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Polymorphism of TNFα with measurement of IL-18, IL-37 and Glutathione in Iraqi patents with Leishmaniasis

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Abstract. The genus Leishmania includes species that cause various clinical disorders to humans especially to immunocompromised people and children. The findings illustrated that mean L. donovani IgG antibody level in the patients was (13.62±0.95) in comparison to control group (0.10±0.02) with highly significant differences (P≤0.001), and the mean IL-18 levels in patients was (31.14±2.96) when compared with the controls (1.23±0.31) with highly significant differences (P≤0.001). Also, the mean IL-37 levels in patients was (23.81±1.34) when compared with the controls (1.73±0.33) with highly significant differences (P≤0.001). Moreover, the mean TNFα levels in patients was (18.21±0.50) as compared with the controls (1.31±0.31) with highly significant differences (P≤0.001). Furthermore, the mean Glutathione levels in patients was (161.18±25.52) when compared with the controls (19.82±1.06) with a highly significant variation (P≤0.001). Moreover, there was a direct correlation between Glutathione and TNFα (r= 00224**) with a highly significant variation (P=0.006). Also, there was a direct correlation between Glutathione and IL-37 (r=0.155) with a significant variation (P=0.05). In addition, a variation occurred in rs767455 SNP to TNF GENE ID 7132, that AA was changed GG in samples number 9,11 and 15 with Leishmania donovani infections.

Highlights:

1. Significant Increase in Cytokines: Patients with visceral leishmaniasis exhibited markedly higher levels of IL-18, IL-37, TNFα, and Glutathione compared to controls.
2. Genetic Variation Identified: A SNP (rs767455) mutation in the TNFα gene was observed in several infected individuals, changing genotype from AA to GG.
3. Correlative Biomarker Insight: Strong correlation found between Glutathione and TNFα/IL-37 levels, suggesting possible biomarker or therapeutic implications.

Keywords: Leishmaniasis, TNFα, IL-18, IL-37, Glutathione, Polymorphism, Iraqi Patents.

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Introduction

The protozoal parasite of genus Leishmania is the causative agent of visceral leishmaniasis (VL). The genus Leishmania includes species that cause various clinical disorders to humans especially to immunocompromised people and children [1]. In the Middle East, Mediterranean littoral and in Iran, L. infantum is the causative agent of most of visceral leishmaniasis cases [2]. In some parts of Iran e.g. Ardabil, East Azarbaijan and Fars province, visceral leishmaniasis is endemic, while it is sporadic in the majority of other areas [2]. leishmania parasites usually the live in the reticuloendothelial system and macrophages. The immunity against Leishmaniasis is mediated through cellular immune response, and T-helper cells play key roles in the resistance of hosts against diseases [3]. The cellular immune responses of Th1-type against leishmaniasis will be stimulated by cytokines like IL-12, IL-18 and IFN-γ. The development of visceral leishmaniasis or its control is based on efficiency of IFN γ-induced by adaptive and innate immune response. It is usually believed that IFN-γ is important to control and protect against Leishmaniasis [4]. Among different essential anti-leishmanial cytokines, the IFN-γ plays special noticeable macrophage-activating roles that encompass macrophage preparation to leishmanicidal molecule secretion. The parasites are killed by macrophages that are activated by IFN-γ [5]. In the inflammatory cascade, the tumors necrosis factor alpha (TNFα) is an important cytokine as it activates (Th-1) immune responses, enhances macrophage activities and important in granuloma maintenance and formation [6]. The TNF-α blockage has been investigated as a therapeutic approach against these diseases because it was implicated in multiple immune-mediated diseases [7]. At present, treatments based on anti-TNF are broadly employed and accepted for treating chronic inflammatory diseases, rheumatoid arthritis, polyarticular juvenile idiopathic arthritis, psoriatic arthritis, ankylosing spondylitis as well as inflammatory bowel disease [8]. Since their first use, the TNF-α blockers were recognized as a risk factor for reactivation of granulomatous infections such as tuberculosis, intracellular infections such as salmonellosis or listeriosis and other opportunistic viral and fungal infectious diseases [9]. The granulomatous Leishmaniasis are parasitic infections and are endemic in South Asia, South America, Africa and South Europe. Leishmania are obligate intracellular protozoa of the cells of mononuclear phagocytic systems. Clinical aspects of leishmaniasis involve subclinical asymptomatic aspect, localized cutaneous as well as disseminated aspects (mucosal, cutaneous as well as visceral infections) [10].

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The clinical expressions of leishmaniasis may be determined by host factor and immune responses or by parasite's species and zimodeme. Leishmaniasis is related to using of TNF-α blocker, although only few cases were mentioned in the literatures, primarily in Mediterranean basins [11]. The biosynthesis of glutathione and activation of arginase are essential in the biology of Leishmania parasite mechanism. For example, the glutathione biosynthesis inhibitions and activities of arginase were highlighted as potential plans in leishmaniasis therapy [12].

Method

In this study, (100) blood samples were collected from patients with Leismaniasis in Qanaqine Hospital, Alrazi Hospital, Jalwla Hospital and Baqubah Teaching Hospital in Diyalaa governorate from March 2024 to March 2025. Blood samples were left to clot for 15 minutes, then centrifuged at 3000 rpm for 10 minutes to obtain serum. The serum Leishmania IgG was estimated by ELISA technique. The serum IL-18, IL-37 and Glutathione were determined using the Double Antibody Sandwich ELISA technique. The TNFα gene was detected by conventional PCR and the primers used were:

rs767455-F GTAAAACGACGGCCAGTCCCTCCTCTCTGCTTTAATTT 55 664

rs767455-R CAGGAAACAGCTATGACACTCCCACTCCCTTCTTT in 55°C at 664bp.

A. Ethical approval

Before beginning this study, each participant provided a written consent. Medical ethics approval certification was approved by the ethics committee number 54/ 219 on March 11, 2024.

B. Statistical analysis

Data were analyzed by Chi-sequare test or using fisher exact probability (F.E.P) test to compare between percentages (qualitative data). T-test was used to compare between two numeric variables. P-Value of ($P<0.05$) was considered as statistically significant (S)..

Results and Discussion

The results revealed no significant difference ($p=0.81$) between the mean age ranges of patients (41.66±17.23) years and the mean ages of controls (40.98±14.96) years. as shown in table (1).

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Table 1. Demographical properties of studied groups

Properties		**Case (n=100)**	**Control(n=50)**	**Total**	**P-value**
Age (M±SD)		41.66±17.23	40.98±14.96	-	0.81
Age range groups (years)	(6-25)	18 (18%)	9 (18%)	27 (18%)	0.93
	(26-45)	40 (40%)	22 (44%)	62 (41.3%)	
	(46-65)	31 (31%)	15 (30%)	46 (30.7%)	
	>65	11 (11%)	4 (8%)	15 (10%)	
Sexes	Male	53 (53%)	25 (50%)	78 (52%)	0.72
	Female	47 (47%)	25 (50%)	72 (48%)	
Residency	Rural	51 (51%)	24 (48%)	75 (50%)	0.73
	Urban	49 (49%)	26 (52%)	75 (50%)	

In their study, Flaih et al, (2023) showed that the age group (5-14) years had the highest proportion of infections (53.0%), and L. tropica was detected in 39 (61.9%) specimens, whereas L. major was detected in 24 (38.1%) specimens in patients with cutaneous leishmaniasis. Despite dermal lesion emerge in all parts of the bodies, a single lesions is most commonly observed, moreover, 13 (33.3%) samples infected with Leishmania tropica were in upper limb, while 9 (37.5%) infected with L. major were in the lower limbs. On contrary to L. major, the majority of L. tropica lesions occurred in urban area in Al-Muthanna/Iraq [13]. The study agreed with (ALTAIE, and AJ ALQAYIM, 2021) who proved that the prevalence rate of visceral leishmaniosis in children males in Baghdad city was higher than females, and the age group (1–3) years was the most affected group. The majority of infections were reported in the cold season in comparison to lower infection rates in the hot season. The hematological picture of the infected children demonstrated a microcytic hypochromic anemia with leukopenia, neutropenia as well as lymphocytosis [14]. Also (Alyasiri, and Ali, 2024) reported that the highest Kala azar rates were observed in females and the highest visceral leishmaniasis cases occurred in January and February, then declined gradually to reach the lowest rate in August. The highest incidence rate of visceral Kala azar was found in the age group (1-4) year, whereas the lowest rate was detected among the elderly patients. A high number

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of Kala azar infection was recorded in Al-Samawa city, and the highest number of cases were reported in rural areas, while the lowest cases were in urban areas [15].

The mean L. donovani IgG antibody level in the patients was (13.62±0.95) in comparison to control group (0.10±0.02) with highly significant differences (P≤0.001), and the mean IL-18 levels in patients was (31.14±2.96) when compared with the controls (1.23±0.31) with highly significant differences (P≤0.001). Also, the mean IL-37 levels in patients was (23.81±1.34) when compared with the controls (1.73±0.33) with highly significant differences (P≤0.001). Moreover, the mean TNFα levels in patients was (18.21±0.50) as compared with the controls (1.31±0.31) with highly significant differences (P≤0.001). Furthermore, the mean Glutathione levels in patients was (161.18±25.52) when compared with the controls (19.82±1.06) with a highly significant variation (P≤0.001) as shown in table (2).

Table 2. Distribution of mean levels of studied parameters in the study groups

Parameters	Groups	Mean	SE	P-value
IgG (ng/ml)	Case	13.62	0.95	≤0.001
	Control	0.10	0.02	
IL18 (ng/ml)	Case	31.14	2.96	≤0.001
	Control	1.23	0.31	
IL37 (ng/ml)	Case	23.81	1.34	≤0.001
	Control	1.73	0.33	
TNFα (ng/ml)	Case	18.21	0.50	≤0.001
	Control	1.31	0.31	
Glutathione (ng/ml)	Case	19.82	1.06	≤0.001
	Control	161.18	25.52	

The mean levels of Leishmaniasis antibodies in the current study was highest and this study matched with (Han, et al, 2023) who found that the total IgG, IgG1 increased and both IgG1/IgG and IgG1/IgG2 ratios significantly decreased following treatment, while no apparent increase was shown in IgG2/IgG ratio. Visceral leismaniasis patients without hyperglobulinemias showed significantly lower IgG1/IgG2 ratio, but higher IgG2/IgG ratio in comparison with patients with hyperglobulinemia. Furthermore, patients with visceral leishmaniasis having (+ve) bone marrow amastigote demonstrated significantly higher IgG1/IgG and higher IgG1/IgG2 ratios, with lower IgG2/IgG ratios. There was a correlation between IgG subclasses and abnormal hematological result [16]. While the level of IL-18 in visceral Leishmaniasis in the current study was highest, and these results agreed with several studies such as Kumar et al, (2014) in India who reported an association between Interleukin-18 Gene Polymorphisms and susceptibility to VL in endemic region of Bihar, and also the study conducted by (Mehrangiz, et al,

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2020) who found a highly significant difference between the mean serum IL-1β levels of the patient and the controls (47.34±23.82) and (20.49±9.38), respectively (p<0.001). in addition, mean serum levels of IL-17 in VL patients was (243.96 ± 73.46) which is twice higher than the controls (106.38 ± 129.06) (p <0.001). Many cytokines contribute to immunity regulation against visceral leishmaniasis. The present results revealed that highl serum IL-1β and IL-17 levels were found in VL patients. More studies are required to support our information on the regulatory roles of such cytokines in leishmanial infections [17]. In addition, (Tadesse et al., 2021) found high circulating IL-10, IFN-γ, and TGF-β1 concentrations prior to treatment. The detected increased levels of serum IL-10 noticeably dropped within 7 days following treatment commencement. Among all VL patients, the Anti-leishmanial antibody index (AI) was high regardless of the grade of spleen aspirates parasite prior to treatment and at various treatment times [18]. The mean level of IL-37 and TNFα were higher in visceral Leishmanisis, and the study was in a harmony with (Hussein, et al, 2022) who reported that (TNFα) which is a cytokine produced by the innate immune responses to visceral leishmaniasis, and is able to affect disease clearances in human hosts. The influence of the pro-inflammatory cytokine (TNFα) in the development of CL ulcer during infections is not clear. In the present study, levels of TNF-α were noticed in patients with cutaneous leishmaniasis, and this cytokine's level was also evaluated in newly-diagnosed people who underwent various pentostam treatment stages. The results remarkably detected a significant increased serum TNF-α levels in the newly infected patient group, in addition to the patients continuing second and third pentostam treatment trials, which was (1125.49, 838.75 and 1264.26) ng/ml respectively when compared with the healthy controls (235.35) ng/ml. Additionally, no significant observations were seen in TNF-α levels among the three groups of patients. The apparent increased levels of serum TNF-α may indicate that this pro-inflammatory cytokine as a biomarker in tracking and prognosis of the disease advancement [19]. Also Kumar et al, (2024) found that serum soluble IL-7 levels were higher in patients with VL in comparison with EC. It is of interest that the IL-7Rα protein expression was higher on the CD4+ T cells of VL patients in comparison with EC, and the activated CD38+ CD4+ T cells showed higher IL-7Rα surface expressions in comparison with CD38- CD4+ T cells among patients with visceral leishmaniasis. The CD4+ T cells of patients with VL showed higher signaling potentials baselines following recombinant human IL-7 (rhIL-7) stimulation in comparison with EC when measured by STAT5 (pSTAT5) phosphorylations [20]. Our study may be the first to measure interleukin 37 and we found that there is a

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very significant increase in its levels with infection with visceral leishmaniasis. On the other hand, Lamberet, et al, (2020) demonstrated that IL-33-KO mice had lower parasite loads and a higher Th1 response than their wt counterparts [21]. The study also found that there is a very significant decrease in Glutathione levels in visceral leishmaniasis infections. Amiri-Dashatan et al,(2020) shared opinion with this study as the levels of reduced glutathione (GSH) were significantly (P<0.001) reduced in cutaneous and visceral Leishmaniasis [22]. Alishvandi et l, (2024) concluded that there is a very significant inhibition of glutathione levels in Leishmania infantum infections, this means that there is inhibition of glutathione, which is accompanied by an increase in the levels of cytokines and interleukins [23].

There was a direct correlations between Glutathione level and TNFα (r= 00224**) with highly significant differences (P=0.006). Also, there was a direct correlation between Glutathione and IL-37 (r=0.155) with a significant variation (P=0.05) as shown in table (3).

Table 3. Correlation analysis between the levels of glutathione with the levels of IgG (ng/ml) and TNFα (ng/ml)

Parametere	Pearson Correlation	P-value	Significant
IgG (ng/ml)	0.148	0.07	Non-significant
TNFα (ng/ml)	00224**	0.006	Highly-significant
IL-18 (ng/ml)	0.096	0.24	Non-significant
IL-37 (ng/ml)	0.155	0.05	Significant

The direct corelation beteen reduced Glutathione and Intrlukine levels was increased in a study by Pinho et, al, (2022) who revealed that it may participate in NADPH pools maintenance and fuels reducing conditions for (GSH) recovery upon the exposure to NO. Hence, the capability of GSH-mediated redox and the elevated glucose consumption may clarify the natural L. braziliensis resistance to NO [24]. Tadesse et al, (2021) said that the parasite's specific enzymes and proteins which are important for the parasite's viability can be targeted for controling these parasites' proliferations and growths. Glycolysis, sterol biosynthesis and purine salvage pathways are some of the druggable targets which were more comprehensively investigated [25]. Furthermore, studies assume that both the antioxidant systems, glutathiones and trypanothione/trypanothione reductases, are involved in Leishmania protection against the toxic impacts of nitrogen-derived reactive species with the rise in levels of these interleukins and cytokines [26].

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Figure (1) showed the amplification of rs7674 to TNFα gene specific region of human blood samples were fractioned on 2% agarose gel electrophoresis stained by Eth. Br. With 100bp ladder marker.

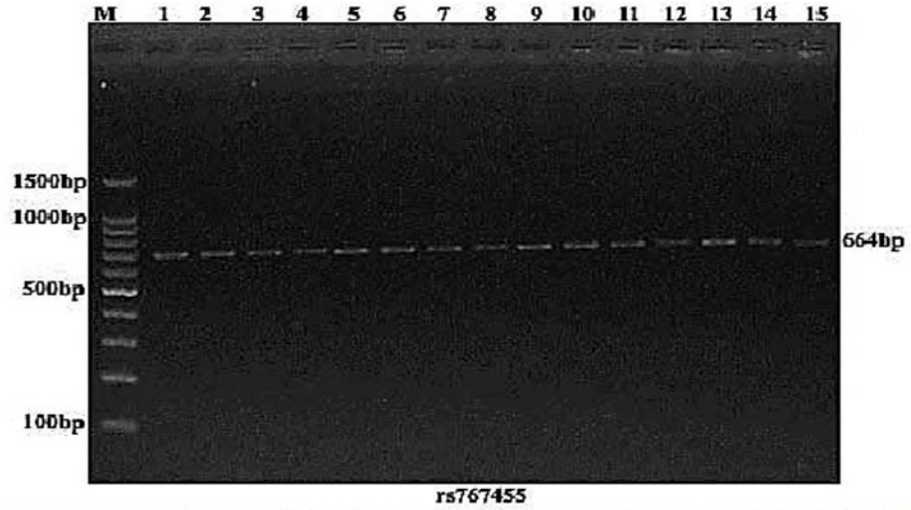

Figure 1: The amplification of rs7674 to TNF gene specific region of human blood samples species were fractioned on 2% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-15 resemble 664bp PCR products

Table (4) and figure (2) revealed that a variation occurred in rs767455 SNP to TNF GENE ID 7132, that AA was changed to AA to GG in samples number 9,11 and 15 with Leishmania donovani infections.

Table 4. The sequence of rs767455 SNP to TNF GENE ID 7132

TNFRSF1A GENE ID 7132	
SNPs	**rs767455**
Wild	**AA**
Variation	**A>G**
Samples	
1	AG
2	AG
3	AG
4	GG
5	AA
6	AG
7	AG
8	GG

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9	AA
10	AG
11	GG
12	AG
13	AG
14	GG
15	AG

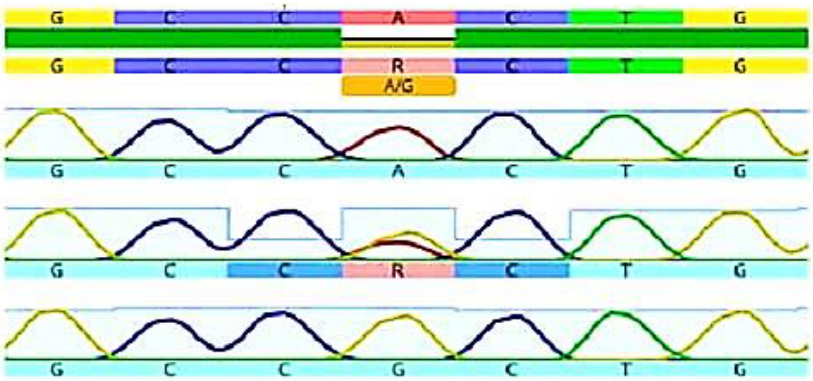

Figure 2: Analysis of SNP rs767455 of TNFRSf1A gene using sanger sequencing. Single "A" Peak indicative of an A homozygous allele. Single "G" peak indicative of G homozygous allele. Presence of the "A" and "G" peak indicative A/G heterozygous allele.

These results agreed with Khudhur, et al, (2022) who showed that their study tried to find if the existence of SNPs in TNF receptor [TNFRSF1A (rs767455), TNFRSF1B (rs1061622)] encoding genes can affect the outcomes of patients [26]. Also, Alhilali, et al, (2023) found that the effectiveness of treatment with infliximab in 58 RA patients may be affected by the variants 676 T>G (TNFR2). Individuals with TNFR1 36 A/A genotypes had the superior European Alliance of Associations for Rheumatology (EULAR) responses following 3 months treatment in comparison with individuals with G alleles in an experiment on 280 patients with RA who were treated with TNF inhibitors. Individuals with TNFR1 36 A/A genotypes showed less activity of the disease following six months than persons with G/G genotypes [27].

Conclusions

Based on our results, there was a direct correlation between Glutathione inhibition and TNFα (r= 00224**) with a highly significant variation P=0.006. Also, there was a direct correlation between Glutathione and IL-37 (r=0.155) with a significant variation. On the other hand, a variation occurred in rs767455 SNP to TNF GENE ID 7132, that AA was changed to GG patients with Leishmania donovani infection.

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