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Immuno-Informatics Insight into Relationship Between Cholesterol and Cytokines in Cutaneous Leishmaniasis

From clinics to computation

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Abstract

***Objectives*:** The role of serum cholesterol and its interactions with cytokines in the pathophysiology of human cutaneous leishmaniasis (CL) is not known. This study was aimed to evaluate the correlation between serum total cholesterol (TC), very-low-density lipoprotein-cholesterol (VLDL-C), low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C) and triglycerides (TG), and cytokines including interleukin-10 (IL10), interleukin-12 (IL12), tumor necrosis factor-alpha (TNF-α), in CL. Moreover, we analyzed the cholesterol-cytokine network to shed light on the pathogenesis of CL. ***Methods*:** A case-control study including CL patients (n = 50) and control subjects (n = 25) ranging between 20-30 years old was conducted from December 2022 to March 2023. The serum samples were analyzed via commercial kits to detect the levels of TC, IL-10, IL-

12, TNF-α, VLDL-C, LDL-C, HDL-C and TG. Computational efforts to dissect cholesterol-protein interaction networks was also employed using STITCH. ***Results*****:** TC, HDL-C, and LDL-C levels were markedly lower (p =0.0001) in CL patients compared to those of control subjects, whereas IL10, IL12, TNF-α, VLDL-C and TG levels were higher in CL patients. Serum cholesterol did not exhibit a correlation with cytokines, however a significant correlation (r = 0.57; p = 0.026) was observed between IL12 and TNF-α. Within the cholesterol-protein network, cholesterol potentially interacted with IL10, connecting cholesterol to modules with immunological significance, including TARAF1, TARAF2, and TNFRSF1B, as well as IL10, IL10RA, and IL12RB1. ***Conclusion*****:** This study showed alteration of lipid and lipoprotein in CL, and it introduced two immunological modules in CL which appreciates attention to the altered cholesterol-cytokine interaction network in CL.
Keywords: Cutaneous Leishmaniasis, Cholesterol, Cytokine, Interaction Network

Advances in Knowledge:

- This study pioneers the exploration of cholesterol-cytokine interactions in cutaneous leishmaniasis (CL), offering novel insights into the immunopathogenesis of this parasitic infection. Employing computational tools, this research reveals a cholesterol-protein interaction network, advancing our understanding of the altered molecular landscape in CL and its potential implications for host immune responses. This study identifies specific immunologically significant modules within the cholesterol-protein network, contributing to the knowledge of key players in CL pathogenesis.

Application to Patient Care:

- The observed alterations in cholesterol and cytokine levels provide potential diagnostic biomarkers for CL, aiding in early detection and targeted intervention. The identified cholesterol-protein network modules present promising therapeutic targets, offering a foundation for the development of novel treatment strategies for CL. Understanding the intricate relationship between cholesterol and cytokines allows for personalized patient care strategies, tailoring interventions based on individual immunological profiles in CL.

Introduction

The alteration of cholesterol metabolism is required for the internalization of pathogenic protozoa to the target cells and their life cycle and proliferation. However, the exact mechanism of this alteration is not defined completely. Alterations in the lipid profile have been observed in patients who have various parasitic infections.[1] Additionally, it is still unknown what types of molecules cause lipid alterations, particularly in membrane proteins, which are linked to parasite infection. Through both *in vivo* and *in vitro* investigations, it has been found that if the serum is replaced with fat or cholesterol in the medium or animal models, parasites cause considerable alterations in the lipid parameters. Subsequently, individuals with active parasite infections showed alterations in their lipid profiles.[1-3]

It is unclear how cholesterol is necessary for eukaryotic pathogens to internalize under complex conditions of tissue distribution and lodging.[4] The intestine, blood, liver, lungs, brain, muscles, and lymphatic tissues are typical habitates for protozoa, helminths, and arthropods, known as common human parasites.[5] Numerous parasite species have intricate life cycles, with developmental stages occurring in soil or water. They may utilize a variety of intermediate hosts, including vertebrates, invertebrates, and both cold-blooded and warm-blooded animals.[6] Parasites have evolved to tolerate a wide range of oxygen, carbon dioxide, and hydrogen ion concentrations, as well as temperatures in these various conditions. They exhibit different dietary needs and employ various strategies to obtain and utilize the necessary nutrients for growth, motility, and reproduction.[7] Cholesterol, as a cardinal component of eukaryotic membranes, is essential for the organization, dynamics, function, and sorting of cellular membranes.[8] It is frequently discovered that cholesterol is dispersed non-randomly in the membrane domains.[9] In this regard, cholesterol performs many of its functions by preserving the functionality of a specific sort of membrane domain known as lipid rafts.[10, 11] Cholesterol and sphingolipids are abundant in lipid rafts which have been proposed to serve as a platform for coordinating signal transduction processes and entering pathogens into the host cells.[10]

The immune response and cytokines released by T helper 1 (Th1) and T helper 2 (Th2) cells determine the etiology and rate of progression of diseases. Although the precise relevance of the Th1 and Th2 cells in the pathogenesis of human cutaneous leishmaniasis (CL) is not yet fully understood, certain animal model studies using BALB/c have provided a clear explanation of the immune response.[12] It is demonstrated that Th2 cells proliferate during the progression of the disease and Th1 cells proliferate during the disease control.[13] Tumor

necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-ɣ), released from Th1 cells, activate macrophages and induce nitric oxide synthase.[14] Meanwhile, Th2 cells suppress macrophages by releasing interleukin 10 (IL10), thereby facilitating parasite proliferation.[13]

This study was carried out to assess the levels of TNF-α, IL12, and IL10 cytokines in CL patients in comparison to those of control subjects to explore their correlation with serum cholesterol. The computational tools were also employed to decipher the cholesterol-cytokine network to shed light on the role of cholesterol in the pathogenesis of CL.

Materials and methods

Subjects

We performed this case-control study from December 2022 to March 2023. The demographical and clinical features of CL patients (n = 50) and CL-free control subjects (n = 25) referred to hospitals within Baghdad and Wasit provinces hospitals, Iraq were recorded (Table 1). The study's purpose was explained to participants who then provided their consent to enrol. Subsequently, CL patients (Figure 1), with no history of prior leishmaniasis management, and healthy volunteers without a history of CL were enrolled as control subjects.

The procedure received approval from the Ethics Review Committee, Department of Experimental Therapy, Iraqi Center for Cancer and Medical Genetic Research, Mustansiriyah University, Baghdad, Iraq. Permission to conduct the study was granted by the administration of Baghdad Hospital. All participants were informed that their involvement was voluntary. Written consent, outlining the purposes and procedures in the native language, was obtained from each adult participant. All information provided by the respondents was kept confidential and used exclusively for the study.

Diagnosis of CL

The diagnosis of CL was conducted through an immune-fluorescent antibody test (IFAT), which relies on the reaction of antibodies in the sample with the antigen (leishmania promastigotes) adsorbed on the slide surface. The emitted fluorescent light was assayed via an immunofluorescence microscope (Etaluma, Inc. USA).

Quantification of serum parameters

The serum levels of IL10 and IL12 were measured using commercially available human sandwich ELISA kits (MyBioSource, USA). Also, the serum total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), and triglycerides (TG) were assessed by a commercial kit (Linear, Spain). All quantification procedures were conducted following the instructions provided in the commercial kit catalogue. Finally, the absorbance of both the sample and standard solutions for each TC, HDL-C, and TG measurement was read at a wavelength of 500 nm using a Spectrophotometer (Agilent 8453, Agilent Technologies, Inc., US). The calculation of serum TC, TG, and HDL-C levels was performed using the following equation.[15]

Serum concentration of each parameter (mg/dl) = (Absorbance of sample/Absorbance of standard) × concentration of the standard (mg/dl)

Serum very-low-density lipoprotein-cholesterol (VLDL-C) and low density lipoprotein-cholesterol (LDL-C) were calculated according to the equation of Friedewald et al. [16], respectively:

Serum LDL-C concentration (mg/dl) = TC- (TG/5)- HDL-C
Serum VLDL-C concentration (mg/dl) = (TG/5)
Serum LDL-C concentration (mg/dl) = TC - VLDL-C - HDL-C

Statistical analysis

The Statistical Analysis System (SAS, 2012) program was utilized for data analysis. The Pearson correlation coefficient and independent T-test were employed to compare biochemical variables between the case and control groups, with significance set at $p \leq 0.05$.[17] Dot plots were created to depict differences in the distribution of biomarker levels between cases and controls.

Immuno-informatics

The immune-informatics analysis was conducted using the Search Tool for Interactions of Chemicals (STITCH) platform (http://stitch.embl.de).[18] Specifically, a components-targets analysis was constructed, considering cholesterol and human cytokines assayed in this study (IL10, IL12, and TNF-α) to explore the network-based relationships of these molecules. The drawn network diagram was dissected to delve deeper into the relationships among these

molecules and to identify new co-players. This exploration aims to propose more impactful avenues for further investigations of putative targets and ligands. The STITCH platform was employed to represent cytokine-cholesterol interactions in this context.

The gene mining of leishmaniosis has been curated from Public Health Genomics and Precision Health Knowledge Base (v8.4) of Phenopedia (Centers for Disease Control and Prevention (CDC); http://www.cdc.gov/).

Before sample collection, all participants received detailed information about the study, and verbal consent was obtained from each one. This research was approved under reference number BMS/0542/06 by the Committee on Publishing Ethics at the College of Science, University of Mustansiriyah, Iraq.

Results

CL infection was confirmed by the positive findings from an immunofluorescence microscope (Figure 2). Figure 4, supplementary Figure 2 and Supplementary Table 1 and 2 indicate significant differences in TC, IL10, IL12, TG, HDL-C, VLDL-C and LDL-C levels between patients and control subjects. The levels of all three cytokines (including IL10, IL12, and TNF-α) were considerably increased in patients compared to normal subjects. In contrast, TC levels were significantly lower in CL patients compared to controls. Dot plots were created to depict differences in the distribution of Cytokines and Lipid profiles levels between cases and controls (supplementary file S2 Figure 3).

The statistical evaluation of the correlation coefficient (r) between the study parameters revealed a strong positive correlation between IL12 and TNF-α, while other parameters did not show significant correlation (Table 2). The dot plot of significant correlation of IL12 and TNF-α is presented in supplementary file S2 Figure 4.

Based on the analyzed data extracted from STITCH through data mining, the condensed interaction network of cholesterol with measured cytokines (as detailed in the supplementary file S1 and Figures 1, 2) did not reveal the presence of any endogenous or exogenous chemicals within the network, aside from cholesterol. Within this network, cholesterol exhibited direct interactions with CYP11A1, CYP7A1, LCAT, HMGCR, APOB, ABCA1, and APOA1, which were not the focal proteins of interest in our study. More specifically,

cholesterol demonstrated a potential direct interaction with IL10, thereby establishing a connection between cholesterol and two modules with immunological significance. In this context, IL10 has been directly interacted with TNFRSF1B, ABCA1, IL12RB1, and IL10RA (Figure 3). The type of interaction of IL10 as an anti-inflammatory cytokine with TNF-α receptor, TNFRSF1B, has been text-mined and involved in the cytokine targets for arthritis therapy.[19] Future investigations are acknowledged to dig deeper in the cytokine profile of CL with a special focus on the TNF-TNFR family.

Based on the Phenopedia (http://www.cdc.gov/), there was not any report regarding genes involved in the CL while just 12 genes including CCR5, COL1A1, Col1a2, Mmp13, St3gal5, FLI1, IL2, IL2RA, IL2RB, JAK3, and CCL2 have been reported for mucocutaneous leishmaniosis. The elevated levels of IL12 in CL patients compared to control subjects, along with the absence of any interactions between IL12 and cholesterol, are noteworthy findings in the present study. IL10 functions as an intermediary node, linking cholesterol to a trio module comprising TARAF1, TARAF2, and TNFRSF1B through data extraction[20] which needs more experiments that shed light on this interaction. However, we did not find any significant correlations between cholesterol with TNF, IL10, and IL12. To the best of our knowledge, we initially discussed avenues for future research to further clarify the impact of TARAF1, TARAF2, and TNFRSF1B trio in the pathogenesis of human CL. Moreover, IL10 has been potentially interacted indirectly with cholesterol with inter-node ABC1 (Figure 3). The KEGG pathway of the cholesterol-cytokine network constructed in this study (Figure 3, supplementary file S1 and supplementary Figure1) presented pathways including fat digestion and absorption, Epstein-Barr virus infection, cytokine-cytokine receptor interaction, and TNF signalling pathway with very low false discovery rate. However, two African trypanosomiasis and toxoplasmosis pathways will give us stronger cues regarding the involvement of IL10, IL10RA, and APOA1 in the pathogenesis of CL as a protozoan infection. In this context, considering another aspect of protozoa's metabolic competition with the host, the significance of APOA1 becomes more pronounced. APOA1 functions as an apolipoprotein, actively participating in the reverse transport of cholesterol and serving as a cofactor for lecithin cholesterol acyltransferase (LCAT). In this regard, Escribano *et al.*[21] highlighted that an increase in serum apolipoprotein-A1 levels could potentially serve as a biomarker for the efficacy of therapy in canine leishmaniosis. The anti-inflammatory property of APOAI represents an additional mechanism reinforcing our hypothesis that APOAI,

functioning as a Trypanosome lytic factor I, may contribute to the evasion of the host innate immune system by Leishmania parasites.[22]

Among the ontology of biological processes computed from the STITCH-constructed network (Supplementary file S1 and Figures 3), ABCA1, CYP11A1, IL10, IL10RA, and TNFRSF1B were identified as participants in the response to other organisms. At the molecular processing ontology level, two prominent pathways were identified: receptor binding and enzyme binding. These pathways involved ABCA1, APOA1, APOB, TNFRSF1B, TRAF1, TRAF2, and IL10 (refer to Supplementary files S1, S2, and Figure 1). On the other hand, the cellular component ontology of our cholesterol-cytokine network primarily centered around the plasma lipoprotein particle, with key involvement from APOA1, APOB, and LCAT (refer to Supplementary files S1, S2, and Figure 3).

In a straightforward analysis of the cholesterol-cytokine network, the statistics included a total of 13 nodes, 22 edges, an average node degree of 3.38, a clustering coefficient of 0.846, an expected number of edges at 11, and a protein-protein interaction (PPI) enrichment p-value of 0.00302. Notably, in an attempt to enhance network enrichment, statins were the only chemicals introduced into our cholesterol-cytokine network, as detailed in Supplementary file S2.

Discussion

While the relationship between blood lipid and lipoprotein profiles and the pathogenesis of leishmaniasis is not yet fully understood, some studies have focused on the role of cholesterol in the pathogenesis of parasitic infections. The effect of HDL-C on leishmaniasis remains not fully understood; however, some studies have suggested that leishmaniasis can decrease the levels of blood HDL-C. For instance, one study reported that patients with visceral leishmaniasis had lower levels of HDL-C (mean = 22.8 mg/dl) than healthy controls (mean = 48.6 mg/dL) and observed that HDL-C levels were inversely correlated with parasite load and disease severity. This suggests that HDL-C may play a protective role against leishmaniasis by inhibiting the entry and replication of Leishmania in macrophages.[23] Another study reported that Leishmania parasites can consume the host's cholesterol to evade the immune response and survive inside the cells.[24] Moreover, the host's lipid droplets, which are storage organelles for lipids, may play a key role in disease progression and parasite development.[25] In summary, the effect of HDL-C on leishmaniasis is multifaceted and involves multiple

factors, including the metabolism of the host and parasite, and the host's immune system. More research is required to fully elucidate the mechanisms and implications of this effect. Additionally, the effect of TG on leishmaniasis is not well understood, but some investigations have suggested that leishmaniasis can increase blood TG levels. For example, one study found that patients with visceral leishmaniasis had higher TG levels than healthy controls.[26] Another study reported that Leishmania parasites can use the host's TG to produce their own lipids and survive inside the cells.[27] According to some results, there may be a link between VLDL-C and leishmaniasis. One study found that Leishmania parasites can bind to VLDL-C receptors on the surface of macrophages, which are immune cells that normally kill the parasites. By binding to these receptors, the parasites can enter the macrophages and avoid being destroyed by the immune system. The study also showed that blocking the VLDL-C receptors reduced the parasite load and improved the outcome of the infection in mice.[28] Another study found that visceral leishmaniasis could trigger hemophagocytic lymphohistiocytosis by causing persistent activation of lymphocytes and histiocytes, leading to hypersecretion of pro-inflammatory cytokines and dysregulation of lipid metabolism.[29] This could potentially impair the ability of the macrophages to kill the parasites and favor their survival and replication.[30] A recent study confirmed that hypertriglyceridemia was correlated with increased levels of inflammatory markers, such as C-reactive protein, interleukin-6, and TNF-α3. It concluded that hypertriglyceridemia could be used as a biomarker of VL severity and prognosis.[31] One study found that hypertriglyceridemia (high levels of TG, which are carried by VLDL-C) was a possible marker of disease severity in visceral leishmaniasis.[32] Lipid formulations of drugs may enhance uptake by macrophages, the cells that the parasite infects.[31] However, more research is needed to understand the exact relationship between VLDCL- and leishmaniasis.

In line with our findings, another study by Oliveira *et al.* 2014 demonstrated a considerable increase in the levels of interferon-gamma (IFN-γ) and TNF-α cytokines in the treated group with soluble leishmania antigen (SLA) and phytohaemagglutinin (PHA) mitogen. Furthermore, in the healed group, the level of IL10 dramatically decreased, while it significantly increased in the unhealed groups. The evidence suggested that Leishmania braziliensis-induced tegumentary leishmaniasis is characterized by increased IFN-γ and TNF-α, the absence of IL10 production, tissue damage, and the development of lesions similar to those observed in CL and mucosal leishmaniasis (ML). Then, in their study, SLA was used to excite peripheral blood mononuclear cells from CL and ML in the presence or absence of

regulatory cytokines (IL10, IL-27, and TGF-α) or other cytokines (TNF-α and IFN-ɣ). TNF-α and IL-17 production was downregulated by IL10, TGF-α, and IL-17 production. However, the IL-27 level was unaffected in these patients. Their study showed that the immune response in CL patients seems to be more modulated by the cytokines IL10 and TGF-α since the neutralization of IFN-ɣ reduces the generation of TNF-α in an IL10-dependent way.[33]

In the present study, the level of IL12 and TNF-α may indirectly show the involvement of Th1 in producing these inflammatory cytokines that play a role in the initial protective immunity for Leishmania. In contrast, since IL10 prevents the generation of mediators like nitric oxide, IFN-ɣ, and the leishmanicidal activity of macrophages, IL10 may be considered as an inhibitory strategy against overt inflammatory responses during the progression of CL and is linked to the disease progression.[34] A systematic review and meta-analysis by Silva *et al.* revealed a relationship between particular polymorphisms and the regulation of IL10 and the emergence of more significant clinical manifestations of leishmaniasis.[35]

Another study supported the hypothesis that the blockade of TNF-α alters the clinical manifestation of leishmaniasis in endemic populations, leading to atypical presentations.[36] According to the cases described, the optimal course of treatment would involve systemic medication and the cessation of TNF-blocker therapy until clinical improvement. In this context, pro- and anti-inflammatory cytokines play distinct roles in resistance/susceptibility, immune pathogenesis, and the temporal and spatial balance of cytokines that may control or predict the clinical manifestation of CL.[36] Another research effort has centered on the elevation of cholesterol in CL patients. It has been demonstrated that cholesteryl esters attached to fatty acids and associated with LDL-C are increasingly retained in subcellular fractions containing parasites during Leishmania infection of macrophages. Host cell cholesterol is transported to the parasitophorous vacuole (PV), where it becomes integrated into the parasites. Meanwhile, filipin staining revealed a halo surrounding the parasites within the PV. The upregulation of mRNA encoding proteins essential for cholesterol production coincided with this dual cholesterol sequestration process.[37]

In alignment with this perspective, Kumar *et al.* 2016[38] reached the conclusion that maintaining a critical level of membrane cholesterol in host cells is essential for CL. Furthermore, they found that chronic statin-induced hypocholesterolemia effectively inhibits the proliferation of *Leishmania donovani*.

Conclusions

In summary, this study identified decreased TC levels and increased levels of IL10, IL12, and TNF-α in CL patients compared to normal subjects. While no significant correlations were observed between cholesterol and cytokines, a positive correlation was found between IL12 and TNF-α in CL. Future investigations are warranted to further explore the cytokine profile in CL, with a particular emphasis on the TNF-TNFR family. Notably, Phenopedia, a disease-centered view of genetic association studies did not report any genes associated with CL, and this computational effort opens new avenues for understanding the pathogenesis of CL. In this context, two immunological modules including TARAF1, TARAF2, and TNFRSF1B, as well as IL10, IL10RA, and IL12RB1 were found, which their involvement in CL should be pursued in future studies.

Conflict of Interest

The authors declare no conflicts of interest.

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Authors' Contribution

EHS contributed to the methodology, data curation, validation, preparation, visualization and investigation. LJM contributed to the supervision, conceptualization, methodology, writing-original draft preparation, writing- reviewing and editing. AHT contributed to the data curation and validation. IK contributed to the writing, visualization, reviewing and editing of the manuscript. All authors approved the final version of the manuscript.

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Table 1. Demographical and Clinical Characteristics of Patients with Cutaneous Leishmaniosis.

	Control subjects (n = 25)		Patients (n = 50)	
	Female (n = 15)	**Male (n = 10)**	**Female (n = 30)**	**Male (n = 20)**
Age range (years)	20-30	20-30	20-30	20-30
Weight range (Kg)	50-75	60-80	50-75	60-80
Location of skin infection				
Face	0	0	10	3
Hand	0	0	15	10
Feet	0	0	5	7
Erythematous	0	0	20	10
Local recurrence	0	0	2	4
Tumors	0	0	0	0

Table 2. The correlation coefficients between measured parameters in this study.

	Correlation coefficient	P-value
IL10 & IL12	0.22	0.414
IL10 & TNF-α	0.16	0.566
IL10 & TC	-0.03	0.902
IL12 & TNF-α	0.57 *	0.026
IL12 & TC	-0.34	0.213
TNF-α & TC	0.11	0.682

*Note: *Significant level: p≤0.05. IL10: Interleukin-10, IL12: Interleukin-12, TNF-α: tumor necrosis factor-alpha, TC: Total cholesterol*

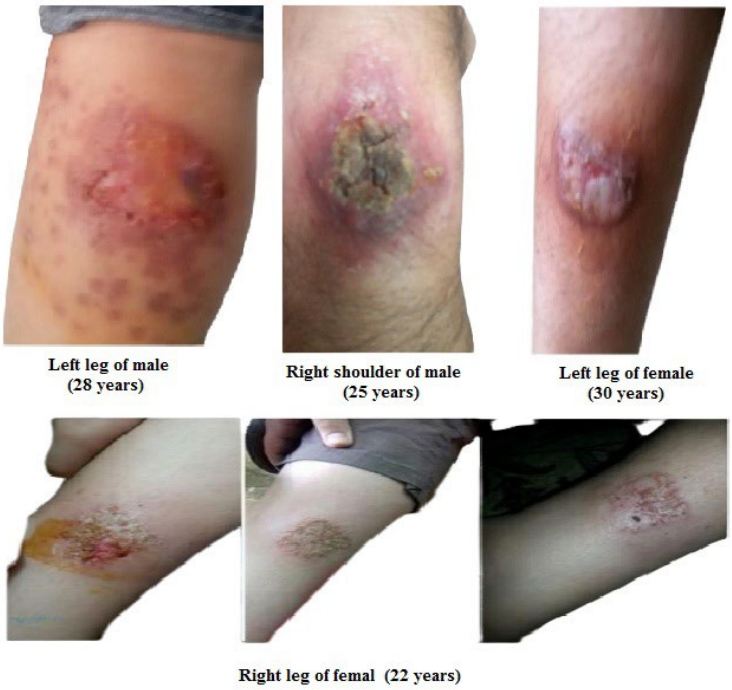


Figure 1. Clinical appearance of some cases of the cutaneous leishmaniosis.

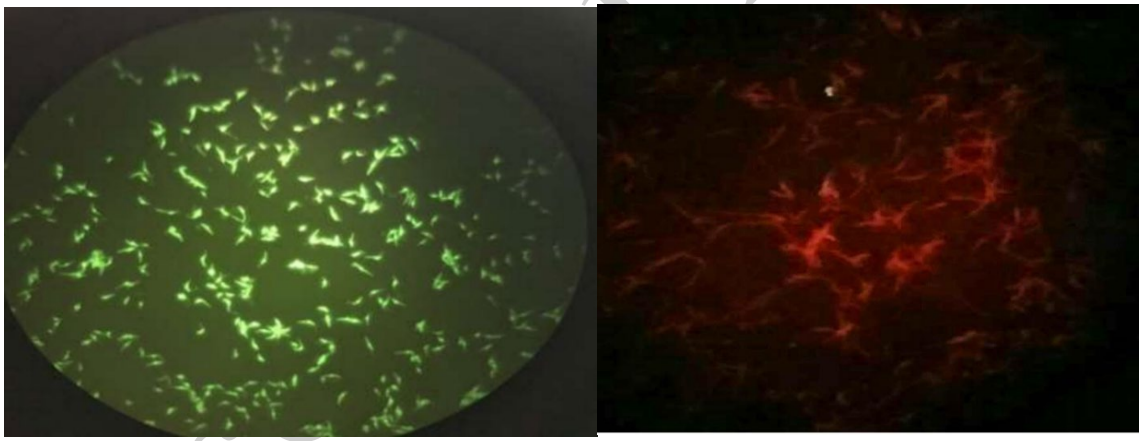


Figure 2. Immunofluorescent photos of leishmanial promastigotes (left photo: positive; right: negative).

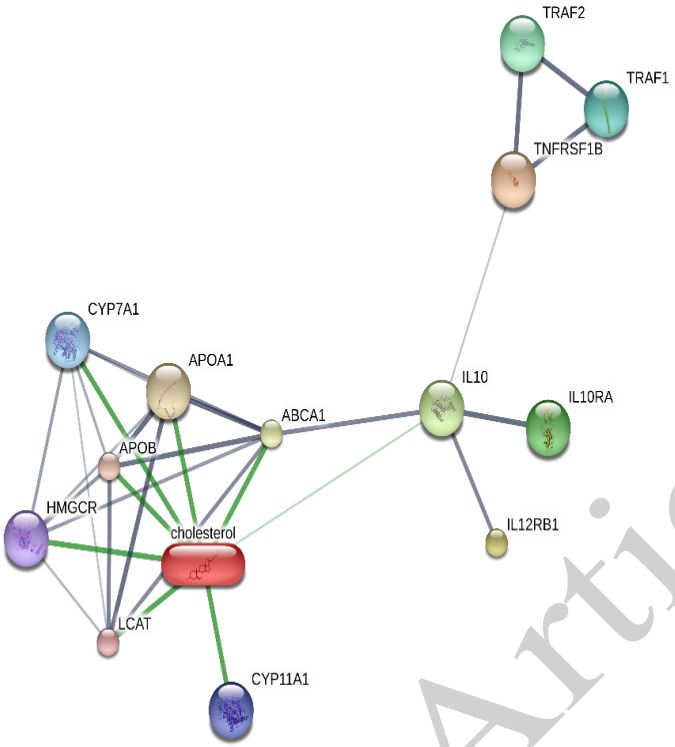
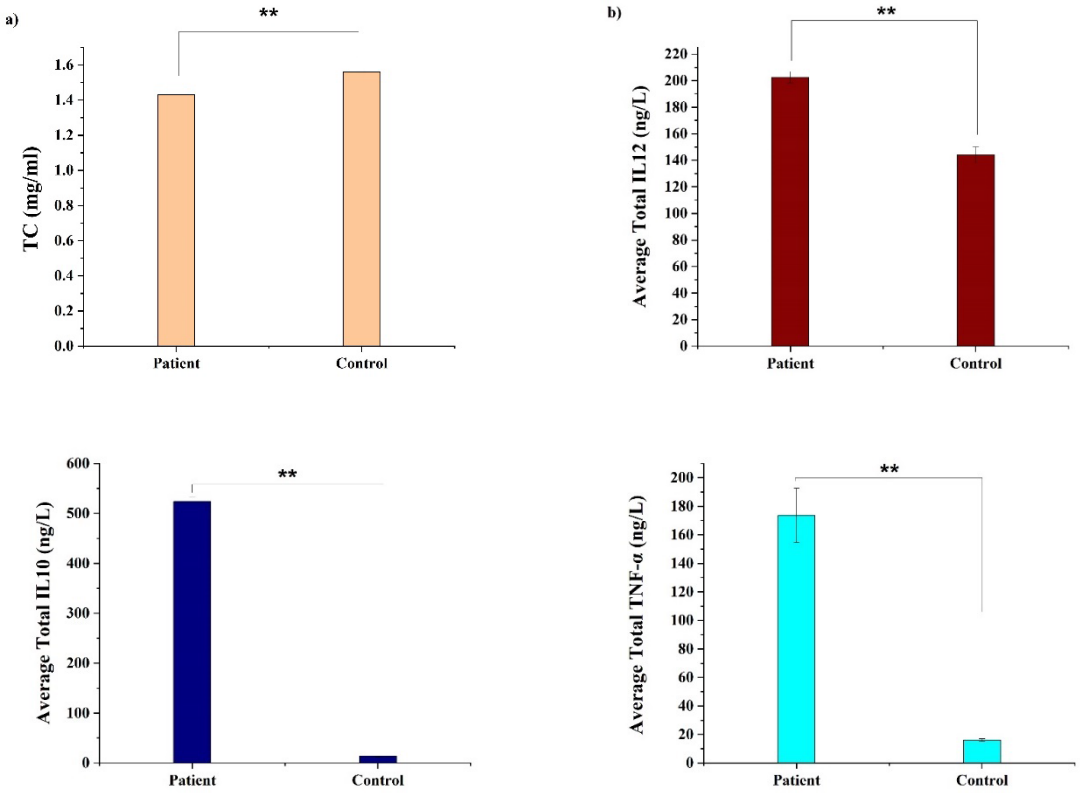


Figure 3. Interactions of the cholesterol with selected cytokines possess diagnostic value in the pathogenesis of cutaneous leishmaniosis. Proteins are demonstrated as spheres while cholesterol is shown as capsule-shaped node. Stronger associations are represented by thicker lines. Protein-protein interactions are shown in grey, chemical-protein interactions in green. *TRAF1: TNF receptor-associated factor 2; TRAF2: TNF receptor-associated factor 2; TNFRSF1B: tumor necrosis factor receptor superfamily, member 1B; IL10: interleukin-10; IL10RA; interleukin-10 receptor, alpha; IL12RB1: interleukin-12 receptor, beta 1; ABC1A: ATP-binding cassette, sub-family A (ABC1); APOA1: apolipoprotein A-I; APOB: apolipoprotein B; CYP7A1: cytochrome P450, family 7, subfamily A, polypeptide 1; HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase; LCAT: lecithin-cholesterol acyltransferase; CYP11A1: cytochrome P450, family 11, subfamily A, polypeptide 1.*



510 **Figure 4:** The serum TC and cytokine profiles in *CL patients (n=50) and Healthy Controls*
511 *(n=25). Average of a) TC, b) IL12, c) IL10, d) TNF-α. TC: Total Cholesterol, IL: interleukin;*
512 *TNF-α: tumor necrosis factor alpha; (**p≤0.01).*