Comparing acute and chronic human cutaneous leishmaniasis caused by Leishmania major and Leishmania tropica focusing on arginase activity

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Comparing acute and chronic human cutaneous leishmaniasis caused by *Leishmania major* and *Leishmania tropica* focusing on arginase activity

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Running title: Comparing Acute and Chronic Cutaneous Leishmaniasis and Arginase Activity

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Abstract

Background: Cutaneous leishmaniasis (CL) in Iran is mainly caused by *Leishmania major* (*L. major*) and *L. tropica* species. Arginase-mediated L-arginine metabolism is an important metabolic pathway in *Leishmania* parasite propagation.

Objective: Arginase activity in human CL caused by *L. major* and *L. tropica* has not been studied so far. Therefore, we aimed to compare the clinical and laboratory aspects of acute and chronic CL, focussing on arginase activity.

Methods: In this case-control study, 30 patients with acute CL, 13 patients with chronic CL, and 11 healthy controls were recruited. The diagnosis of CL and determination of the infecting species were done by direct smear and nested PCR methods, and the patients' skin biopsies were pathologically characterized. Arginase activity was measured in skin biopsies from lesions, peripheral blood polymorphonuclear cells (PMNs), peripheral blood mononuclear cells (PBMCs) and plasma by standard methods.

Results: The median of arginase activity in the acute lesions was higher than in chronic samples and significantly higher than that in healthy controls (*p*-value= 0.008). However, no difference was observed in lesion arginase activity and chronicity between *L. tropica* and *L. major* as causative agents. PMNs of both acute and chronic patients showed higher levels of arginase activity as compared to the levels in PBMCs and plasma. The median of arginase activity in the PMNs of patients with chronic CL was higher than that of patients with acute CL and significantly higher than that of the healthy controls (*p*-value= 0.010).

Conclusion: The level of arginase activity in lesions of patients with acute and chronic CL was higher than the skin of healthy controls. The highest level of arginase activity was observed in PMNs from patients with chronic CL. This suggests that the high level of arginase activity in PMNs of patients with chronic CL may contribute to the chronicity.

Keywords: cutaneous leishmaniasis, *L. major*, *L. tropica*, chronic, acute, arginase activity.

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1. Introduction

Cutaneous leishmaniasis (CL) is a neglected tropical disease that is caused by *Leishmania* parasites. The disease phenotype comprises of a wide spectrum of clinical manifestations and is highly endemic in tropical and sub-tropical parts of the world.[1] CL in Iran is mainly caused by *L. major* and *L. tropica.*[2] CL lesions due to infection by *L. major* are zoonotic and usually heal within 2-8 months. CL lesions caused by *L. tropica* are anthroponotic and usually heal spontaneously within about one year, though they may persist longer.[3] CL patients with disease duration of more than two years are considered as chronic or non-healing.[4-5]It has been proposed that a combination of immunological and clinical factors contribute to chronicity or non-healing nature of cutaneous lesions.[5]

Macrophages are the main reservoir for the obligate intracellular *Leishmania* parasites and their activation is a critical step for killing and control of the parasites. Activated macrophages produce two key enzymes that regulate the killing or survival of *Leishmania* parasites, inducible nitric oxide synthase (iNOS) and arginase, respectively.[6] Both enzymes metabolize L-arginine; iNOS catabolizes L-arginine into nitric oxide (NO) and citrulline. NO is a major effector molecule in cell-mediated immune reactions and also crucial for killing and/or inhibiting parasite growth.[7] Two distinct isoforms of arginase exist in mammalian hosts that differ in their subcellular localization and tissue expression. Both isoforms hydrolyze arginine into urea and ornithine, and the latter is the first building block for polyamine synthesis. Polyamines are essential for the growth of all eukaryotic cells as well as *Leishmania* parasites.[8-9]

In experimental models of leishmaniasis, arginase-expressing macrophages have been shown to promote parasite growth *in vitro*. In addition, increased arginase activity at the site of infection is a hallmark of non-healing infection.[8] Several studies have been carried out to investigate the role of arginase in visceral and cutaneous leishmaniasis in Ethiopia.[10-11] Visceral leishmaniasis (VL) in Ethiopia is characterized by marked immunosuppression, and it has been reported that in patients with active disease arginase levels are significantly higher than those in healthy endemic controls.[12] Moreover, co-infection of VL patients with HIV results in a further increase in arginase activity and may contribute to poor disease outcome.[10] Patients with CL due to infection with *L. aethiopica* had increased arginase levels in their lesions, and this might play a role in the pathogenesis of the disease by impairing T cell function.[11]

In the present study, the clinical and immunopathological factors contributing to chronicity of CL in Iran were investigated. We compared clinical and laboratory aspects of acute and chronic CL, focussing on arginase activity in skin lesions, peripheral blood mononuclear cells (PBMCs), polymorphonuclear cells (PMNs) and plasma from CL patients and healthy controls.

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2. Material and Methods

2.1 Patients and healthy controls

For this case-control study, 30 CL patients with short duration of disease (less than one year) and 13 chronic (more than two years) CL patients were recruited, from June 2013 to February 2015 (Table 1). CL patients were admitted to Razi Hospital, the skin hospital affiliated with Tehran University of Medical Sciences. The duration of disease was determined using as starting point the time the patients noticed the appearance of the lesions, confirmed by a dermatologist. The clinical diagnosis of CL was confirmed by direct smear and nested PCR as well. In addition, 11 healthy controls were also recruited. Inclusion criteria were an age of between 10 and 70 years and confirmed CL. Pregnant patients and patients with other skin diseases or underlying diseases were excluded.

2.2 Ethical approval

All individuals agreeing to participate were informed about the nature of the study and signed an informed consent form. This project was approved by the Ethics Committee of the Pasteur Institute of Iran (ID 8916, May 2013) and Deputy of Research, Tehran University of Medical Sciences (TUMS, ID 20895, June 2013).

2.3 Determination of parasite species by nested PCR

In order to isolate the DNA from each biopsy, 3mm of lesions were collected and homogenized with 150 µl of 10% chelex resin (BioRad 100-200mesh). After vortexing, the samples were incubated at 100°C for 15 min, followed by centrifugation at 13000 rpm for 2 min. The supernatant was collected and stored at 4°C. The nested PCR method was applied in order to confirm the infection and determine the species of *Leishmania* parasites.[13] External primers CSB2XF and CSB1XR and internal primers 13Z and LIR were used in the first and second round of PCR respectively. As positive controls, genomic DNA from standard strains of *L. major* (IR75) and *L. tropica* (MOHM/IR/09/Khamesipour-Mashhad) was used in parallel.

2.4 Sample collection and preparation

We used biopsies, peripheral blood mononuclear cells (PBMCs), polymorphonuclear cells (PMNs) and plasma from patients with acute and chronic CL patients and healthy controls. The biopsies from healthy controls were obtained from individuals who had plastic surgery. For biopsy collections, 3-4 mm of samples were collected and homogenized in 150µl of phosphate-buffered saline (PBS) and frozen immediately at -70°C until used.

For PBMCs, PMNs and plasma samples, 10 ml of blood was taken into tubes containing EDTA. Plasma was separated from blood after 10min centrifugation at 400xg. PBMCs and PMNs were

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isolated based on dextran sedimentation and density gradient using Histopaque 1077 (Sigma) as previously described.[14] The purity of the granulocytes was always above 98% as determined microscopically after Kimura staining. This staining method enables us to discriminate neutrophils from eosinophils.[15] The viability of PBMCs and PMNs was 98% as assessed by trypan blue dye exclusion method. All samples were kept at -70 °C until used.

2.5 Protein quantification

The protein content of different samples (25µl of each sample) including biopsy, PBMCs and PMNs was measured by BCA Protein Assay Reagent (Pierce) following supplier's instructions.

2.6 Determination of arginase activity

The enzymatic activity of arginase in biopsies, PBMCs and PMNs was determined as described previously.[8, 10] Twenty five microliter of each sample was resuspended in lysis buffer (0.1% Triton X-100, 10mM $MnCl_2$ and 25mM Tris-HCl) and incubated for 7 min at 56°C. Arginine hydrolysis was done by incubating the lysate with 50µl of 0.5 M L-arginine (pH 9.7) at 37°C for 60 min. The reaction was stopped with 400µl of acid mix (H_2SO_4 (96%), H_3PO_4 (85%) and H_2O in a ratio of 1/3/7, v/v/v). Urea concentration was determined by measuring the optical density of the mixture at 540 nm wavelength after addition of 20µl α-isonitrosopropiophenone (ISPF) (dissolved in 100% ethanol), followed by heating at 100°C for 45 min.

One unit of enzyme was defined as the amount of enzyme that catalyzed the formation of 1µmol of urea. For plasma, the urea concentrations were determined without the activation and hydrolysis steps.[10] Arginase activity was normalized to the total amount of protein used in the measurement.

2.7 Preparation of lesion biopsies for histology

A biopsy specimen was obtained from each patient and the slides were prepared by hematoxylin and eosin (H&E) staining.

2.8 Statistical Analysis

The values of arginase activity are presented as median (interquartile range (IQR)). The statistical analyses were performed using the IBM SPSS statistics and GraphPad Prism software 5.0. Kruskal-Wallis H test and Mann-Whitney U test were performed to determine the statistical significance. *P*-values less than 0.05 were considered as significant.

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3. Results

3.1 Clinical data

Forty-three CL patients were enrolled in the study. The data of CL patients including age, gender, duration of disease, the species of *Leishmania*, and number of lesions (single or multiple) are shown in table 1. In total, 28 patients were infected with *L. major*, 12 with *L. tropica* and for 3 patients the species could not be determined.

The clinical and pathological features of patients with acute and chronic CL are summarized in table 2.

3.2 Histopathological features in lesion biopsies from patients with acute versus chronic CL

Microscopic examination of H&E stained sections of acute and chronic CL lesions revealed the presence of Leishman bodies in 53% and 23% of acute and chronic lesions respectively. The high number of parasite infected macrophages (red arrow) indicates the high level of parasite contamination in acute patients. In addition, the granuloma formation is usually observed in the chronic patients (Fig. 1 and Table 2).

3.3 Arginase activity in skin lesions, PBMCs and PMNs

3.3.1 Arginase activity in lesions of patients with acute and chronic CL and in skin of healthy controls

We observed a significant difference in arginase activity between chronic and acute lesions and healthy skin biospsies (P=0.033, Table 3). When compared individually, the median of arginase activity was significantly increased in acute lesions of CL patients in comparison with healthy controls (P=0.008, Fig. 2). No significant differences were observed between the levels of arginase activity measured in lesions of patients with chronic CL and in the skin of healthy controls (P= 0.284), or between chronic and acute lesions (P=0.267, Fig.2). These results showed that the levels of arginase activity were increased in lesions of patients with acute CL. Furthermore, there were no significant differences in arginase levels in lesions of CL patients caused by *L. major* or *L. tropica* (Table 4).

3.3.2 Arginase activity in PBMCs of patients with acute and chronic CL and of healthy controls

The median level of arginase activity in PBMCs isolated from patients with acute CL was higher than those of chronic CL patients and healthy controls (Table 3). However, the difference was not significant between three groups (p = 0.154) (Fig. 3, Table 3).

3.3.3 Arginase activity in peripheral blood PMNs of patients with acute and chronic CL and in healthy controls

The median arginase activity in PMNs was significantly different (P=0.048) between acute CL with median of 827.11 mU/mg (IQR 558.74), chronic CL with median of 1070.85 mU/mg (IQR

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609.83), and healthy controls with median of 585.65 mU/mg (IQR 378.00) (Table 3). When compared individually, only arginase activity of patients with chronic CL, not with acute CL, was significantly higher than healthy controls (*P*= 0.010 vs. *P*= 0.239) (Fig. 4).

3.3.4 Arginase activity in plasma from patients with acute and chronic CL and in plasma from healthy controls

The median level of arginase activity in the plasma of patients with chronic CL was higher than those of patients with acute CL and healthy controls (Table 3). There was no significant difference between three groups (*P* = 0.091, Table 3, Fig. 5).

3.3.5 Arginase activity in lesions, PBMCs, PMNs and plasma of patients with acute CL

In patients with acute CL, the highest arginase activity was present in PMNs and lesion samples as compared to PBMCs and plasma (Table 3). When arginase activity of lesions was compared with PBMCs and plasma, the difference was significant. Similarly, the differences between PMNs with plasma, and PBMCs with PMNs were significant (Fig. 6).

3.3.6 Arginase activity in lesions, PBMCs, PMNs and plasma of patients with chronic CL

In patients with chronic CL, the highest arginase activity was measured in PMNs (Table 3), and it was not significantly different from arginase activity in lesions (*P*=0.12) (Fig. 7). In chronic CL patients, arignase activity in PBMCs and plasma were significantly lower than that in PMNs and lesions (Fig. 7).

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4. Discussion

Our main objective was to investigate clinical and biological factors contributing to chronicity of cutaneous leishmaniasis. With this aim, we compared acute and chronic CL patients, focusing on arginase activity.

Both *L. major* and *L. tropica* are able to cause chronic lesions (Table 1). This is in accordance with a study in Iran which showed that there is no relationship between *Leishmania* species and the chronicity of the disease.[16]

In the present study, both ulcerative and non-ulcerative clinical features were observed in patients with acute and chronic CL (Table 2). In patients with acute CL, non-ulcerative features such as simple plaque, tumoral, nodular, erysipeloid and sportrichoid were observed. In patients with chronic CL the non-ulcerative clinical features including lupoid, tumoral, plaque, sportrichoid, and erysipeloid were also observed (Table 2). Classically lupoid, erysipeloid and non-ulcerating lesions (plaque and tumoral) get a chronic or non-healing course. These clinical pictures are in agreement with previous reports from Iran.[4, 17, 18]

The histological analysis of acute lesion biopsies showed a spectrum of inflammation with diffuse parasitized macrophages (presence of Leishman bodies in 16 out of 30 cases, Table 2). In contrast, in patients with chronic CL, only few parasitized macrophages and granuloma formations were observed (Table 2, Fig. 1). Similarly, other studies have shown that with increasing chronicity of CL lesions, the number of parasitized macrophages decreases, and consequently, few Leishman bodies could be observed.[19-21]

In the present study, we focused on arginase and compared the activity of this enzyme in different samples including lesions, PBMCs, PMNs and plasma obtained from CL patients infected with *L. major* or *L. tropica*, with different duration of disease, namely acute and chronic, for the first time (Table 3 and 4). The synthesis of polyamines from ornithine following the catabolism of arginine by arginase is essential for *Leishmania* proliferation. *Leishmania* parasites have their own L-arginine metabolism pathways. The single-copy gene encoding arginase is essential for parasite growth, and the enzyme is localized in their unique organelle called glycosome.[22] Arginase activity has been indicated to be a significant marker of disease severity during leishmaniasis, but it is difficult to distinguish whether this difference originates from the host macrophages or the *Leishmania* parasites.[8] A plausible explanation of the function of parasite-derived arginase is that this enzyme may be involved in the suppression of host iNOS. On the other hand, parasites trigger macrophages' arginase for synthesis of polyamines, which are essential nutrients for parasite survival and growth in the host cells. A study by Roberts and colleagues on *L. mexicana* arginase confirms the presence of the arginase and shows that parasite-derived arginase closely resemble mammalian arginase isoform II.[23] Thus, the availability of arginase in the infected host promotes parasite growth.[8, 24]

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Our results showed that the species of the *Leishmania* parasites does not make significant differences in arginase levels in the lesion of CL patients (Table 4). These results suggest that both *L. major* and *L. tropica* can create similar environment in the lesion of CL patients and like other reported species such as *L. aethiopica,* they may also induce high levels of arginase at the site of infection.[12]

There was an increased activity of arginase in the lesions of CL patients (acute and chronic) versus healthy controls (Fig. 2). Our results are in agreement with those obtained in lesional biopsies of patients with CL in Ethiopia.[11] The lower level of arginase activity we measured in lesional biopsies from patients with chronic CL (in comparison with acute CL) may be due to lower parasite numbers as compared to patients with acute CL. With decreasing number of parasites in chronic lesions the arginase activity would decrease and this would explain why the arginase activity in chronic lesions was lower than that measured in acute lesions. However, it is noteworthy that the arginase activity in chronic lesions was still higher than the arginase activity in healthy skin (Fig. 2 and table 2).

The levels of arginase activity in PBMCs and plasma of CL patients (acute and chronic) and healthy controls were not significantly different (Fig. 3, Fig. 5). This is in agreement with the report of Abebe *et al* showing that there was no difference between arginase activity of PBMCs from CL patients and healthy controls.[11] This is likely to be due to the concept that CL is a localized disease. However, our results showed that the level of arginase was higher in the peripheral blood PMNs of CL patients as compared to healthy controls (Table 3). Importantly, the arginase level in PMNs from patients with chronic CL was significantly higher than in healthy controls (Fig. 4).

PMNs are likely to play an important role here and can induce more immunosuppression following cell death and release of arginase in the environment.[25] It has been shown that arginase released by PMNs induces a profound suppression of T cell and NK cell proliferation and cytokine synthesis.[26] In the present study, we showed that PMNs of patients with both acute and chronic CL contained high levels of arginase compared to PBMCs (Table 3, Fig. 6, and Fig. 7). Moreover, as mentioned above, arginase activity of peripheral blood PMN of chronic CL patients was significantly different from that of healthy control (Fig. 4, Table 3). Therefore, PMNs may have a negative role in healing and could exacerbate the disease. It has been shown that monocytes and neutrophils are recruited into lesions of *L. major*-infected mice, and that they are responsible for arginase production.[27, 28] Therefore, PMNs not only may have a role in circulation but also in the lesions and contribute to the local increase of arginase levels.

It is also possible that higher level of arginase activity in the lesion is due to the presence of both cell types, the macrophages and neutrophils. High levels of arginase activity are also reported in other diseases such as psoriasis and diabetes.[29, 30] Therefore, controlling arginase activity at the site of the skin lesion could help to control and improve the wound healing.[31]

There are some approaches for intervening with the L-arginine metabolism such as induction of host NOS2, application of nitrosating agents, and inhibition of L-arginine uptake into the parasitophorous vacuole.[24] It is clear that L-arginine is an essential amino acid for parasite growth which lacks a biosynthetic pathway and is brought into the parasitophorous vacuole by transporters.[31] Thus, L-arginine transporters should be considered as potential targets for treatment.[24] There are some reports on using Pentamidine on promastigotes of *L. donovani* and *L. amazonesis* which resulted in significant decrease in the intracellular L-arginine pool and in arrest of the cell growth.[32, 33] Pharmacological interference of macrophage arginase by nor-hydroxy-L-arginine (NOHA) in *L. major* and *L. infantum* infection induced a dramatic decrease in the number of intracellular amastigotes per cell and the percentage of infected cells.[6, 8] So far, different compounds have been shown to target the metabolic pathways of L-arginine as anti-*Leishmania* agents.

In conclusion, both *L. major* and *L. tropica* could cause chronic CL lesions. Granuloma formations were observed in H&E stained sections of most of chronic CL patients. To the best of our knowledge, this is the first report that analyzes the level of arginase activity in lesions, PMNs, PBMCs, and plasma from patients with acute and chronic CL due to infection with *L. major* and *L. tropica*. The level of arginase activity in lesions of patients with acute and chronic CL was higher than the arginase levels in the skin of healthy controls. The highest level of arginase activity was observed in peripheral blood PMNs from patients with chronic CL. Our results indicate that the high level of arginase activity in PMNs of patients with chronic CL may contribute to the chronicity and non-healing of CL. In this regard, further studies with larger sample size are needed.

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Figure legends:

Figure 1. Histological presentations of two acute lesions due to *L. major* and *L. tropica* (left panel) and chronic cases (the right panel). In the acute cases, parasitized macrophages (red arrow) with variable number of parasites can be easily seen. In the cases of chronic cases, hardly are any parasites seen in the biopsy sections; however, an epitheloid granulomatous response with multi-nucleated giant cells with a mixture of macrophages, lymphocytes, plasma cells and histocytes is observed.

Figure 2. Arginase activity in skin lesions of patients with acute (n=30) and chronic (n=13) cutaneous leishmaniasis versus its levels in skin of healthy controls (n=11). Statistical significance was determined by two-tailed Mann-Whitney test. The arginase activity is significantly different between acute, chronic and control individuals with p= 0.033. The p values between different groups are indicated in the graph. The straight line represents the median and each symbol is representative of one individual.

Figure 3. Arginase activity in PBMCs of patients with acute and chronic cutaneous leishmaniasis versus its levels in PBMCs of healthy controls. PBMCs were isolated by density gradient from the blood of each individual. There is no significant difference between the arginase levels in the PBMCs of patients and controls. The straight line represents the median and each symbol represents one individual. NS=not significant

Figure 4. Arginase activity in PMNs of patients with acute and chronic cutaneous leishmaniasis versus its levels in PMNs of healthy controls. PMNs were isolated from blood as described in Materials and Methods. The arginase activity is significantly different between acute, chronic and control individuals with p= 0.048. The p values between different groups are indicated on the graph. The straight line represents the median and each symbol representsone individual.

Figure 5. Arginase activity in plasma of patients with acute and chronic cutaneous leishmaniasis versus its levels healthy controls. Plasma was isolated by centrifugation of the blood of each individual. Statistical significance was determined by two tailed Mann-Whitney test. NS=not significant. The straight line represents the median and each symbol representative of one individual.

Figure 6. Arginase activity in lesion, PBMCs, PMNs and plasma of patients with acute CL. The activity of arginase was measured by enzyme assay in different samples (n=30). The straight line represents the median and each symbol is representative of one individual. Not significant shown as “NS”, $p<0.01$ as “**” and $p<0.001$ as “***”.

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Figure 7. Arginase activity in lesion, PBMCs, PMNs and plasma of patients with chronic CL. The activity of arginase was measured by enzyme assay in different samples (n=13). The straight line represents the median and each symbol represents one individual. Not significant shown as "NS", $p<0.01$ as "**" and $p<0.001$ as "***".

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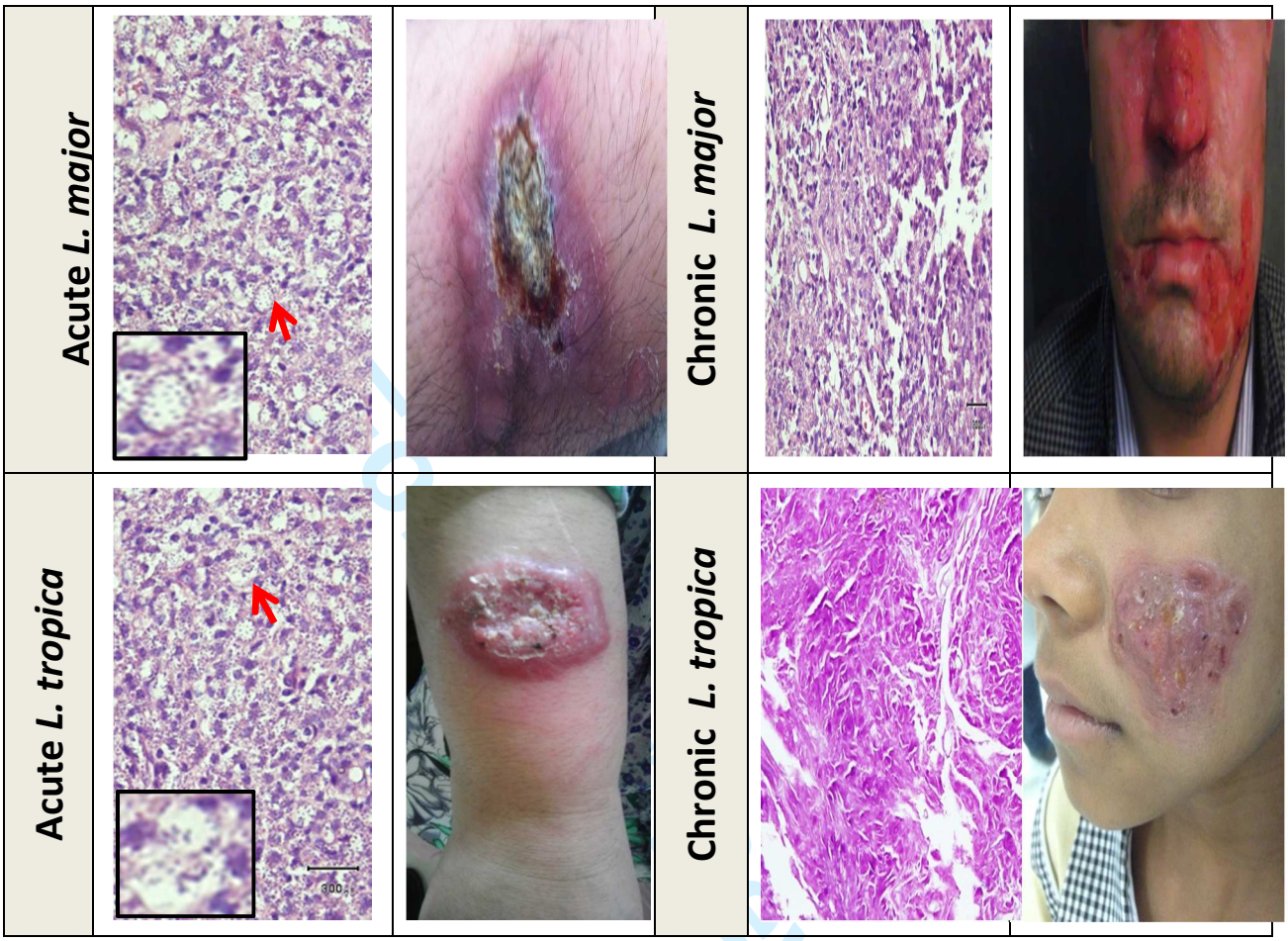

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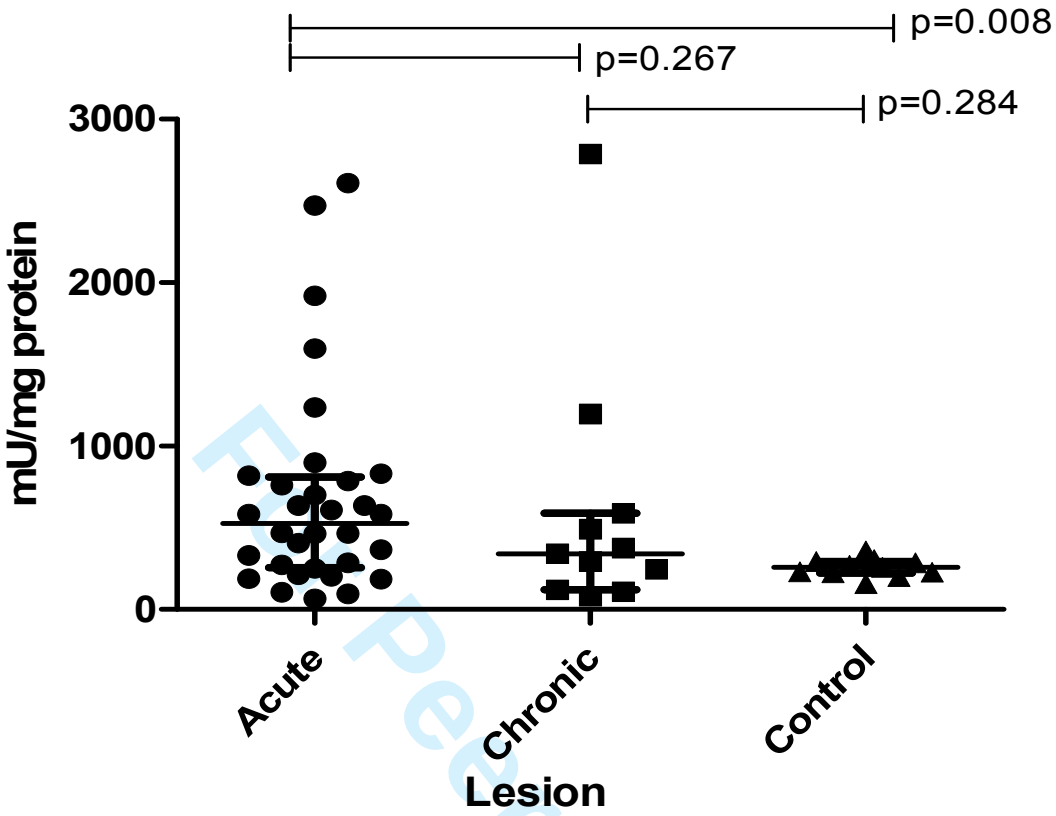

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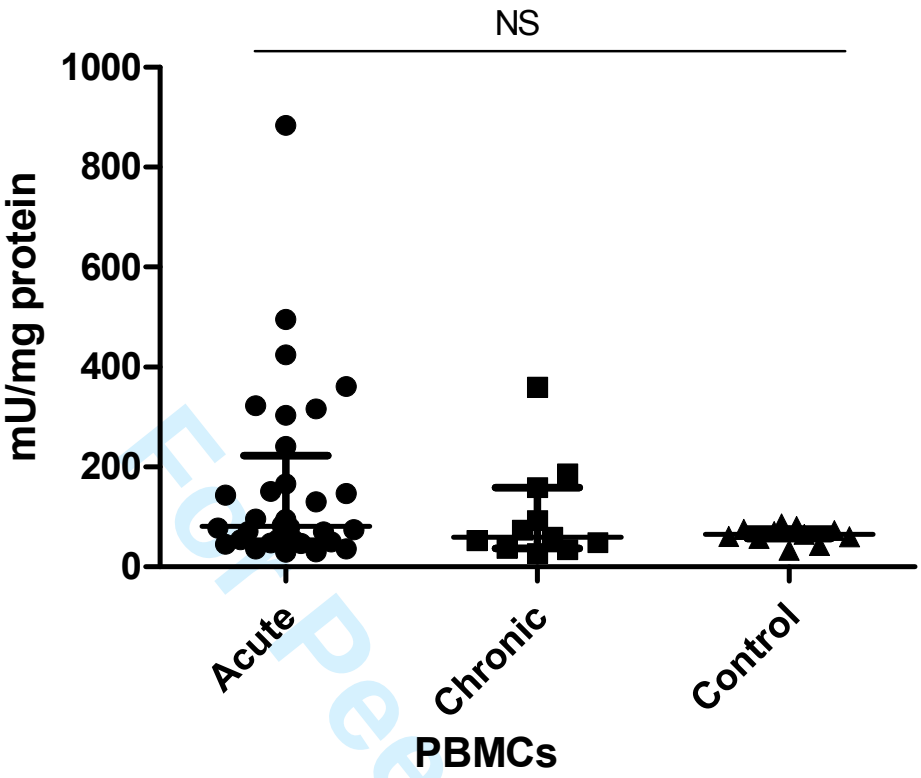

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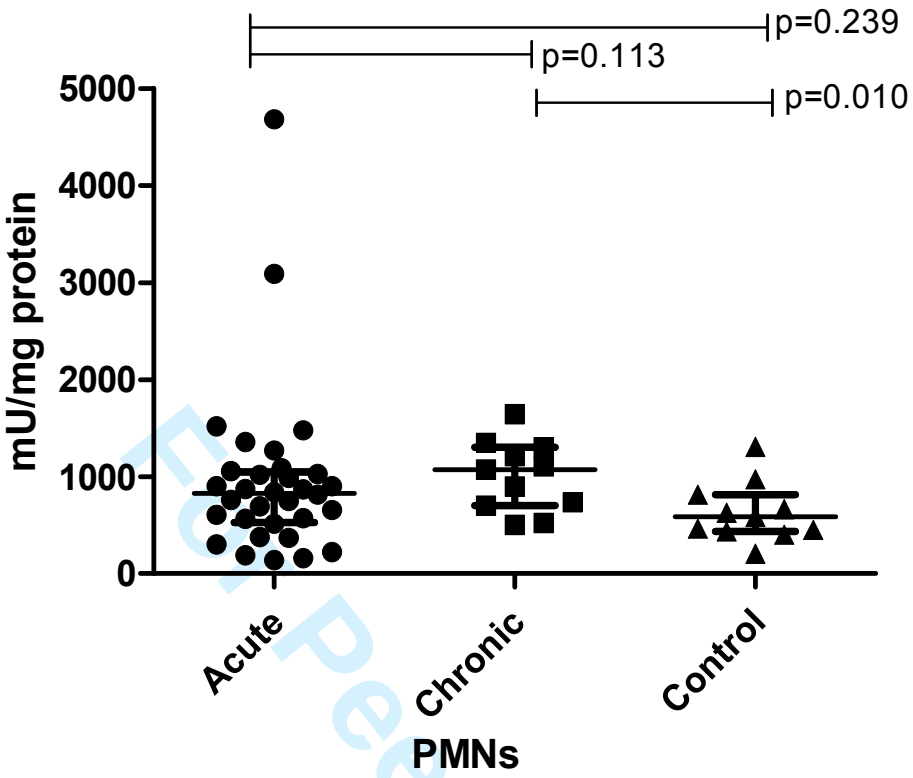

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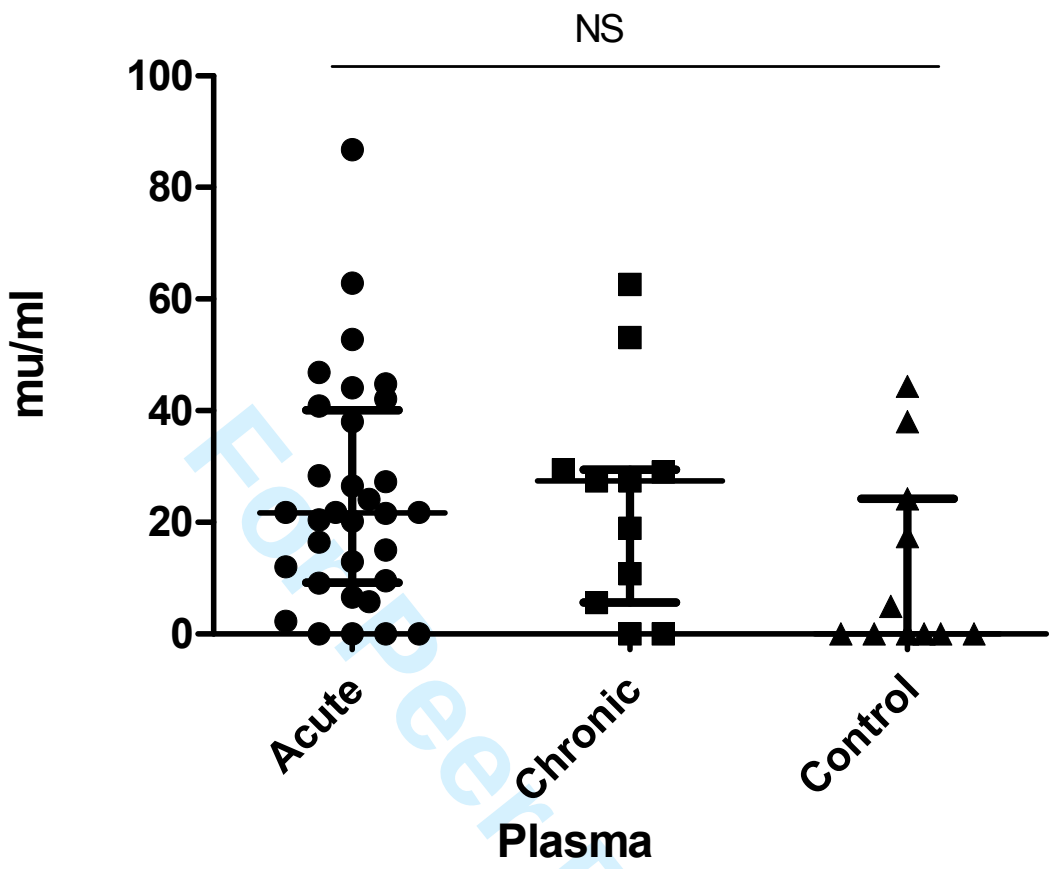

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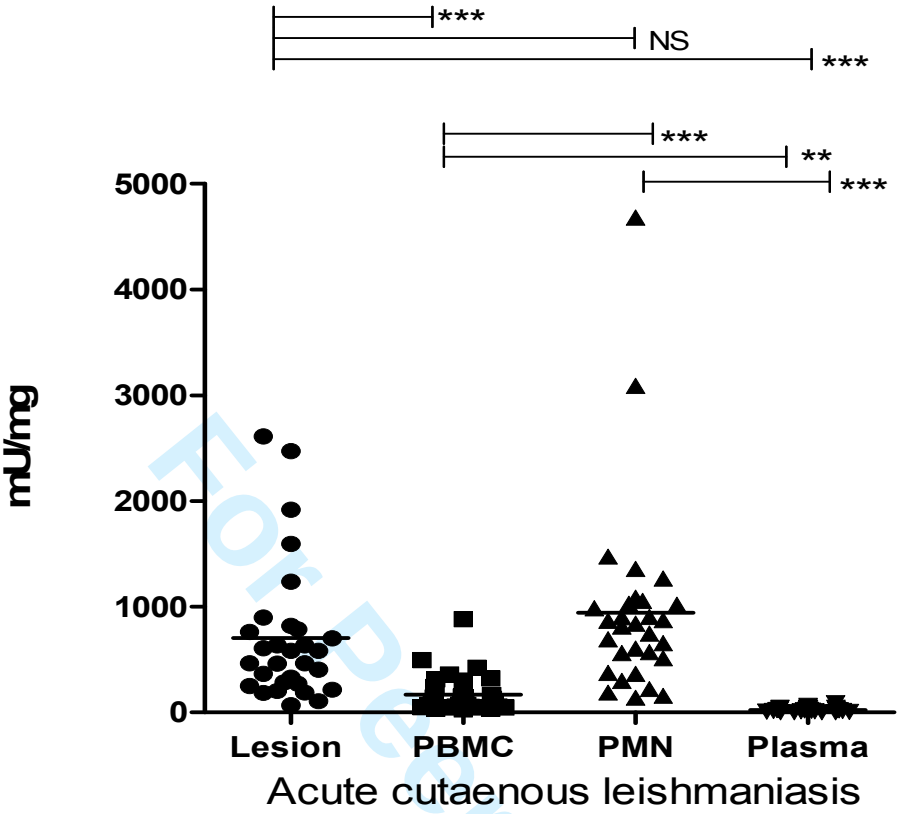

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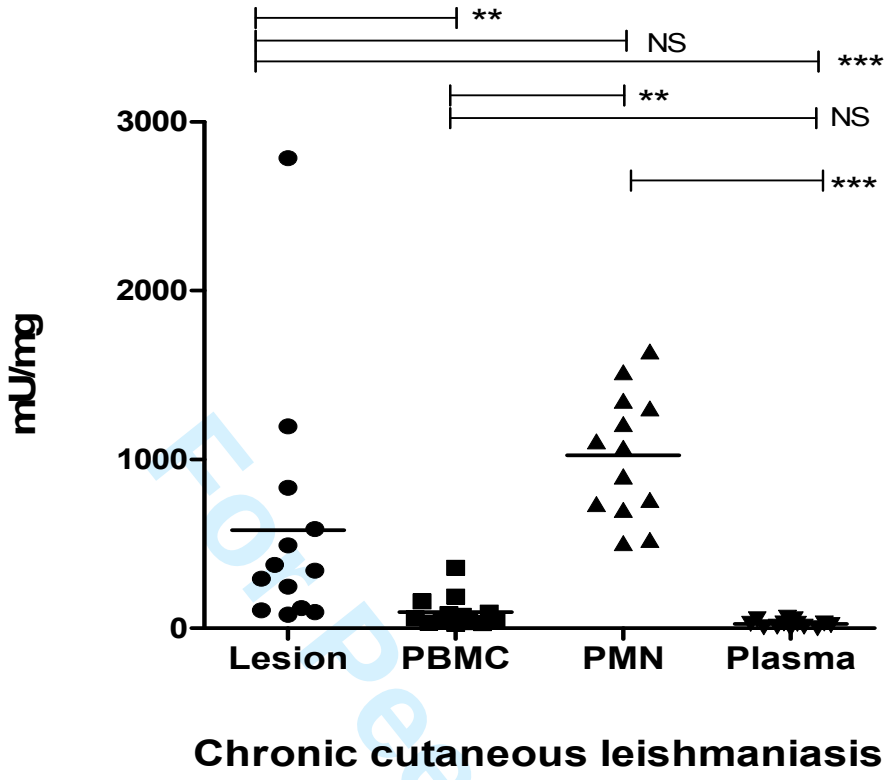

Figure 7

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Table 1. Characteristic of patients with acute and chronic cutaneous leishmaniasis

	Age (year)		Gender		Duration of disease (month)		Species			Lesions	
	Median	Range	Male	Female	Mean	Range	*L. major*	*L. tropica*	undetermined	Single	multiple
Acute (30 patients)	45.50	[21,76]	20	10	4.4	[0.6,12]	21	7	2	12	18
Chronic (13 patients)	27.50	[10,70]	10	3	90.6	[24,360]	7	5	1	5	8
Total (43 patients)	42.50	[10,76]	30	13	30.5	[0.6,360]	28	12	3	17	26

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Table 2. Clinical and pathological features of patients with acute and chronic cutaneous leishmaniasis

	Clinical feature		Lesion location	Pathologic feature			
	Ulcerative	Non-ulcerative		Granuloma	Non-granuloma	Leishman body positive	Leishman body negative
Acute (30 patients)	Tumoral ulcerative: 4 Ulcerative: 8 Plaque ulcerative: 4 **53%**	Plaque: 4 Tumoral: 2 Nodular: 3 Nodular Plaque: 1 Erysiploid: 3 Sportrichoid: 1 **47%**	Face: 5 Neck: 1 Upper extermities: 24 Trunk: 1 Lower extremities: 15	5	25	16	14
Chronic (13 patients)	Tumoral ulcerative: 2 Plaque ulcerative: 2 **30%**	Lupoid: 1 Sporotrichoid (nodular): 1 Tumoral: 2 Plaque: 3 Nodular: 1 Erysipeloid: 1 **70%**	Face: 9 Upper extremities: 11 Lower extremities: 4	10	3	3	10

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Table3. The median (IQR) levels of arginase activity in lesion, PMNs, PBMCs, and plasma of patients acute and chronic CL in comparison with healthy controls.

Arginase activity / Patients and control	**Lesion mU/mg**	**PMNs mU/mg**	**PBMCs mU/mg**	**Plasma mU/ml**
Acute (n=30)	525.75(IQR 526.12)	827.11(IQR 558.74)	87.56(IQR 203.84)	21.69(IQR 30.24)
Chronic (n=13)	342.00(IQR 596.40)	1070.85(IQR 609.83)	59.47(IQR 90.14)	27.49(IQR 33.52)
Healthy control (n=11)	258.62(IQR 69.46)	585.65(IQR 378.00)	65.33(IQR 18.94)	0.00(IQR 24.21)
P value	0.033	0.048	0.154	0.091

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Table 4. Comparison of arginase activity in lesions of patients with acute and CL due to infection with *L. major* and *L. tropica*.

Infecting Parasite species	Acute lesion median(IQR) (mU/mg protein)	Chronic lesion Median(IQR) (mU/mg protein)	P value
L. major	464.70(IQR 487.03)	376.46(IQR 1075.36)	0.058
L. tropica	728.04(IQR 1260.25)	247.26 (IQR 460.94)	0.291

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