most severe form of leishmaniasis with an estimated 30,000 new cases annually and is often fatal if not treated appropriately. It is caused by infection with primary vector-borne Leishmania species including L. donovani (Ld) that spread to internal organs such as the spleen and bone marrow **[3].**
Biomarkers play a vital role in the aforementioned challenges by expanding the follow-up of patient information including immune status, determining patient response to treatment, as well as epidemiological feedback, exposure of humans and animals to vectors, and reservoir hosts model **[4].**Some macrophage-secreted cytokines are considered indirect biomarkers for leishmaniasis infection, including IFN-γ, TNF-α and IL-10 **[5].**Recently, leishmaniasis investigation in humans has been associated with the detection of several biomarkers, including TNF-α, and/or other circulating proteins **[6].**

1.1. Immune response to Leishmania parasite:

different key players of the host immune system. As the promastigotes enter into the blood stream following a sand fly bite, until the amastigotes reside within the macrophages, the tussle between the eradication of parasites and establishment of disease will be somewhat decided by the ability of Leishmania to modulate and/or evade host immune defenses. The components of host defense that are necessary for parasite clearance include elements of the innate and adaptive immune systems **[7].**

1.2. Cytokines:

The host immune response against Leishmania infection involves coordinated contribution from innate and adaptive immune response. Cytokines and chemokines participate rigorously in early protection against the infection as they recruit subsets of leukocytes that are activated through increased adhesion, degranulation and the respiratory burst. Therefore, these molecules play a crucial role in determining the outcome of the infection through mediating, regulation and subdiverting the host immune response against leishmaniasis. This group of cytokine genes contributes to the virulence of the parasite and favors the persistence of the infection and parasite survival, as in L. major **[8].**

2. Materials and Method

2.1 The study subjects

(74) blood samples were collected from various Iraqi hospitals that were diagnosed by a specialist doctor according to the following clinical symptoms:(the presence of long-lasting intermittent fever, large abdominal size, loss of appetite, splenomegaly, hepatomegaly, loss of weight and appetite, pale and darkening of the skin, jaundice, anemia, Cough, fatigue, vomiting). And (60) healthy people not infected with visceral leishmaniasis, aged between (9-65) years A venous blood sample was drawn from each patient after confirming his infection with visceral leishmaniasis using a 5 ml medical syringe and placing it in tubes containing an anticoagulant. EDTA, then placed in gelatinous tubes for the purpose of blood separation using a centrifuge (3000 rpm), where the serum was isolated and distributed into four Eppendorf tubes according to the number of immunological tests conducted in the study, and the samples were stored at a temperature of (-20C°). until use.

2.2..mmunological study

2.2.1. (IL-2,10,12,TGF-β- ELISA)

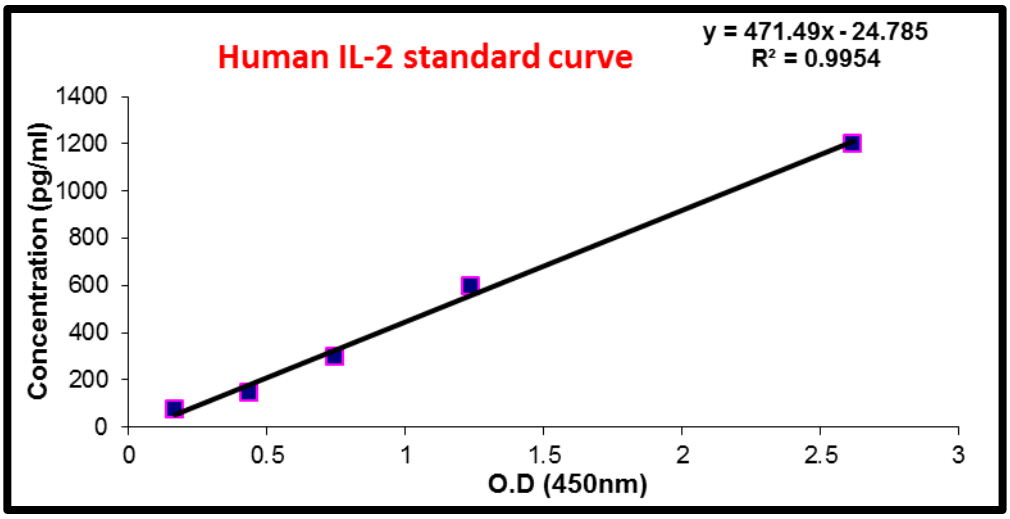
Human Interleukin 2,10,12,TGF-β (ELISA) was used in this study for quantitative determination of IL-2,10,12,TGF-β from in patient and healthy control serum samples and done according to company instruction (BT-LAB) as following:

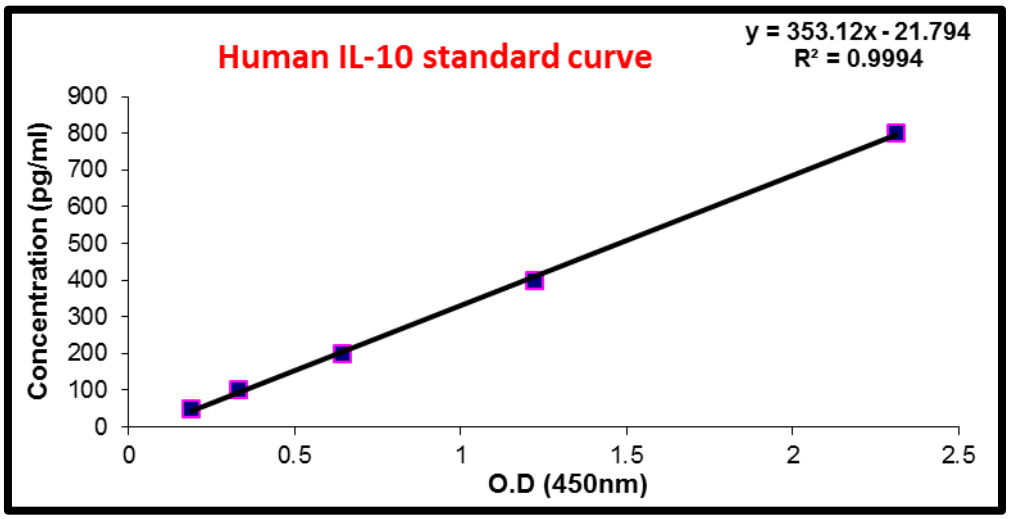
A. ELISA components:

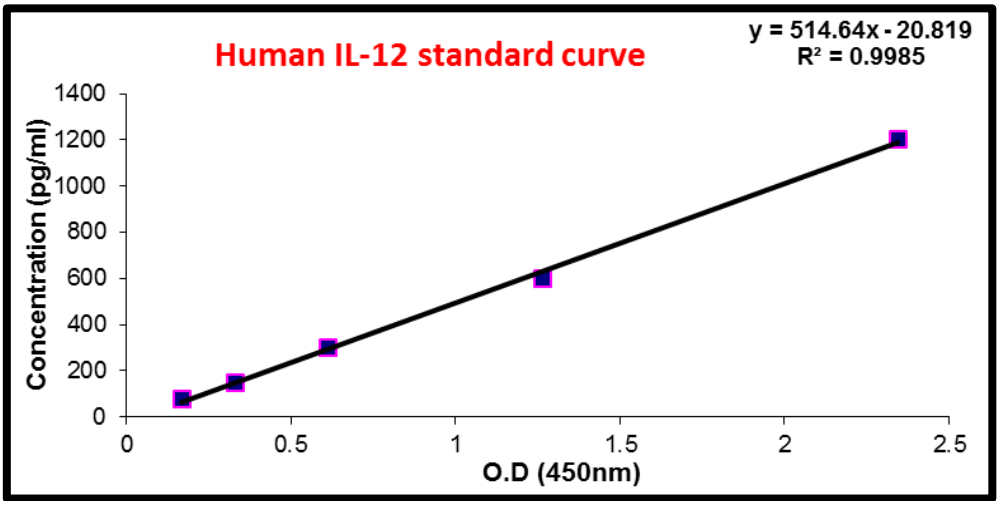
Reagent	**Quantity**
Standard Solution IL-2,IL-12 (1200ng/L) Standard SolutionIL-10 (800ng/L) Standard Solution TGF-β (2400ng/L)	0.5ml x1
Pre-coated ELISA Plate	12 * 8 well strips x1
Standard Diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
Wash Buffer Concentrate (25x)	20ml x1
Bio tinylated Human IL-2,10,12,TGF-β Antibody	1ml x1

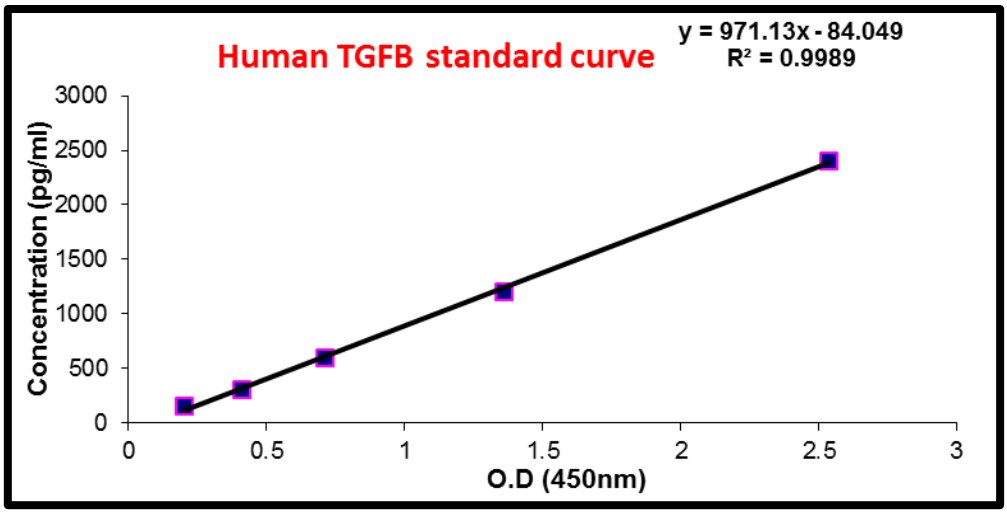
B. Assay procedure:

1. All reagents, standard solutions and samples were prepared according to kit instruction and bring at room temperature before use.
2. A 50µl standard serial dilution was added into to standard well. **Without** added antibody to standard well because the standard solution contains bio tinylated antibody.
3. A 40µl sample was added to sample wells and then add 10µl anti-IL-2 ,10, 12, TGF-β antibody to sample wells, then 50µl streptavidin-HRP added in to sample wells and standard wells (Not blank control well).
4. The ELISA mixed well and covered the plate with a sealer. Then incubated at 60 minutes at 37°C.
5. The sealer was removed and wash the plate 5 times with wash buffer. wells socked
6. with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For
7. A 50µl substrate solution A was added to each well and then 50µl substrate solution B added to each well.
8. The plate was covered with a new sealer and incubated for 10 minutes at 37°C in the dark.
9. A 50µl Stop Solution was added to each well, then the blue colour will change into yellow immediately.
10. The optical density (OD value) was determined using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.


Human IL-2 standard curve
y = 471.49x - 24.785
R² = 0.9954
Concentration (pg/ml)
O.D (450nm)
0
200
400
600
800
1000
1200
1400
0
0.5
1
1.5
2
2.5
3


Human IL-10 standard curve
y = 353.12x - 21.794
R² = 0.9994
Concentration (pg/ml)
O.D (450nm)
0
100
200
300
400
500
600
700
800
900
0
0.5
1
1.5
2
2.5


Human IL-12 standard curve
y = 514.64x - 20.819
R² = 0.9985
Concentration (pg/ml)
O.D (450nm)
0
200
400
600
800
1000
1200
1400
0
0.5
1
1.5
2
2.5



C. Calculation of results:
The ELISA results were calculated depend on the optical density reading for each standard and samples optical density. Then the standard curve was plotted by the mean OD value for each standard on the X-axis against the concentration on the Y-axis and draw a best fit curve through the points on the graph.

3. Results

3.1. Level Interleukin-2 (IL-2) in patients and healthy control.

The comparison of Interleukin-2 (IL-2) levels between patients with Kala-azar and healthy control subjects has been carried out and the results were demonstrated in table (3-1) Mean levels of IL-2 were 131.58 ± 15.92 and 168.63 ± 21.61, in patients with Kala-azar and healthy control respectively; the mean level was highly significant lower than in patients with Kala-azar in comparison with healthy control (P< 0.001).

Table (3-1): Interleukin-2 concentration in the studied groups.

	Cases –control comparison		**P**
	Patients n = 74	**Healthy control n = 60**	
Interleukin-2 (IL-2) levels			
Mean± SD	**131.58 ± 15.92**	**168.63 ± 21.61**	**< 0.001 † HS**
Range	50.29 – 197.90	104.68-258.81	

n: number of cases; **SD**: standard deviation; †: independent samples t-test; HS: Highly significant at P ≤ 0.001.
To evaluate the Interleukin-2 cutoff value as well as to predict Kala-azar disease as diagnostic tests or adjuvant diagnostic tests, receiver operator characteristic (ROC) curve analysis was carried out and the results are shown in table (3-2) . The Interleukin-2 cutoff value was >142.67-fold with sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and area under curve of 81.1%, 80.0%, 83.3%, 77.4% and 0.813 (0.727- 0.900). The present results indicates IL-2 is considered as a good diagnostic marker

Table (3-2): Sensitivity and specificity of IL-2 (> 142.67-fold) in Kala-azar disease.

IL-2 levels	**Kala-azar patients n = 74**	**Healthy control n = 60**
> 142.67	60	12

< 142.67	14	48
Sensitivity %	81.1 %	
Specificity %	80.0 %	
PPV %	83.3 %	
NPV %	77.4%	
AUC (95% CI)	0.813 (0.727- 0.900)	

CI: Confidence interval, AUC: Area under curve.

4.5.2. Level Interleukin-10 (IL-10) in patients and healthy control.

The comparison of Interleukin-10 (IL-10) levels between patients with Kala-azar and healthy control subjects has been carried out and the results were demonstrated in table (3-3) . Mean levels of IL-10 were 335.68 ± 15.57 and 260.93 ± 16.79, in patients with Kala-azar and healthy control respectively; the mean level was highly significant greater than in patients with Kala-azar in comparison with healthy control (P< 0.001).

Table (3-3): Interleukin-10 concentration in the studied groups.

	Cases –control comparison		
	Patients n = 74	**Healthy control n = 60**	**P**
Interleukin-10 (IL-10) levels			
Mean± SD	**335.68 ± 15.57**	**260.93 ± 16.79**	**< 0.001 † HS**
Range	247.91 – 488.66	190.00-380.41	

n: number of cases; **SD**: standard deviation; †: independent samples t-test; HS: Highly significant at P ≤ 0.001.

To evaluate the Interleukin-10 cutoff value as well as to predict Kala-azar disease as diagnostic tests or adjuvant diagnostic tests, receiver operator characteristic (ROC) curve analysis was carried out and the results are shown in table (3-4) . The Interleukin-10 cutoff value was <271.82-fold with sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and area under curve of 83.6%, 83.3%, 86.1%, 80.6% and 0.837 (0.751- 0.922). The present results indicates IL-10 is considered as a good diagnostic marker.

Table (3-4): Sensitivity and specificity of IL-10 (< 271.82-fold) in Kala-azar.

IL-10 levels	**Kala-azar patients n = 74**	**Healthy control n = 60**
< 271.82	62	10
> 271.82	12	50
Sensitivity %	83.8 %	
Specificity %	83.3 %	
PPV %	86.1 %	
NPV %	80.6%	
AUC (95% CI)	0.837 (0.751- 0.922)	

CI: Confidence interval, AUC: Area under curve.

4.5.3. Level Interleukin-12 (IL-12) in patients and healthy control .

The comparison of Interleukin-12 (IL-12) levels between patients with Kala-azar and healthy control subjects has been carried out and the results were demonstrated in table (3-5) .Mean levels of IL-12 were 341.16 ± 15.19 and 458.28 ± 18.14, in patients with Kala-azar and healthy control respectively; the mean level was highly significant lower than in patients with Kala-azar in comparison with healthy control (P< 0.001).

Table (3-5): Interleukin-12 concentration in the studied groups.

	Cases –control comparison		
	Patients n = 74	**Healthy control n = 60**	**P**
Interleukin-12 (IL-12) levels			
Mean± SD	**341.16 ± 15.19**	**458.28 ± 18.14**	**< 0.001 † HS**
Range	186.07 – 457.80	317.81-674.97	

n: number of cases; **SD**: standard deviation; †: independent samples t-test; HS: Highly significant at P ≤ 0.001.
To evaluate the Interleukin-12 cutoff value as well as to predict Kala-azar disease as diagnostic tests or adjuvant diagnostic tests, receiver operator characteristic (ROC) curve analysis was carried out and the results are shown in table (3-6). The Interleukin-12 cutoff value was > 371.62-fold with sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and area under curve of 83.6%, 83.3%, 86.1%, 80.6% and 0.832 (0.749- 0.916). The present results indicates IL-12 is considered as a good diagnostic marker.

Table (3-6): Sensitivity and specificity of IL-12 (> 371.62-fold) in Kala-azar disease

IL-12 levels	**Kala-azar patients n = 74**	**Healthy control n = 60**
> 371.62	62	10
< 371.62	12	50
Sensitivity %	83.8 %	
Specificity %	83.3 %	
PPV %	86.1 %	
NPV %	80.6%	
AUC (95% CI)	0.832 (0.749- 0.916)	

CI: Confidence interval, AUC: Area under curve.

4.5.4. Level Transforming growth factor-β in patients and healthy control

The comparison of Transforming growth factor-β (TGF-β) levels between patients with Kala-azar and healthy control subjects has been carried out and the results were demonstrated in table (3-7) . Mean levels of TGF-β were 823.62 ± 98.75 and 693.14 ± 68.31, in patients with Kala-azar and healthy control respectively; the mean level was highly significant greater than in patients with Kala-azar in comparison with healthy control (P< 0.001).

Table (3-7): Transforming growth factor-β concentration in the studied groups.

	Cases –control comparison		**P**
	Patients n = 74	**Healthy control n = 60**	
Transforming growth factor-β (TGF-β) levels			
Mean± SD	**823.62 ± 98.75**	**693.14 ± 68.31**	**< 0.001 † HS**

Range	463.20 – 1179.39	498.0-885.14	

n: number of cases; **SD**: standard deviation; †: independent samples t-test; HS: Highly significant at P ≤ 0.001.
To evaluate the TGF-β cutoff value as well as to predict Kala-azar disease as diagnostic tests or adjuvant diagnostic tests, receiver operator characteristic (ROC) curve analysis was carried out and the results are shown in table (3-8). The TGF-β cutoff value was < 730.24-fold with sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and area under curve of 70.3%, 70.0%, 74.3%, 65.6% and 0.712 (0.611- 0.814). The present results indicates TGF-β is considered as an acceptable diagnostic marker.

Table (3-8): Sensitivity and specificity of TGF-β (< 730.24-fold) in Kala-azar disease

TGF-β levels	**Kala-azar patients n = 74**	**Healthy control n = 60**
< 730.24	52	18
> 730.24	22	42
Sensitivity %	70.3 %	
Specificity %	70.0 %	
PPV %	74.3 %	
NPV %	65.6%	
AUC (95% CI)	0.712 (0.611- 0.814)	

CI: Confidence interval, AUC: Area under curve.

4. Discussion

4.1.Immunological Analysis Results

4.1.1.Level Interleukin-2 (IL-2) in patients and healthy control.

Proinflammatory cytokines are mainly produced to amplify the immune response -17,IL-18,IL-2,IL-12), while anti-inflammatory cytokines that counteract the effect of proinflammatory cytokines include (IL-4 ,IL-6 ,IL-10 ,TGF-β) **[9].**
Interleukin 2 (IL-2) is an important cytokine that influences T-cell behavior **[10].**It is required for the survival, proliferation, and differentiation of $CD4^+$ T cells, $CD8^+$ T cells, and natural killer (NK) cells by**[11],** binding to either the high-affinity trimeric IL-2 receptor (IL-2R), made up of IL-2Rα (CD25), IL-2Rβ (CD122), and IL-2Rγ (CD132), or the dimeric IL-2R (comprising β and γ chains) **[12].**The trimeric IL-2R receptor is highly expressed on activated $CD4^+$ T cells and $Foxp3^+CD4^+$ T regulatory (Treg) cells, while memory $CD8^+$ T cells and NK cells express high levels of the dimeric IL-2R**[10].**
Leishmania donovani–infected mice receiving IL-2-blocking monoclonal antibodies (mAbs) failed to control hepatic parasite growth, associated with impaired granuloma development, whereas infected mice treated with exogenous IL-2 had reduced liver parasite burdens and increased granuloma development, relative to controls **[13].** Similarly, intranodular injection of recombinant IL-2 in patients with disseminated cutaneous leishmaniasis reduced parasite numbers, associated with $CD4^+$ T-cell infiltration **[14].** However, the therapeutic application of IL-2 has had limitations because of its short half - life **[15]** and adverse side effects **[16].**
A study **[17]** found that IL-2 is a good indicator for diagnosing visceral leishmaniasis, as the AUC was 0.9613 (0.8767-1.000), and in studies it was confirmed that IL-2 is the most sensitive and specific biomarker in L.infantum **[18].**

4.1.2.Level Interleukin-10 (IL-10) in patients and healthy control.

Previous studies have shown a direct role for IL-10 in the pathogenesis of human VL **[19,20].**Previously thought to be a Th2 cytokine, IL-10 is produced by CD4+ FoxP3+ CD25 regulatory T cells (Treg) as well as IFN-γ-producing Treg+ cells (Tr1 cells) in humans **[21, 22,23, 24].** The advancement of the disease is mainly related to Th 2 type of immune response along with an increase in levels of interleukin-10, IL-5, IL-4 and transforming growth factor **[25].** The results agreed with a study by **[26]**To evaluate the predictive value of levels of inflammatory markers to identify patients with cutaneous leishmaniasis, IL-10 showed that the (ROC) curve

showed the best discrimination. AUC 1(1.00-1.00), cut off 81.13, SE(1.000), SP(1.000). Likewise, **[17]** IL-10 was found to be a good diagnostic marker for diagnosing Kala azar, as it was (0.9574-1.000) AUC 0.9881.

4.1.3. Level Interleukin-12 (IL-12) in patients and healthy control .

Once the initial infection starts, monocytes are recruited to infected tissue and begin to differentiate into macrophages **[27].**T cells in lymphoid tissues and spleen are activated both by dendritic cells and the macrophages. The naïve T cells get activated into Th0 cells (intermediate stage) which then travel to the liver and get triggered as Th1 cells on coming in contact with dendritic cells and macrophages in IL-12 environment (Siewe et al. 2016). During this attachment, CD4+ T cells recognize the antigens which are bound to Major Histocompatibility Complex (MHC) **[28].** CD4+ T cells then produce IL-12, which in turn triggers CD8+ T cells and enhance CD4+ T cell multiplication. Both CD8+ T cells and CD4+ T cells yield IFN-γ, which in turn activates the macrophages to wipe out the parasites **[29].**

Leishmania reduces dendritic cell-dependent immune responses by inhibiting maturation, migration, antigen presentation capabilities, and IL-12 production. **[30]**, These influence on dendritic cells effects the development of effectiveTh1 response required to the infection clearance **[31].**Infection with VL results in subclinical infection, leading to protective immunity or, in clinical cases, fatal outcomes if untreated. The development of protective immunity appears to depend on , host genetic factors, the infecting species **[30].**

A study **[17]** found that IL-2 is a good indicator for diagnosing visceral leishmaniasis, as the AUC was 0.9613 (0.8767-1.000), and in studies it was confirmed that IL-2 is the most sensitive and specific biomarker in L .infantum **[18].**

4.1.4. Level Transforming growth factor-β in patients and healthy control.

TGF-β is a potent regulatory cytokine that suppresses expression of inducible NO synthase and IFN-γ, and suppresses Th1 and Th2 cell development, plays an important role in the progression of leishmaniasis in rodents **[32].**In the study conducted by **[33]** on the role of TGF-β which aimed to examine whether functionally active TGF-β may be present in the local environment surrounding Leishmania promastigote invasion in murine models, high levels of total TGF-β were found. -β in tissues and cultured immune cells from mice and hamsters infected with L.donovani.

In other studies, it was found that TGF-β promotes progression or prevents the treatment of leishmaniasis in mouse models **[34].**Active TGF-β is augmented during infection of inflammatory mouse peritoneal macrophage cultures with several different Leishmania sp. in vitro **[35].**

In a study of**[36]** it was found that TGF-β alters the phenotype of innate macrophages by inhibiting TNF-α, IFN-γ, inducible NO synthesis (iNOS), and enhancing arginase expression. Conversion of arginine from iNOS to arginase results in decreased NO. and enhancing polyamines, which in turn can promote parasite growth **[37].**

The results agreed with a study by **[26]** To evaluate the predictive value of levels of inflammatory markers for identifying patients with cutaneous leishmaniasis based on TGF-B, the (ROC) curve showed that the best discrimination of TGF-B was at 72 hours, and the AUC ratio was 0.957 (0.901-1.00) and cut off (3834.55),SE(0.95)

5. Conclusion:

Inflammatory cytokines such as interleukin 10, 2 and Transforming growth factor-β, are increased in patients with visceral forms. Indicates IL-2, IL-12 and IL-10 can be used as markers for leishmaniasis infection, especially in endemic areas of patients at different stages of the disease. The present results indicates TGF-β is considered as an acceptable diagnostic marker.

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