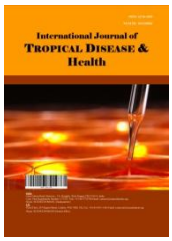

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Histopathological Aspects of Cutaneous Leishmaniasis due to Leishmania-Major in Libya

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To describes the histopathological characteristics of skin lesions from patients with cutaneous leishmaniasis caused by Leishmania major in northwestern Libya and correlate with clinical presentation.
Study Design: case series study.
Place and Duration of Study: This study was carried on patients referred by the region's healthcare institutions and those presented at the Tripoli Central Hospital or the Libyan National Centre for Disease Control between July 2017 to January 2018.
Methodology: The study included 38 patients, aged between 1-73 years, of both sexes, and came from 18 endemic areas in North-Western of Libya. The inclusion criteria were clinical symptoms and microscopic visualization of the parasite on a Giemsa-stained skin smear, in addition, clinical by the slit and smear technique, polymerase chain reaction for L. major. In addition, statistical analysis was conducted for the Histopathological examination.

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Results: The study found that 36 (94%) of the cases studied were positive by the slit and smear technique, and 32 (88.9%) were positive by PCR for L. major. Five histopathological patterns were observed: (i) diffuse cellular reaction without necrosis (25%); (ii) diffuse cellular reaction with necrosis (31.3%); (iii) exudative and necrotic granulomatous reaction (25%); (iv) exudative granulomatous reaction without necrosis (9.3%); (v) exudative-tuberculoid reaction with typical tuberculoid granuloma (organized) (9.3%). Inflammatory cellular infiltration ranged from mild to severe. Lymph plasmacytosis and lymph histiocytosis were predominant (34.4% and 21.7%, respectively). Necrosis was diffuse or local. The clinical features were correlated with this histological pattern. Epidermal changes included acanthosis, exocytosis, spongiosis, hyperkeratosis, and atrophy.

Conclusion: The histopathological changes observed in CL caused by L. major in North-Western of Libya are characterized by an intense diffuse inflammatory reaction in the dermis with the predominance of lymphoplasmacytic infiltration. Overall, the granulomatous presentation was the main one. Various clinical forms, including papule, plaque, erythematous nodule with hemorrhagic crust, or violaceous nodule with adherent crust and ulcerated nodule, are significantly correlated with the histopathological stages, whereas disease progression could be related to age. The histopathological diagnosis of CL caused by L. major has a sensitivity of 78% relative to PCR.

Keywords: Leishmania major; Libya; histopathological patterns.

1. INTRODUCTION

Cutaneous leishmaniasis (CL) is a vector-borne disease [1,2] endemic in various regions, including Libya. [3,4] Only a few published molecular studies on CL in Libya revealed that the disease was caused by L. major and L. tropica. Amro et al., [5] used PCR-RFLP method on 195 archived samples from 49 areas in Northwestern Libya to identified and determined distributions of leishmania species. No extensive published studies on the histopathological aspects of CL in Libya were found; only two cases reports were found [6,7]. These cases studies were finally diagnosed with CL after being treated for a long time for a different skin condition. The previous studies also observed that the epidemiological patterns in the different foci were the same as in other Mediterranean foci of CL. WHO is concerned about an outbreak of CL in places that are not historical hotspots; such change is likely related to civil wars and consequent refugee crises. A similar situation seems to be unfolding in Libya, the prevalence since 2011 gradually started to rise, and the cases reached 6744 cases in 2019. [8] This study aims to contribute to knowledge on CL in Libya by describing the histopathological characteristics of the lesions in skin specimens caused by L. major obtained from geographically diverse regions to inform clinical practice.

2. MATERIALS AND METHODS

Leishmania public health clinics in northwestern Libya were asked to refer patients with leishmania to the Dermatology Department of Tripoli Central Hospital and the Reference Leishmaniasis Clinic of the Libyan National Center for Disease Control (NCDC), where tissue samples were taken. The patients were recruited between June 2017 and January 2018. Both institutions granted ethics approval for the study, and the informed written consent was obtained from all adults who participated and the parents/guardians of the children who participate in the study.

This is a case series study. The inclusion criteria were clinical symptoms of cutaneous leishmaniasis and microscopic visualization of the parasite in Giemsa-stained skin smears. Exclusion criteria included chronic illness, secondary infection on the lesion, and application of any medication to it. A total of 38 CL patients were recruited from 18 towns and cities in the northwestern region of Libya: Zliten, Tarhona, Gharyan, Yefran, Nalut, Alruhaibat, Alhesha, Alarban, Shakshouk, Alharaba, Tripoli, Alhera, Kabaw, Algmail, Tiji, Tandamera, Ragdalin, and Tamzein.

A questionnaire was used to collect information from the patients: age, sex, residence, time of appearance of the lesion before diagnosis, and whether they had been medicated for the lesion, as well as the date of diagnosis.

2.1 Slit and Smear Technique

This technique was used to obtain samples for examination of tissue smears and PCR. The

lesions were sterilized with 70% ethanol. Small incisions were made in the lesion margin with a sterile, disposable surgical scalpel blade size 15 (Vimodone, Italy) to obtain skin samples, which were then smeared on a clean glass microscope slide. After the smears dried completely, they were fixed with 100% methanol and allowed to dry again. At least two specimens were prepared from each case. One was stained with Giemsa stain for microscopic examination, and the other was used for PCR analysis. The stained slides were examined for the presence of amastigote bodies by light microscopy under an oil immersion lens (100 x objectives) [9].

2.1.1 Molecular characterization of the parasites

DNA was extracted from the tissue specimens by using the QIAamp DNA mini kit according to the manufacturer's recommendations (Qiagen, Hilden, Germany). The purity of the DNA was assessed with a microvolume Nano Drop™ Lite spectrophotometer (Thermo Scientific, Germany). The extracted DNA was tested for Leishmania spp. by PCR using the primer pair 5'GCAGCTGGATCATTTTCC3' and 5'ATATGCAGAAGAGAGGAGGC3' designed to amplify a 390-bp sequence of the ITS1 gene. All PCR reactions were performed in a total of 50 μL containing 2.5 μL of DNA template, 25 μL of Hot Start Taq Master Mix (Qiagen, Hilden, Germany), five μL of 10x (2 μM of each primer), and 17.5 μL of RNase-free water. DNA of Leishmania spp. obtained from Pasteur Institute, Tunisia, was used as a positive control.

2.2 RFLP Analysis

PCR product (10 µL) was combined with 10 U of Hae III (Sigma, St. Louis) and 2 µL of the appropriate reaction buffer in a total volume of 20 µL and digested overnight in a 37°C water bath.[10]. All 20 µL of the digest were used for electrophoresis in 2.5% agarose gel. DNA samples of L. major, L. tropica, and L. infantum (Pasteur Institute, Tunisia) were used as positive controls. RNase-free water was used as a negative control.

2.2.1 Histopathological examination

After injection of the lesion site with 2% lidocaine, skin biopsies were taken from the margin of the lesion (avoiding ulcerated and heavily crusted parts) with a 2-mm disposable punch (Whatman International, Germany). The material was fixed in 10% formalin for < 48 h and processed for paraffin embedding. The tissue was sectioned at 5 μm and stained with hematoxylin and eosin.[11].

2.3 Statistical Analysis

The data were analyzed by using Statistical Package for the Social Sciences (SPSS version 16, IBM). Frequencies and percentages were calculated, and Pearson's correlation test was used to study the relation between histopathological staging, clinical stages, histopathological types and clinical appearance. P values < 0.05 were considered statistically significant.

3. RESULTS AND DISCUSSION

Of the 38 patients recruited into the study, two were excluded because microscopic examination of the smear did not reveal the presence of the parasite. Over half of the cases (52.8%) were recorded in October. The ages of the 36 patients ranged from 1 to 73 years, and almost two-thirds of them (23, 63.9%) were 1-15 years old. Eleven of the patients were aged 16-40 years and 11 were aged 41-73. There were 11 patients in the 16-40 years age group and likewise in the 41-80 years age group. The 23 males represented 63.9% of the patients. The duration of the lesions before diagnosis was 2-10 weeks, and none of the patients were medicated. Of the 36 patients, 32 (88.9%) were confirmed by restriction fragment polymorphism analysis to be infected with *L. major.*

Most of the patients (72.2%) sought medical advice the duration of the lesion was 4-8 weeks. Lesions appeared mainly on exposed parts of the body, with 66.7% of them on the extremities. The most frequent clinical presentation of the lesions was as an erythematous nodule with hemorrhagic crust or violaceous nodule with adherent crust (41.6%) (Table 1).

Multiple lesions were present in 32 cases (88.9%). The number of lesions per patient ranged from 1 to 13, with a mean of 6 lesions. The diameter of the lesions ranged from 0.5 x 0.5 cm to 4 x 6 cm. In one-third of the patients, it was 1.5 x 2 cm (Table 2).

Histopathological examination of the skin biopsies revealed *L. major* amastigotes in the superficial dermis of 25 of the 32 samples (78.1%) that were positive in the scanned slit

skin smear slides. The presence of *Leishmania* amastigote forms were more obvious in early-stage lesions, mainly in macrophage cytoplasm and, in one sample, within neutrophils.

Histopathological examination showed epidermal changes related to *L. major* infection in all 32 cases. The most frequent was acanthosis (56.2%), followed by hyperkeratosis (46.8%), spongiosis (34.3%), parakeratosis (28.1%), granulosis (18.7%), exocytosis (21.8%) and atrophy (6.2%). In all cases, the dermis showed mononuclear dermal infiltration consisting of lymphocytes, histiocytes, plasma cells and, occasionally, eosinophils.

Histopathological findings were grouped in patterns according to the staging proposed by Magalhães. [12] Five histopathological patterns were identified. Stage I consisted of a dermal infiltrate composed predominantly of histiocytes with a few lymphocytes; eosinophils were rare, and plasma cells were seen occasionally. The infiltration reaction was distributed in diffuse forms extending in some cases to the subcutaneous layer, and localized forms with mild infiltration that tended to perivascular and periadenxeal distribution. Parasites in this stage were present in all the specimens, mainly in the upper dermis. Stage II consisted of a mixture of diffuse infiltration of histiocytes, polymorphs and plasma cells, with few lymphocytes. The striking features of this pattern were the presence of necrosis, seen as hematophilic necrotic material and nuclear debris, but no granulomatous response. Stage III consisted of early granuloma formation mainly in the lower dermis, with focal collection of epithelioid cells, lymphocytes and a few plasma cells. There were no giant cells and a few amastigotes were seen. Necrosis was present. This pattern was seen in 8 of 32 cases (25%). Stage IV showed disorganized epithelioid granuloma, with lymphocytes, histiocytes and a few giant cells. Stage V showed well-formed epithelioid granuloma in the dermis. There were plenty of lymphocytes, epithelioid cells and a few giant cells. It was difficult to identify parasites and no caseation was seen. Other dermal features included edema, vasodilatation, and endothelial hyperplasia.

Stage II was the most common (10/32 patients, 31.3%), followed by Stage I (25.0%), Stage III (25.0%), stage IV (9.4%) and stage V (9.4%). There was a significant direct correlation between clinical presentation of the lesions and histopathological staging (Pearson's correlation coefficient = 0.545, $p = 0.001$).

All three histopathological patterns described by Gonzalez et al. [13] were found (Fig. 1, Table 3).

Table 1. Clinical presentation of lesions

Clinical appearance	No. of cases	Frequency %
Papule	7	19.4
Plaque with crust	10	27.8
Erythematous nodule with central hemorrhagic crust	8	22.2
Violaceous nodule with central adherent crust	7	19.4
Ulcerated nodule	4	11.1
Total	36	100.0

Table 2. Lesion size

Size*	No. of cases	Frequency %
0.5 x 0.5 cm	7	19.4
1 x 1.5 cm	9	25
1.5 x 2 cm	12	33.3
2 x 3 cm	5	13.9
4 x 6 cm	3	8.3
Total	36	100.0

** Largest diameter x smallest diameter*

Table 3. The frequency of histopathological patterns according to the Gonzales staging

Stage	Frequency	Percent
Lymphohistiocytosis	7	21.7
Lymphoplasmacytosis	11	34.3
Granulomatous reaction	14	43.7
Total	32	100

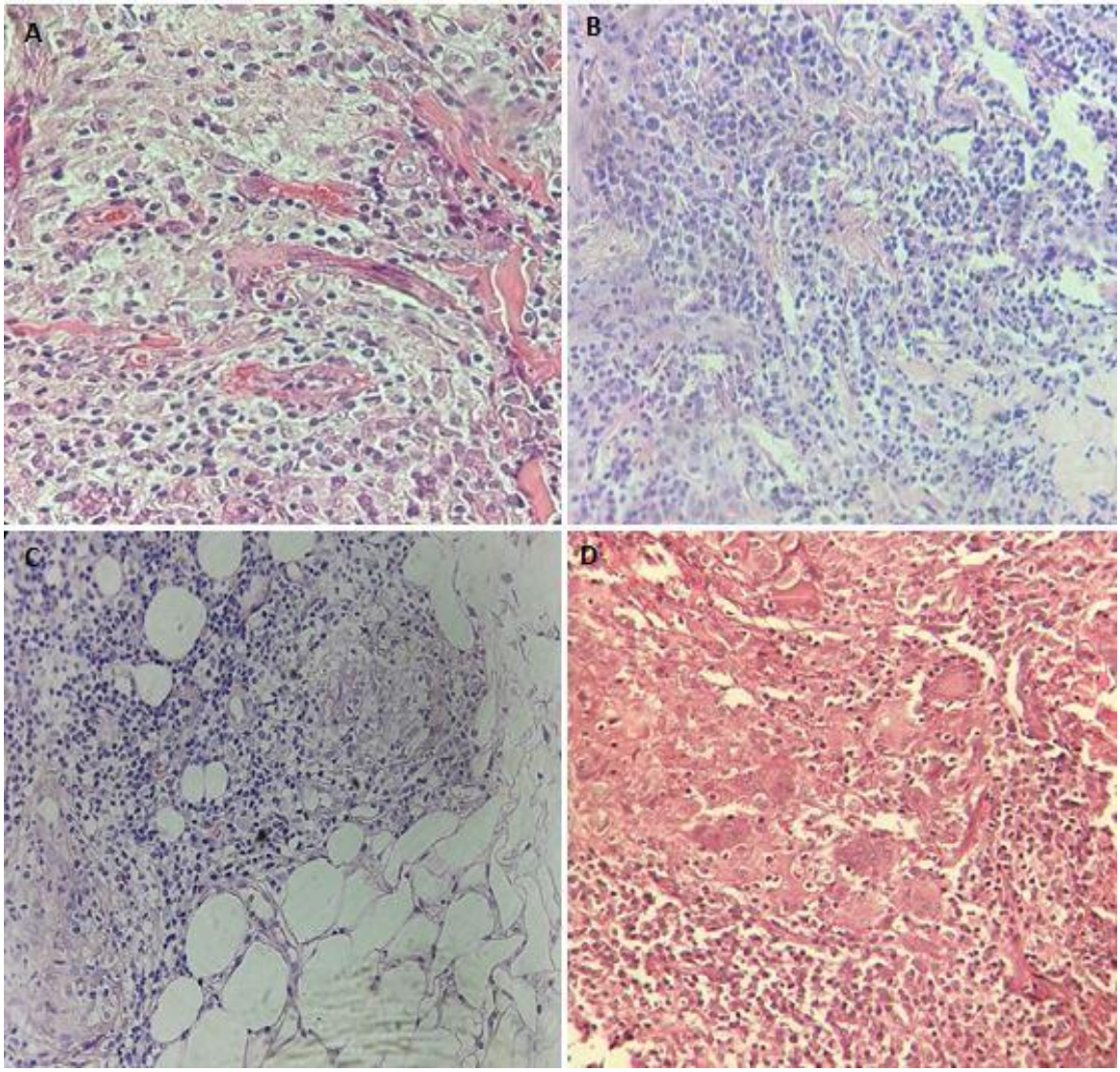

Fig. 1. Inflammatory patterns observed in cutaneous lesions caused by *L. major*. (A) Intense and diffuse lymphohistiocytic inflammatory pattern; (B) Intense lymphoplasmocytic inflammatory pattern; (C) Granulomatous inflammatory pattern; (D) Presence of multinucleated giant cells observed in the granulomatous reaction (hematoxylin-eosin staining) (all x40)

A granulomatous outline or well-formed epithelioid granulomatous reaction was the main histopathological presentation granulomatous were observed in 43.7% (14/32) of the cases, followed by lymphoplasmacytic inflammatory infiltrate, and lymphohistiocytosis. Diffuse infiltration of inflammatory cells was seen in 11 of 32 patients (34.3%) and local distribution in 65.6%. The inflammatory infiltrate was intense in 5 of 32 cases (15.6%), moderate in 22 (68.7%) and mild in 5 cases (15.6%). The inflammatory infiltrate was characterized by predominance of lymphocytes (18/32, 56.2%), followed by histiocytes (9/32, 28.1%) and plasma cells (5/32, 15.6%), with the presence of multinucleated giant cells in 5 of 32 cases (15.6%). Photomicrographs of the inflammatory patterns are shown in Fig.1.

Stages I and II could appear at any time, but stage III, which indicates a granulomatous reaction, started to appear after four weeks of infection or later. Moreover, the age of the patient could affect disease progression, with the granulomatous reaction appearing earlier in younger people (Table 4).

Clinical appearance as classified by Gonzalez *et al.* was significantly correlated with the histopathological stage (R = 0.545, p = 0.001) (Fig 2).

Table 4. Relation of reaction degree, inflammatory type and Gonzales staging

Stage 2	Age (years)	Date of appearance (weeks)						Total
		2	3	4	6	8	10	
Lympho-histocyte	1-15	2	1	2	0	1		6
	16-40	0	0	0	1	0		1
	Total	2	1	2	1	1		7
Lymphoplasma cell	1-15	0		0	0	1	0	1
	16-40	0		1	1	2	0	4
	41-80	1		0	3	1	1	6
	Total	1		1	4	4	1	11
Granulomatous reaction	1-15			4	1	2		7
	16-40			3	1	0		4
	41-80			0	1	2		3
	Total			7	3	4		14

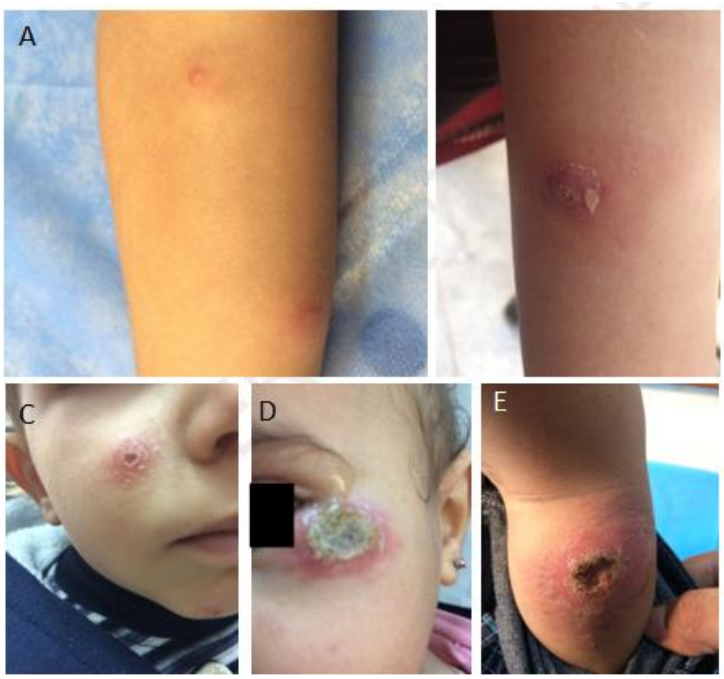

Fig. 1. Clinical presentation according to second histopathological staging; (A) Erythematous papule on forearm in stage I. (B) Erythematous plaque on leg regarded as stage II. (C, D, E) Granulomatous reaction that is in stage III, with erythematous nodule on cheek, ulcerated nodule with crust below left eye, and ulcerated nodule on left leg

However, there was no significant correlation between histopathological staging and duration of lesions or between clinical appearance and the area of residence (mountain, plain) ($p > 0.05$, Table 5*)*.

In the present study regarding sex and age with the prevalence of CL, males represented 63.9% of the patients and could be related to the fact that the men work outdoors for longer hours, which agrees with similar studies [5,14]. In addition, two-thirds of the patients represented age group (1-15 years) (23, 63.9%), similar to other studies. [5,15], which indicates that Libya is an old endemic area for CL. The majority of the patients sought medical advice in October and December, which resembles the seasonal pattern reported by El-Buni in 2000.[16] Such a result also agrees with another study reporting that transmission of L. major by the phlebotomine sand fly occurs during the summer months in Tunisia [17], with active lesions in humans tending to emerge during the autumn and winter.

Table 5. Relation of inflammatory type and Gonzales staging to residence

Stage 2	Terrain	Inflammatory type		Total
		Localized	Diffuse	
Lymphohistiocytes	Mountain	2	3	5
	Plain	2	0	2
	Total	4	3	7
Lymphoplasma cells	Mountain	5	3	8
	Plain	2	1	3
	Total	7	4	11
Granulomatous reaction	Mountain	8	2	10
	Plain	2	2	2
	Total	10	4	14

The diagnosis was confirmed as CL, based on the presence of amastigotes in slit skin smear samples; only 2 out of 38 samples (5.3%) were negative. This result could have been due to insufficient tissue on the slide. Nevertheless, this rate is lower than observed in another study [18], which reported a negative slit-skin smear in 33.3% of the study sample. Our higher positivity rate could be related to the fact that our patients had not been medicated. Of the 36 patients, 32 (88.9%) were confirmed by restriction fragment polymorphism analysis to be infected with L. major. On histopathological examination, amastigotes were detected intra or extra macrophages in 25/32 biopsies (78.1%), which is higher than the rate of detection found in the Iraqi study was 10/35 (10.25%). Amastigotes were found within neutrophils in only one specimen, which might point to the initial uptake of L. major by neutrophils. [19,20]. Parasites were more prevalent in early lesions and were more difficult to find in older lesions. According to the literature, histopathological examination is usually described as having the lowest diagnostic sensitivity (30-60%) because of the difficulty of identifying parasites in late lesions [21]. Histopathological presentation of L. major shows considerable variability. In this study, the predominant pattern was characterized by the presence of diffuse cellular inflammatory infiltration by histiocyte, plasma cell, and lymphocytes were predominant in the inflammatory infiltrate in early lesions. In addition, necrosis was mainly associated with infiltration of plasma cells, suggestive of plasma cell-induced necrosis [22]. The necrosis was observed in both non-granulomatous and granulomatous cellular reactions, and the lymphocyte and plasma cell reactions were more frequent in the presence of well-formed epithelioid granulomas. The granulomatous reaction was seen in acute lesions after four weeks when the epithelioid granuloma started to appear in the lower dermis, as reported previously [21]. Also observed were well-organized epithelioid granulomas, which are related to the immune system's attempt to eliminate the parasites [23]. In this study, 65.6% of the biopsies showed a local inflammatory response, in contrast to findings showing a diffuse inflammatory response in 62.5% of the biopsies from patients with CL caused by L. panamensis in Colombia [23]. Nevertheless, based on the Magalhães classification, diffuse necrosis was observed in 31.3% of the specimens. This result agrees with histopathological findings in Nicaragua and Guyana but is different from that in Sudan and Saudi Arabia, where focal necrosis was reported as the main presentation, [24] that compared the pathological patterns of cutaneous leishmaniasis in different geographical regions. Based on the Magalhães classification, a granulomatous reaction was observed in 14 patients (43.7%). This observation is in agreement with that reported on 73 biopsies from patients infected with L. mexicana. [11] The essential feature of CL pathology is a chronic granulomatous inflammatory response resulting from the colonization of cells of the mononuclear phagocytic system by amastigotes and is usually accompanied by features such as necrosis. [12,25-27].

At the epidermal level, our study exocytosis was present in 21.8% of the samples. Exocytosis was observed at a much higher frequency (69.5%) in another study.[23] The presence of exocytosis emphasizes the importance of the epidermis in the immunoregulation of the disease. The sample we took from a patient's face clearly showed acanthosis, exocytosis, and atrophy.

In the present study regarding clinical features, lesions were mostly multiple (88.9%) and reached up to 13 lesions in one patient, higher than previously reported [28]. Such a result could

be related to a higher parasite count in the sand fly or a high prevalence of the vector in the endemic areas. Our study observed lesions in different stages of progress, from papules to soft, boggy, crusted plaques/nodules that became ulcerated. This clinical polymorphism may reflect variability in the host immune response and/or variability of parasite virulence and size of the initial inoculums [29]. Papule plaque erythematous, violaceous, and ulcerated nodules were significantly correlated with the histopathological classification ($p = 0.001$). Such findings have differed from the descriptions of the histopathology of cutaneous leishmaniasis in Oman [21]. However, interestingly, there seems to be an inverse association between the clinical features and the duration of the lesion before diagnosis and more rapid progression of lesions in younger patients.

The results obtained by applying the classification of Magalhães and that of Gonzalez yielded similar results. However, the Gonzalez classification could be favored because it is easier for clinical application and illustrates the different stages with images, facilitating diagnosis.

4. CONCLUSION

The histopathological changes observed in CL caused by L. major in northwestern Libya are characterized by an intense diffuse inflammatory reaction in the dermis with predominance of lymphohistiocytic and lymphoplasmacytic infiltration, and overall, the granulomatous presentation was the main one. The various clinical forms, including papule, plaque, erythematous nodule with hemorrhagic crust or violaceous nodule with adherent crust and ulcerated nodule, are significantly correlated with the histopathological stages, whereas disease progression could be related to age. The histopathological diagnosis of CL caused by L. major has a sensitivity of 78% relative to PCR.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

All authors declare that written informed consent was obtained from the patients and the parents of the children for publication of the cases report and accompanying images.

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the authors

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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