Sultan Qaboos University Med J, November 2024, Vol. 24, Iss. 4, pp. 507–514, Epub. 27 Nov 24
Submitted 21 Jan 24
Revision Req. 2 May 24; Revision Recd. 13 May 24
Accepted 2 Jul 24
https://doi.org/10.18295/squmj.7.2024.043

CLINICAL & BASIC RESEARCH

Immuno-Informatics Insight into the 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 human cutaneous leishmaniasis (CL) pathophysiology is unknown. This study aimed to evaluate the correlation among serum total cholesterol (TC), very-low-density lipoprotein cholesterol (VLDL-C), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG) and cytokines (including interleukin [IL] 10), IL-12 and tumour necrosis factor-alpha [TNF-α]) in CL. The cholesterol–cytokine network was analysed to illuminate the pathogenesis of CL. ***Methods:*** This case-control study was conducted from December 2022 to March 2023 in hospitals within Baghdad and Wasit provinces, Iraq, and included CL and CL-free subjects ranging between 20–30 years of age. The serum samples were analysed via commercial kits to detect TC, IL-10, IL-12, TNF-α, VLDL-C, LDL-C, HDL-C and TG levels. Computational efforts to dissect cholesterol-protein interaction networks were employed using STITCH. ***Results:*** A total of 50 CL and 25 control subjects were included. The TC, HDL-C and LDL-C levels in CL patients were markedly lower (P = 0.0001) than in control subjects, whereas the IL-10, IL-12, TNF-α, VLDL-C and TG levels were higher in CL patients. Serum cholesterol showed no correlation with cytokines; however, a significant correlation (r = 0.57; P = 0.026) was observed between IL-12 and TNF-α. Within the cholesterol-protein network, cholesterol potentially interacted with IL-10, connecting cholesterol to modules with immunological significance, including TRAF1, TRAF2 and TNF receptor superfamily member 1B, as well as IL-10, IL-10RA and IL-12RB1. ***Conclusion:*** This study showed the alteration of lipid and lipoprotein in CL and introduced 2 immunological modules in CL, highlighting the importance of the altered cholesterol-cytokine interaction network in CL.

Keywords: Cutaneous Leishmaniasis; Cholesterol; Cytokine; Protein Interaction Network.

Advances in Knowledge

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

Applications 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 developing novel treatment strategies for CL.*
- *Understanding the intricate relationship between cholesterol and cytokines allows for personalised patient care strategies, tailoring interventions based on individual immunological profiles in CL.*

The alteration of cholesterol metabolism is required for the internalisation of pathogenic protozoa to the target cells and their life cycle and proliferation. However, the exact mechanism of this alteration has not been defined completely. Alterations in the lipid profile have been observed in patients with 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. Both *in vivo* and *in vitro* investigations have 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 internalise under complex

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conditions of tissue distribution and lodging.[4] The intestine, blood, liver, lungs, brain, muscles and lymphatic tissues are typical habitats 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 utilise a variety of intermediate hosts, including vertebrates, invertebrates and both cold-blooded and warm-blooded animals.[6] Moreover, 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 utilise the necessary nutrients for growth, motility and reproduction.[7] Cholesterol, as a cardinal component of eukaryotic membranes, is essential for the organisation, 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 aetiology 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 has been demonstrated that Th2 cells proliferate during the progression of the disease, while Th1 cells proliferate during the disease control.[13] Tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), released from Th1 cells, activate macrophages and induce nitric oxide synthase.[14] Meanwhile, Th2 cells suppress macrophages by releasing interleukin 10 (IL-10), thereby facilitating parasite proliferation.[13]

This study aimed to assess the levels of TNF-α, IL-12 and IL-10 cytokines in CL patients in comparison to those of control subjects to explore their correlation with serum cholesterol. Computational tools were also employed to decipher the cholesterol–cytokine network to illuminate the role of cholesterol in the pathogenesis of CL.

Methods

This case-control study was conducted from December 2022 to March 2023 and included CL and CL-free subjects referred to hospitals within Baghdad and Wasit provinces, Iraq. CL patients with no history of prior leishmaniasis management and healthy volunteers without a history of CL were enrolled as control subjects. The diagnosis of CL was conducted through an immune-fluorescent antibody test, 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., Carlsbad, California, USA).

The serum levels of IL-10 and IL-12 were measured using commercially available human sandwich ELISA kits (MyBioSource, San Diego, California, USA). Moreover, the serum TC, high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG) were assessed by a commercial kit (Linear Chemicals S.L.U., Barcelona, 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, Santa Clara, California, USA). The calculation of serum TC, TG and HDL-C levels was performed using the following equation:[15]

serum concentration of each parameter in mg/dL = (absorbance of sample/absorbance of standard) × concentration of the standard in mg/dL

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

serum LDL-C concentration in mg/dL = TC - (TG/5) - HDL-C

serum VLDL-C concentration in mg/dL = (TG/5)

serum LDL-C concentration in mg/dL = TC - VLDL-C - HDL-C

The Statistical Analysis System (SAS) programme, Version 9 (SAS Institute Inc., Cary, North Carolina, USA) was utilised for data analysis.[17] The Pearson correlation coefficient and independent t-test were employed to compare biochemical variables between the case and control groups, with significance set at $P \leq 0.05$. Dot plots were created to depict differences in the distribution of biomarker levels between cases and controls.

The immune-informatics analysis was performed using the Search Tool for Interactions of Chemicals (STITCH) platform.[18] Specifically, a components-targets analysis was constructed, considering cholesterol and human cytokines assayed in this study (IL-10, IL-12 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 identify new co-players. This exploration aimed 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 leishmaniasis has been curated from the Public Health Genomics and Precision Health Knowledge Base, Version 8.4 of Phenopedia (Centers for Disease Control and Prevention, Atlanta, Georgia, USA).

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. This research was approved by the Committee on Publishing Ethics at the College of Science, University of Mustansiriyah, Iraq (BMS/0542/06).

Results

A total of 50 CL patients and 25 CL-free control subjects were included in this study. The participant age ranged from 20–30 years [Table 1]. CL was found in various locations on the body [Figure 1]; CL infection was confirmed via immunofluorescence microscopy results [Figure 2]. Significant differences in TC, IL-10, IL-12, TG, HDL-C, VLDL-C and LDL-C levels between patients and control subjects were found ($P \leq 0.01$) [Figure 3, Supplementary Figure 2 and Supplementary Tables 1 & 2]. The levels of all 3 cytokines (IL-10, IL-12 and TNF-α) were considerably increased in patients compared to normal subjects. In contrast, TC levels were significantly lower in CL patients than in controls. Dot plots were created to depict differences in the distribution of cytokine and lipid profile levels between cases and controls [Supplementary Figure 3].

Table 1: Characteristics of patients with and without cutaneous leishmaniosis

Characteristic	Control subjects (n = 25)		Patients (n = 50)	
	Female (n = 15)	Male (n = 10)	Female (n = 30)	Male (n = 20)
Age range in years	20–30	20–30	20–30	20–30
Weight range in 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
Tumours	0	0	0	0

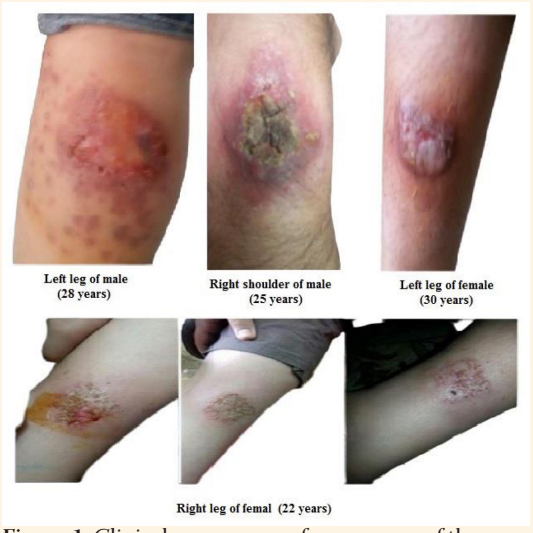

Figure 1: Clinical appearance of some cases of the cutaneous leishmaniosis.

The statistical evaluation of the correlation coefficient (r) between the study parameters revealed a strong positive correlation between IL-12 and TNF-α ($P = 0.026$) while other parameters did not show a significant correlation [Table 2 and Supplementary Figure 4].

Based on the analysed data extracted from STITCH through data mining, the condensed interaction network of cholesterol with measured cytokines did not reveal the presence of any endogenous or exogenous chemicals within the network, aside from cholesterol [Figure 4, Supplementary Dataset] Within this network, cholesterol exhibited direct interactions with CYP11A1, CYP7A1, lecithin cholesterol acyltransferase (LCAT), HMGCR, APOB,

Table 2: Correlation coefficients between measured parameters in this study

Parameter	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

IL = interleukin; TNF-α = tumour necrosis factor-alpha; TC = total cholesterol.

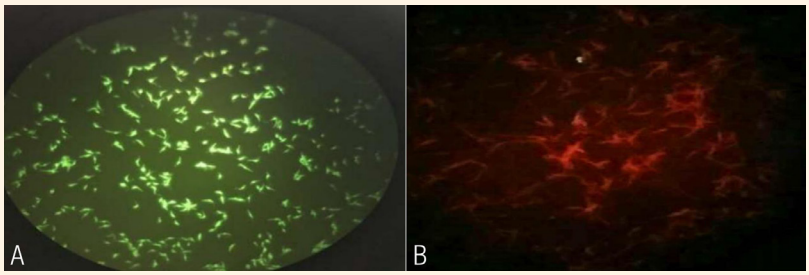

Figure 2: Immunofluorescent photos of *Leishmanial promastigotes* showing (**A**) positive and (**B**) negative result.

ABCA1 and APOA1, which were not the focal proteins of interest in this study. More specifically, cholesterol demonstrated a potential direct interaction with IL-10, thereby establishing a connection between cholesterol and 2 modules with immunological significance. In this context, IL-10 has directly interacted with TNF receptor superfamily member 1B (TNFRSF1B), ABCA1, IL-12RB1 and IL-10RA [Figure 4]. The type of interaction of IL-10 as an anti-inflammatory cytokine with TNF-α receptor TNFRSF1B has been text-mined and involved in the cytokine targets for arthritis therapy.[19]

Based on the Phenopedia (Centers for Disease Control and Prevention), there was a lack of reports regarding genes involved in the CL while only 12 genes, including CCR5, COL1A1, Col1a2, Mmp13, St3gal5, FLI1, IL-2, IL-2RA, IL-2RB, JAK3 and CCL2, have been reported for mucocutaneous leishmaniasis. The elevated levels of IL-12 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. IL-10 functions as an intermediary node, linking cholesterol to a trio module comprising TRAF1, TRAF2 and TNFRSF1B through data extraction.[20] However, the present study did not find any significant correlations among cholesterol with TNF, IL-10 and IL-12.

Moreover, IL-10 has potentially interacted indirectly with cholesterol with inter-node ABC1 [Figure 4]. The KEGG pathway of the cholesterol–cytokine network constructed in this study presented pathways that include fat digestion and absorption, Epstein–Barr virus infection, cytokine–cytokine receptor interaction and TNF signalling pathway with very low false discovery rate [Figure 4, Supplementary Figure 1, Supplementary Dataset].

Among the ontology of biological processes computed from the STITCH-constructed network, ABCA1, CYP11A1, IL-10, IL-10RA and TNFRSF1B were identified as participants in the response to other organisms [Supplementary Dataset]. At the molecular processing ontology level, 2 prominent pathways were identified: receptor binding and enzyme binding. These pathways involved ABCA1, APOA1, APOB, TNFRSF1B, TRAF1, TRAF2 and IL-10 [Supplementary Figure 1, Supplementary Dataset].

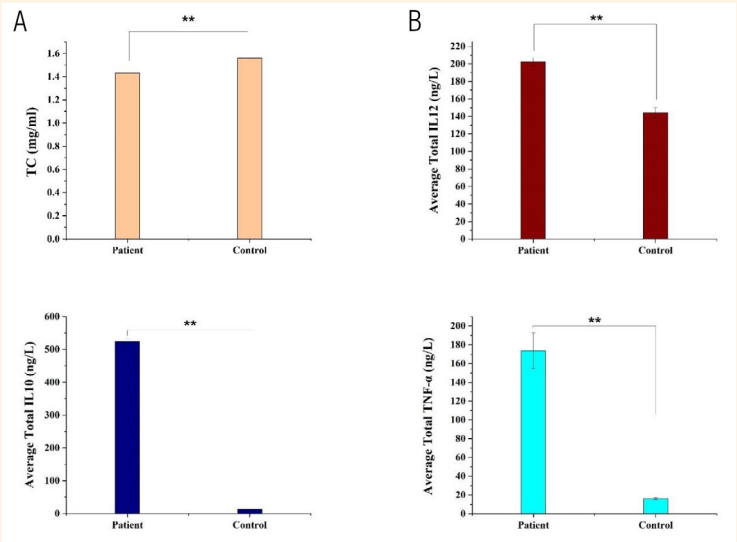

Figure 3: The average serum total cholesterol and cytokine profiles in cutaneous leishmaniasis patients (n = 50) and healthy controls (n = 25).

TC = total cholesterol; IL = interleukin; TNF-α = tumour necrosis factor alpha.

***P ≤0.01.*

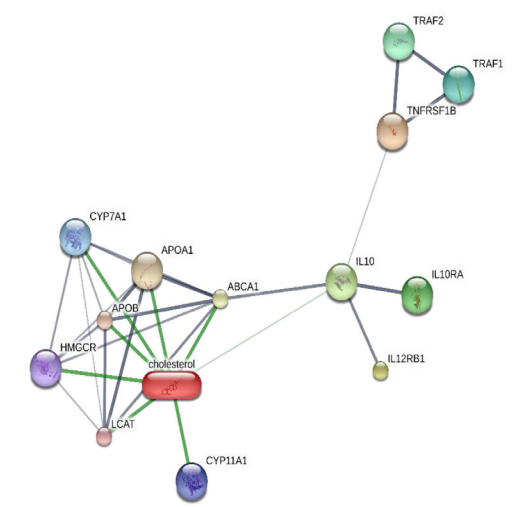

Figure 4: Diagram showing the interactions of cholesterol with selected cytokines that possess diagnostic value in the pathogenesis of cutaneous leishmaniosis. Proteins are demonstrated as spheres while cholesterol is shown as capsule-shaped node. Stronger associations are represented by thicker lines and protein–protein interactions are shown in grey, chemical-protein interactions in green.

TRAF = tumour necrosis factor receptor-associated factor; TNFRSF1B = tumour necrosis factor receptor superfamily member 1B; IL = interleukin; IL10RA = interleukin-10 receptor alpha; IL12RB1 = interleukin-12 receptor beta 1; ABC1A = adenosine triphosphate-binding cassette sub-family A; APO = apolipoprotein; 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.

On the other hand, the cellular component ontology of this study's cholesterol–cytokine network primarily centred around the plasma lipoprotein particle, with key involvement from APOA1, APOB and LCAT [Supplementary Figure 1, Supplementary Dataset].

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 this study's cholesterol-cytokine network [Supplementary Figure 1].

Discussion

While the relationship between blood lipid and lipoprotein profiles and the pathogenesis of leishmaniasis is not yet fully understood, some previous studies 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, Martínez and Ruiz 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.[21] Pucadyil and Chattopadhyay reported that *Leishmania* parasites can consume the host's cholesterol to evade the immune response and survive inside the cells.[22] Moreover, the host's lipid droplets, which are storage organelles for lipids, may play a key role in disease progression and parasite development.[23]

However, 2 African trypanosomiasis and toxoplasmosis pathways would provide stronger cues regarding the involvement of IL-10, IL-10RA 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 LCAT. In this regard, Escribano *et al.* highlighted that an increase in serum apolipoprotein-A1 levels could potentially serve as a biomarker for the efficacy of therapy in canine leishmaniasis.[24] 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.[25]

The effect of HDL-C on leishmaniasis is multifaceted and involves multiple factors, including the metabolism of the host and parasite, as well as the host's immune system. More research is required to fully elucidate the mechanisms and implications of this effect. Furthermore, although the effect of TG on leishmaniasis is not well understood, some investigations have suggested that leishmaniasis can increase blood TG levels. For example, a study found that patients with visceral leishmaniasis had higher TG levels than healthy controls, while another study reported that *Leishmania* parasites can use the host's TG to produce lipids and survive inside the cells.[26,27] According to some results, there may be a link between VLDL-C and leishmaniasis. Additionally, a 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 haemophagocytic 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 favour their survival and replication.[30]

A recent study confirmed that hypertriglyceridemia was correlated with increased levels of inflammatory markers, such as C-reactive protein, IL-6 and TNF-α3. It concluded that hypertriglyceridemia could be used as a biomarker of visceral leishmaniasis severity and disease prognosis.[31] Moreover, 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 VLDL-C and leishmaniasis.

In line with the current study's findings, Oliveira *et al*. demonstrated a considerable increase in the levels of IFN-γ and TNF-α cytokines in the treated group with soluble leishmania antigen (SLA) and phytohemagglutinin mitogen. In the healed group, the level of IL-10 dramatically decreased while significantly increasing in the unhealed groups. The evidence suggested that *Leishmania braziliensis*-induced tegumentary leishmaniasis is characterised by increased IFN-γ and TNF-α, the absence of IL-10 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 (IL-10, IL-27 and TGF-α) or other cytokines (TNF-α and IFN-γ). While the TNF-α and IL-17 production was downregulated by IL-10, TGF-α and IL-17 production, 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 IL-10 and TGF-α since the neutralisation of IFN-γ reduces the generation of TNF-α in an IL-10-dependent way.[33]

In the current study, the level of IL-12 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 IL-10 prevents the generation of mediators such as nitric oxide, IFN-γ and the leishmanicidal activity of macrophages, IL-10 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 da Silva *et al*. revealed a relationship between particular polymorphisms and the regulation of IL-10 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 centred on the elevation of cholesterol in CL patients, demonstrating that cholesteryl esters bound 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, where it becomes integrated into the parasites. Meanwhile, filipin staining revealed a halo surrounding the parasites within the parasitophorous vacuole. 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*. concluded that maintaining a critical level of membrane cholesterol in host cells is essential for CL.[38] Furthermore, they found that chronic statin-induced hypocholesterolemia effectively inhibits the proliferation of *Leishmania donovani*.

Conclusions

This study identified decreased TC levels and increased levels of IL-10, IL-12 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 IL-12 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. Initially, the authors discussed avenues for future research to further clarify the impact of TRAF1, TRAF2 and TNFRSF1B trio in the pathogenesis of human CL. Notably, Phenopedia (Centers for Disease Control and Prevention), which presents a disease-centred 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, 2 immunological modules including TRAF1, TRAF2 and TNFRSF1B, as well as IL-10, IL-10RA and IL-12RB1 were found, the involvement of which in CL should be pursued in future studies.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

FUNDING

No funding was received for this study.

AUTHORS' CONTRIBUTION

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

ACKNOWLEDGEMENT

The authors would like to thank Mustansiriyah University, Babylon University and Razi University for their support in the present work and express heartfelt gratitude and appreciation to His Excellency, Dr Naim Abdel Yasser Al-Aboudi, Minister of Higher Education, for his unwavering support and encouragement of higher education certificates.

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