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Detection of role the enzyme adenosine deaminase in leishmaniasis as biomarkers during of infection

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ABSTRACT: Leishmaniasis is a global illness that is endemic in many countries, including Iraq. The infection is caused by the injection of the parasite *Leishmania* through a female fly bite's belonging to the genus Phlebotomus when feed on the mammal host. The hallmark of cutaneous *Leishmania* is skin ulcers that are likely to be effectively enhanced by the immune system to control the growth and the development of the parasite, while other species of *Leishmania* are more severe and the parasite infect the visceral organs of the host rather than local skin infection According to statistics and available database of Iraqi Center of Disease Control, the number of recorded cases of cutaneous leishmaniasis (CL) in Iraq were 16134 cases in 2020, while for visceral leishmaniasis (VL), it was 95 cases. The current study was conducted on 88 individaul of both sexes, they were from Diyala province and the capital Baghdad, during the period from October 2020 to February 2021. All suspected cutaneous or visceral patients involved in this study were prediagnosed in the laboratory before processing Adenosine Deaminase Enzyme (ADA) by ELISA colorimetric detection. Total number of confirmed leishmaniasis patients were (n=57) cutaneous patients and(n=16) visceral patients, collected from different hospitals in Diyala province and Baghdad. Positive CL or VL samples were confirmed by microscopic Giemsa staining, rapid immune chromatographic strip assay and indirect fluorescent antibody test (IFAT), in addition to the clinical signs and symptoms, which were evaluated by a consultant physician. the ADA enzyme level was also increased in the studied groups where significant difference was recorded in the 2nd trail Pentostam treatment, which was 4.275 nanomol/minute/µg in comparison to control subjects, which was 3.138 nanomol/minute/µg. Although no significant correlation was seen in the ADA enzyme concentration in the newly infected and 3rd trial treatment groups, which were 3.899 nanomol/minute/µg and 3.474 nanomol/minute/µg, respectively.Furthermore, the highest serum ADA enzyme concentration was observed in the newly visceral leishmaniasis groups, which was 5.238 nanomol/minute/µg. However, there was difference seen among the test groups for both studied parameters.The results of this study highlighted a new concept of investigating immunological molecules, such as ADA enzyme, as possible biomarkers for cutaneous or visceral leishmaniasis prognosis and development.

Keywords: Leishmaniasis, enzyme adenosine deaminase

1. INTRODUCTION

Leishmaniasis is a parasitic infection caused by the *Leishmania* parasite protozoan of *Leishmania* spp [1].It is spread to humans by the bite of a female vector belonging to the Phlebotomus genus in the old world and Lutzomyia genus in the new world [2].Leishmaniasis is endemic in tropical and subtropical regions, in addition, in South America, South Asia, Africa[3].The protozoon is a mononuclear phagocytic system cell's obligatory intracellular parasite. At least 20 parasitic species cause visceral and cutaneous leishmaniasis in humans, with clinical characteristics differing depending on the *Leishmania* species and the host's immunological response[4]. Leishmaniasis is a zoonotic infection that affects animals other than humans. It is possible that transmission between people will occur, and that it will even predominate or become exclusive. Cutaneous leishmaniasis (CL) appears as an open sore at the bite site that may heal on its own but

leaves a scar. Cutaneous leishmaniasis (CL) can be asymptomatic and self-resolving, but it usually has a long-term course [5].

The predominant effector Infection-induced acute inflammatory reactions include cells are polymorphonuclear leukocytes (PMN). As primary effectors or phagocytic cells, the first cells to move to the site of infection or injury serve as primary effectors or proteolytic enzymes stored in specific granules and the generation of reactive oxygen species after they have been ingested destroy phagocytic cells, phogocytosing *Leishmania* foreign particles. Neutrophils *Leishmania* interaction can trigger secretion of chemokines such as IL-8 [6].

The Visceral leishmaniasis is mostly linked to the cellular immunological response mediated by CD4+ T cells[7]. The Th1-mediated response is linked to macrophage activation, host resistance, and protection against *Leishmania* parasites, all of which lead to disease control. The Th2-mediated response, on the other hand, is linked to a reduction in macrophage activity and subsequently, disease development [8].

Due to several etiological types of leishmaniasis, a sensitive and fast methods for diagnosis are needed, in addition, follow up of patients status undergoing treatment is very important to pursue the patient's systemic responses to different kinds of drugs and support to anticipate the probability of relapse [9].

Furthermore, leishmaniasis might be asymptomatic in some individuals, existence of many domestic animals (which can act as reservoirs for the parasite because of the zoonotic character of *Leishmania* spp.) co-infection with other pathogens, exclusively in visceral forms, which sometimes results in higher mortality rates in immunocompromised patients, and most prominently is the absence of active and efficient human vaccine against *Leishmania* [10,11].

All the above challenges, and others, have recently been taking a growing attention from scientists around the world, focusing on neglected diseases, including *leishmaniasis* [12].

Biomarkers play a vital role in the mentioned challenges by expanding patient's information follow-up including, immune status, determining patient's response to treatment, in addition, epidemiological feedback, exposure of humans and animals to vectors, and the model of reservoir hosts [13]. Different types of proteins, including cytokines can be followed as biomarkers for its main role in catalysis as structural elements in signaling pathways and molecular systemic parameters, which gives insights for disease developmental stages, acute or chronic, and novel drug discovery[14].. Identification of the sand fly proteins targeted by mammalian antibody responses will improve understanding of vector-host interactions and could also aid in the development of novel epidemiological tools to link host exposure to vector sand flies with leishmaniasis susceptibility [15,16].

Adenosine Deaminase enzyme or Adenosine Aminohydrolase (ADA) has also been considered as a biomarker in some infectious and hereditary diseases, such as Tuberculosis (TB) and squamous cell carcinoma in terms of clinical confirmation and diagnostic prognosis ([17,18]. Similar studies showed that ADA level is increased or decreased during VL which, in turn, reveals better understanding of pathology and physiology of the internal systemic infection [19]. Growing evidences that ADA is increased in serum and lymphocytes in cutaneous leishmaniasis infection via its role in cell-mediated immunity [20].

Aims of the study:

1-Survey of cutaneous leishmaniasis infection in some hospital of the endemic area of Diyala province and Baghdad detection of TNF-alpha and Adenosine Deaminase enzyme in the infected patients.

2-Investigation their levels as possible biomarkers in CL and VL disease,

1.2 Materials and methods:

Patient's collection:

The participants in this study had cutaneous leishmaniasis assembled between October 2020 to February 2021 from Baqubah General Hospital, Diyala provenance north of Baghdad. All 73 patients were suffering from cutaneous ulcer/s and were diagnosed by the resident dermatologist depending on clinical manifestations and laboratory diagnosis through Giemsa stain preparation of suspected ulcers; amastigotes were screened under light microscope 100x oil immersion [21].

Blood samples:

Samples of 5 ml venous blood from each patient were collected in a gel tube; in addition, personal information was documented including: age, sex, the number of lesions, and the location of the lesions of family, duration of infection and number of Pentostam treatment. Blood samples were centrifuged and the serum was divided into an Eppendorf tube each containing at least 500 µl of pure serum and stored at -20°C for later investigation [22].

Experimental design:

OF the total 52 patients, the experimental groups were divided according to Pentostam treatment, as the following:

Group-1: 22 CL patients of a new infection, no Pentostam treatment.
Group-2: 22 CL patients with 2nd trial-treatment of Pentostam.
Group-3: 12 CL patients with 3rd trial-treatment of Pentostam.
Group-4: 4: 16 VL patients of a new infection, no Pentostam treatment
In addition, 15 blood samples were taken from healthy people in the area
Adenosine Deaminase (ADA) Activity Assay Kit (Colorimetric)
This Adenosine Deaminase (ADA) Activity were ordered from Abcam® Company, USA and stored at -20°C. ADA activity is an assay where inosine formed from the breakdown of adenosine is detected via a multi-step reaction, resulting in the formation of an intermediate that reacts with the ADA convertor and developer to generate uric acid that can be easily quantified at OD293 nm. The kit measures total activity of Adenosine Deaminase with limit of quantification of 1 mU recombinant Adenosine Deaminase.

1.3 Results and Discussion:

Parasite infection and ulcers:
All suspected CL patients involved in this study were pre-diagnosed in the laboratory before processing ADA ELISA detection. Clinical features of cutaneous ulcer were first examined for each patient, in addition, Giemsa stained slides from samples of at least one visible ulcer were investigated under microscope oil immersion and the invaded amastigotes were seen, figure 1 and 2.

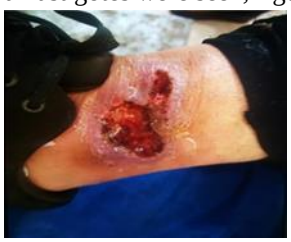

Figure-1: Patient with multiple cutaneous leishmaniasis lesions

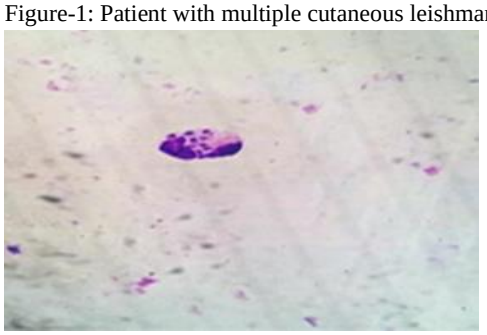

Figure-2: Macrophage showing intra-cellular amastigotes from ulcer swab, light microscope 100X.

Adenosine deaminase enzyme activity result:

Serum concentration of adenosine deaminase enzyme (ADA) activity was investigated in the studied groups of CL pre and post treatment and VL patients with comparison to control group. The findings revealed a significant increase in serum ADA activity in the VL which was before treatment stage when compared to the CL post-treatment stage and healthy controls. subjects However, following the successful treatment of CL subjects, the increased ADA activity in serum was lowered. However, we found that active VL cases had much higher serum activity than CL cases
ADA activity in new CL infections.
Results of patients with new infection (no treatment trials) shows that the average of ADA concentration activity level was higher than that of the control group, which were 3.899 nanomol/minute/ug and 3.138 nanomol/minute/ug, respectively, figure (3-1). However, statistical analysis did not record a significant difference, in comparison with control group ($p \geq 0.05$).

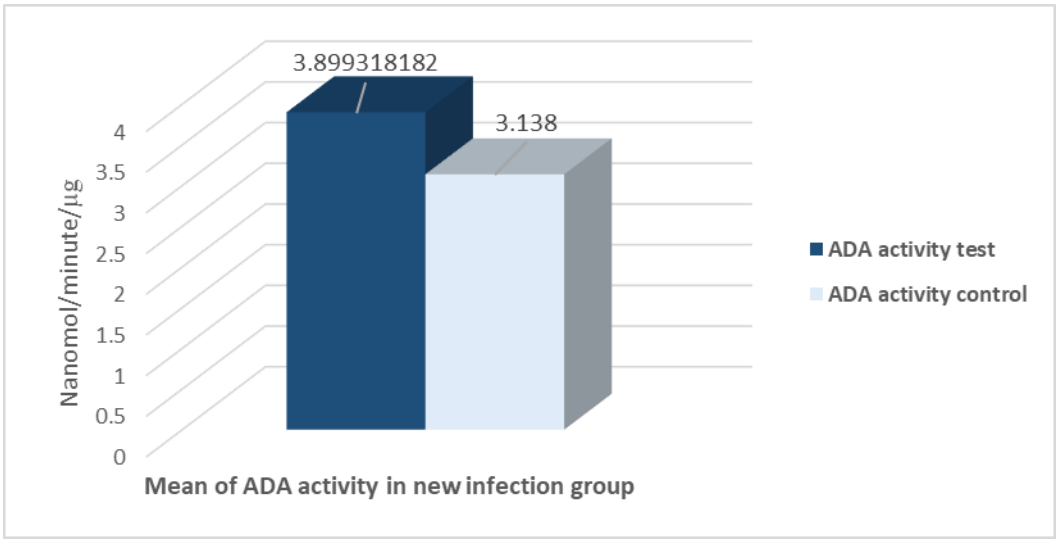

Figure (3-1): this figure shows ADA activity level in the New CL trail pentostam treatment patients, there was Non significance difference between the test and control group (p value < 0.05).

ADA activity in 2nd and 3rd trials treatment:

The result of both patient groups received two or three doses of pentostam also revealed an elevated mean concentrations of ADA activity (4.275) and (3.4745) Nanomol/minute/ug, respectively, when correlated to the mean of control group, which was equal to 3.138 Nanomol/minute/ug. However, the statistical analysis reported a significant difference only in the 2nd trial treatment group, figure (3-2)

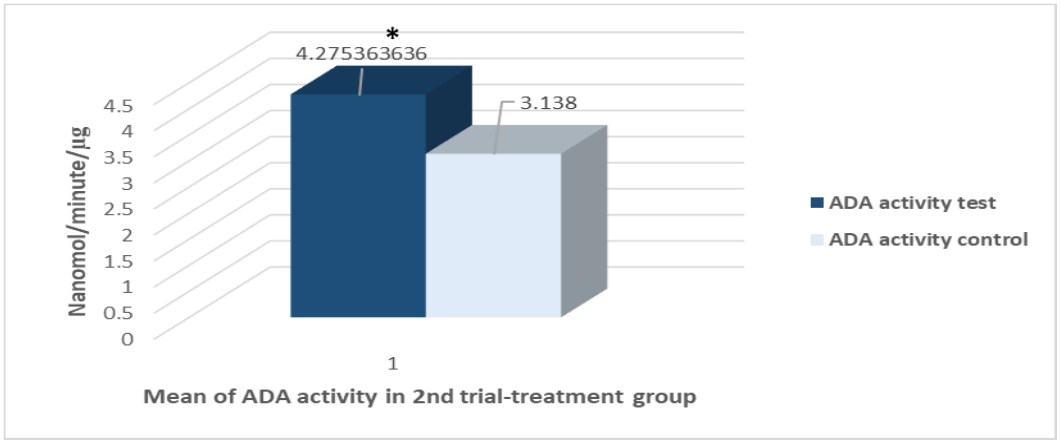

Figure (3-2): this figure shows ADA activity level in the 2nd trail pentostam treatment patients was seen * = significance difference between the test and control group (p value < 0.05).

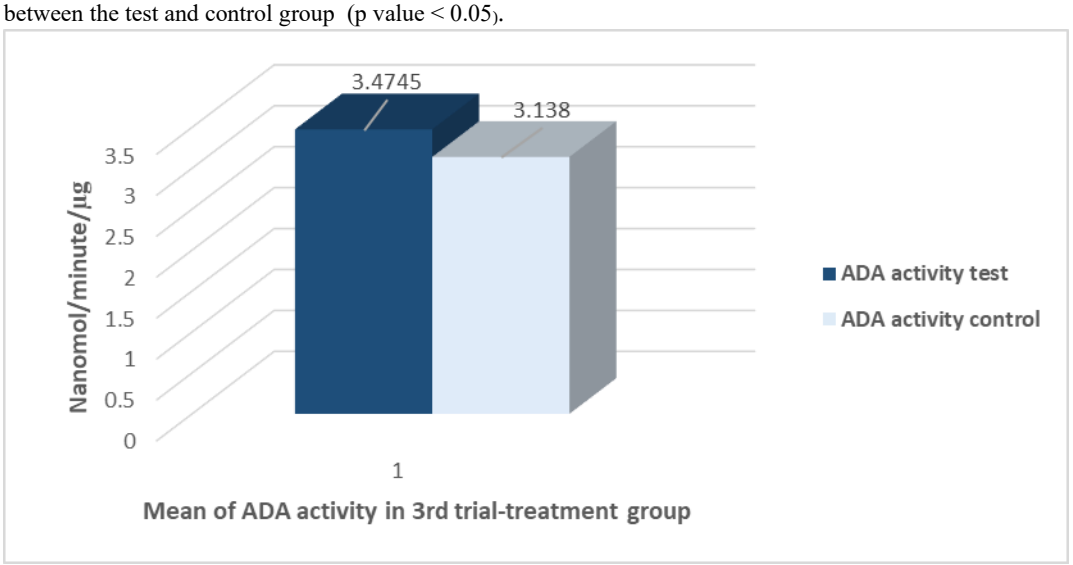

Figure (3-3): There was seen non significance of ADA activity level in the 3rd trail treatment cutaneous of *Leishmania* stages in comparison with control healthy.

ADA activity level in New visceral infection stage:

Visceral leishmaniasis patients showed a significant increase in ADA concentration activity level which was the highest in the studied groups, the mean concentration of ADA was (3.4745) Nanomol/minute/ug. while comparison with the healthy group a lower value in the control group was detected, which was equal to (3.138. Nanomol/minute/ug) as in Figure (3-4).

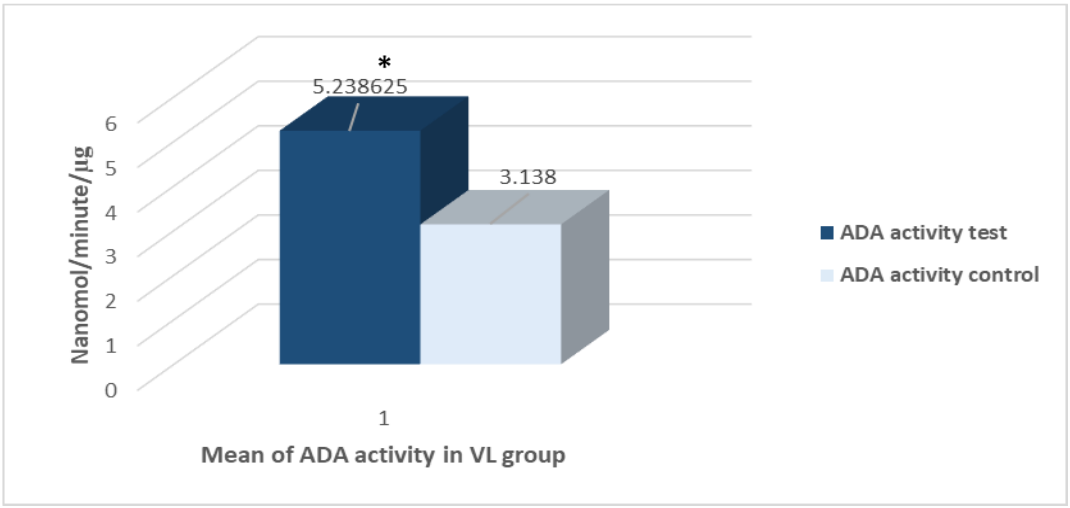

Figure 3-4: this figure shows ADA activity level in the New visceral *leishmania* infected stages in comparison with control healthy** =significance (p value=0.009).

Furthermore, there was no significant difference between the four test groups of new infection, 2nd trial-treatment and 3rd trial-treatment, New visceral in which p value was > 0.05 according to ANOVA data analysis Figure (3-5)

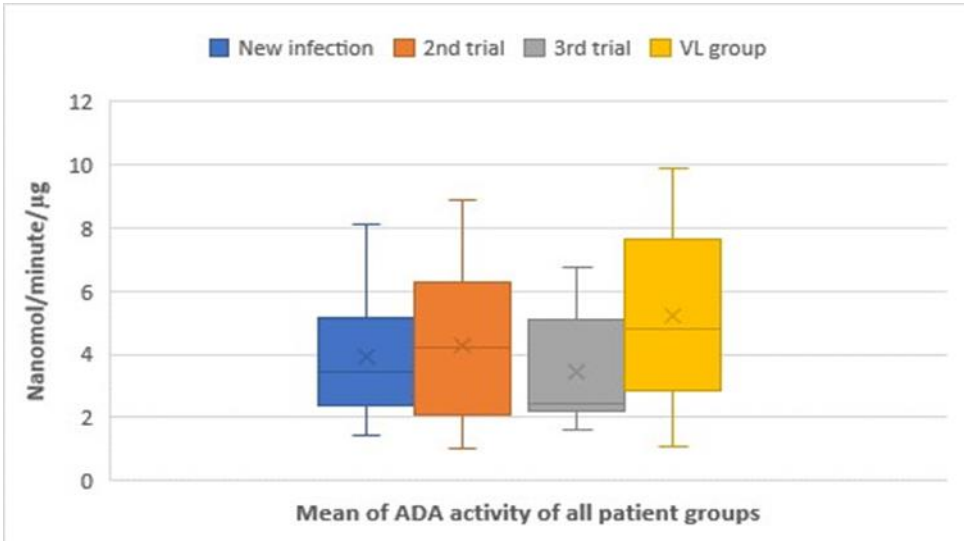

Figure 3-5: The mean concentration of ADA activity Nanomol/minute/ug in the sera of different groups, Non significance, there was seen significant the highest new visceral difference between the three group. p- value = 0.197883

The serum level of ADA activity in CL and VL infected patients were determined in the current study. Group G1 (new CL infections) was recorded (3.89 ±0.38 Nanomol/minute/µg) of ADA, group G2 (2nd trail of CL treatment) was recorded (4.27 ±0.50 Nanomol/minute/µg) of ADA, group G3 (3rd trail of CL treatment) was recorded (3.47 ±0.49Nanomol/minute/µg) of ADA, while, group G4 (new visceral infections) was recorded (5.23 ±0.77 Nanomol/minute/µg) of ADA as revealed in and table (3-1),there were a significant differences between them and with healthy subjects. Adenosine deaminase enzyme activity is commonly circulated in human tissues and its maximum activity was found in lymphoid tissues (Singh et al., 2011).

Table 3-1: Comparison between difference groups in ADA activity

Comparison between difference groups in ADA activity	**p value of different groups**
G1: New infection G2:2 trail treatment	significance (p value < 0.05).
G1: New infection G3:3 trail treatment	significance (p value < 0.05).
G1: New infection G4: New Visceral infection	significance (p value=0.009).
G2:2 trail treatment G3:3 trail treatment	significance (p value < 0.05).
G2:2 trail treatment G4: New Visceral infection	significance (p value=0.009).
G3:3 trail treatment G4: New Visceral infection	significance (p value < 0.05).
Means having with the different letters in same column differed significantly. *, (P≤0.05).**	

Two ADA isozymes have been identified as ADA1 and ADA2, with ADA1 being the primary, component of human tissue extracts and ADA2 being the main component of total serum ADA. ADA activity has been found to be significantly enhanced in the sera of individuals with hepatic disorders, hematological malignancies and other viral diseases, Also in acute leukemias, chronic myeloid blast crisis leukemia and acute liver injury. patients all had high of ADA1 levels in their blood with adult T-cell leukemia, multiple myeloma (B–J type), infectious mononucleosis, rubella, acquired immunodeficiency syndrome, and tuberculosis. had higher Serum ADA2 levels [23,24,25]. ADA has been considered as a marker of cell mediated immunity [26,27]. increased serum ADA activities have been observed in many As a result, Biomarkers for monitoring patients coinfected with *Leishmania*, and HIV, as well as those who are another type of immunodeficiency have gotten a lot of interest [28]. that ADA levels reflects the activity of stimulated T lymphocytes and its levels are raised when ever cell mediated immunity is stimulated. Its activity has been shown to be elevated in disease characterized by T lymphocytes proliferation and activation. In visceral leishmaniasis, cell mediated immunity is the major immunological response. Hence, ADA level have been estimated in these patients[29]. Studied that found, in individuals with cutaneous leishmanasis there was a rise in lymphocytic specific ADA activity T cells activated by antigen proliferate and produce lymphokines capable of activating macrophages. Because *Leishmania* are obligate intracellular parasites of macrophages. lymphokine mediated macrophage activation.is though to be the key effector mechanism driving parasite growth.in leishmaniasis there is a strong link between interferon gamma (IFN-γ) and macrophages in phagocytic activity [30,31]. The ADA activity in PKDL was shown to decreased gradually during therapy stages suggesting that this parameter could be used as a marker of pathophysiology and prognosis[32].The parasite transforms the protein profile of infected cells, making them suitable for proteomic studies and identifying new molecular biomarkers that might reveal the destiny of the host cell and pathogen[33].A previous study by compared serological, parasitological and cellular response biomarkers in patients with different forms of leishmaniasis caused by L. infantum; their work was performed on patients with active disease, plus, others who had been cured, revealed significant differences in the results of pre- and post-treatment parasitological tests it .is also important to access the serum ADA of asymptomatic subjects because in order to diagnosis of asymptomatic leishmanial [34,35].Similar

studies investigated the level serum ADA in VL and PKDL patients in their pre and post treatment stages, found that the level of serum ADA was significantly greater in patients with VL and PKDL at pre-treatment stage compared to healthy controls [36].Parallel studies investigations found a increased ADA level in active VL compared to active PKDL patients; this could be to the immuno-pathogenesis of the two disease, being different VL is more aggressive and systemic disease, whereas PKDL is milder and disease limited to the skin lesions[37]. Furthermore, [38,39]. found higher rise in ADA activity in Nepalese VL patients as compared to healthy controls. Other studies for VL, serum ADA activity was
increased at diagnosis and returned to almost normal concentrations at the end of therapy (day 30) in Nepalese and Indian patients[40]. At diagnosis, activity appeared higher in VL patients than in malaria, leprosy, or tuberculosis patients [41]. Elevated serum ADA activity may be due to mononuclear cells that secrete more ADA in response to *Leishmania* intracellular infection, hence, this elevation of the activity of ADA in the blood can be used as prognostic marker to track how well a patient is responding to treatment [42].Notably, this clarification may be useful in biomarker evaluation of the effector mechanism of macrophages and clinical manifestation of patients due to decrease in parasite load, which in turn, led to less antigenic stimulation a decrease level in ADA activity after 4 weeks of chemotherapy was reported, this is likely because of the reduction in the immune activation level; [43,44]. A parallel study on another intraceullar parasite, Plasmodium vivax, indicated an increase level of ADA activities in the serum samples, erythrocytes, leukocytes and plasma hemoglobin concentrations with in comparison to control group [45]. In addition, similar studies on other infectious agents proved significant high serum ADA levels in multibacillary leprosy and in patients of leprosy which may due to increased lymphoreticular activity during the reactional phases[46]. Many studies have demonstrated alterations of ADA activity in the tumor tissue and serum in patients with lung, head and neck, breast and ovarian cancer [47]. Furthermore, serum ADA level was increased significantly in Oral Squamous Cell Carcinoma (OSCC) which suggested an elevation in serum ADA activity that helps in the diagnosis and follow-up of head and neck cancers. [48,49].In this research, the increased level of ADA found in new infection CL groups and patients undergoing different trials of Pentostam treatment, these parameters give an insight of CL infection in the studied groups. Although there is little known about this concept, the studied parameters of CL patients and individuals undergoing treatment can be used as markers for disease progression.

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