Author’s response to reviews

Title: Clinical and laboratory profiles of patients with early spontaneous healing in cutaneous localized leishmaniasis: A historical cohort study

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Author’s response to reviews:

Dear Sirs,

We are pleased to resubmit for publication the revised version of the original manuscript “Clinical and laboratory profiles of patients with early spontaneous healing in cutaneous localized leishmaniasis: A historical cohort study.” We have made a number of extensive
revisions and considerably improved major sections of the paper (highlighted in yellow in manuscript).

We would like to thank the reviewers both for the positive and negative comments which we found very helpful for improving our manuscript.

We have addressed each of their concerns as outlined below.

We also prepared a manuscript with the track changes as requested.

Fátima Conceição-Silva

REVIEWER 1 COMMENTS

Comment 1: The study could have benefited from a more thorough investigation of the immune mechanisms associated with the spontaneous healing, although this was not the main focus of the study.

Answer 1: We thank the comment. The verification of immune response profile of patients with early spontaneous healing (ESH) in cutaneous leishmaniasis is currently being developed in our research group. Besides that, as this approach is still under the experimental part and there are few studies describing clinical, epidemiology and laboratory profile of these cases, therefore we chose to describe these characteristics in more detail, comparing ESH with non-ESH leishmaniasis. The study of the in situ immunological status in ESH versus non-ESH cutaneous leishmaniasis remains a real concern for us and a subject of experiments that will be published as soon as possible.

Comment 2: Abstract

Background: ACL patients were divided into ESH and CL-treated patients. If all were CL patients, perhaps it would be clearer to name them ESH and non ESH
Answer 2: We greatly appreciate the reviewer's comment pointing out this problem. We agree with the reviewer's opinion and choose to rename the groups into early spontaneous healing (ESH) and non-early spontaneous healing (NESH) in the whole manuscript (including figures and tables). All changes were highlighted in the revised text. The list of abbreviations was modified accordingly.

Comment 3: Abstract

Results: ESH had a scar on initial examination. This is a recurring point in the text. It is not clear if ESH is the presence of a scar at the time of patient recruitment or if it is a fast-resolving lesion for which treatment is not required.

Answer 3: We appreciate the reviewer's comment and regret our misuse of the term “scar”. We apologize for the inconvenience and tried to better explain in the manuscript the used terminology. What we previously named as “scar” is not a previous atrophic lesion, but a epithelialized (non-ulcerated) lesion still showing signs of inflammatory activity (erythema, crusting and/or induration for example) in a context of the current history of disease, at the first medical appointment and physical examination of the patient. We emphasize that all patients were examined in the first appointment and their lesions, even if not ulcerated, were biopsied and analyzed. If in the period between the first medical appointment and the patient's return to check the results (on average after 3–4 weeks) the lesion showed signs of progression to healing (fast-resolving lesion), such as reduction of its diameter, erythema and/or induration, the patient was followed-up without treatment and with periodic clinical exams, as well as considered an ESH case. We also emphasize that only patients with parasitological confirmation of leishmaniasis were included in ESH and non-ESH groups. To clarify the misunderstanding, we replaced the term “scar” for “epithelialized lesion” in the whole manuscript.

Comment 4: Abstract

Conclusion: The results suggest lower parasite load in ESH cases.

This is highly speculative. The results suggest that ESH patients may have cleared the parasites more effectively, for various reasons, including a lower parasite load.

Answer 4: We thank the reviewer's observation and to remedy the problem it has been corrected as follows: ESH group apparently presented a lower parasitic load suggested by the difficulty of
parasitological confirmation, which was in the majority of the cases obtained only by PCR method (page 3, lines 14-17).

Comment 5: Abstract

Conclusion: The treatment of cases which progress to ESH is controversial.

The key point would be to differentiate an ESH case from a non-ESH. How do authors propose this could be done in the field, in order to make the decision no to treat?

Answer 5: We also thank the reviewer for this comment. It is known that many individuals evolve with early healing of the skin lesion of the cutaneous localized leishmaniasis (LCL), often without seeking medical attention. Others remain months to several years with the lesion in activity and the healing process is slow (non-ESH). Such phenomenon may be explained by the early or late establishment of an efficient specific immune response to achieve parasite elimination. We have followed the patients for a long period (median follow-up of 803 days) in a pre-established clinical and laboratory care routine, including dermatological and ear, nose and throat specialist evaluation; that is why we conclude the non-treatment of ESH cases is safe as it is easy to observe possible non-treatment complications, which in the studied cohort were null. Due to the potential toxicity of the drugs used, when clinical signs of early spontaneous resolution are observed the option of the non-treatment seems to be safe. Another point is that regarding spontaneous resolution in field conditions, not to treat is a reasonable possibility since the chance to perform a parasitological diagnosis is weak and many of these cases are misdiagnosed as non-leishmaniasis. We include this explanation in the Discussion section page 11, lines 10-15.

Comment 6:

Background: Line 14: How long does it take, in the present setting, between diagnosis and start of treatment? Is that long enough for the lesion to show signs of healing? It does seem that way but authors should give a time frame for the reader.

Answer 6: We are grateful for the reviewers comments and contributions to improve the manuscript. In the aforementioned cohort, patients were examined at a first medical appointment where clinical data and laboratory tests were performed for diagnostic purposes. The median time between the first appointment and the returning visit to the health service was 29 days. This length of time is usually necessary to perform all the exams (including scarification, imprint, culture isolation, PCR, conventional histopathology and anti-Leishmania immunohistochemistry), since they are required to conclude the diagnosis. In this second appointment, the ESH group showed clear signs of progression to cure, such as spontaneous
epithelialization, reduction of lesion diameter, less infiltration of the borders of the ulcers leading
the attending physician to choose not to treat the patient and to follow up the case within the
recommended routine of the institution. If there were no clinical signs of healing of the lesion
(NESH group) the treatment was instituted and the patient was followed up in the same way as
the ESH group. To remedy the problem, we added the following statement to the results session
(page 8, lines 18-19): “The median time between the first and the second medical appointment
was 29 days in the whole studied cohort.”

Comment 7:
Methods: Line 14: Did inclusion criteria include presence of a scar?
Answer 7: We thank the reviewer for the observation. As explained in the comment 3, what we
previously named as “scar” is not an atrophic lesion, but a non-ulcerated lesion still showing
signs of inflammatory activity (erythema and induration for example) in a context of the current
history of disease, at the first physical examination of the patient. If in the period between the
first medical appointment and the patient’s return to check the results (on average 3-4 weeks) the
lesion showed signs of progression to healing (fast-resolving lesion), such as reduction of its
diameter, of the erythema and / or of the induration, the patient was followed-up without
treatment with clinical and laboratories exams, as well as consider an ESH case. To clarify the
misunderstanding, we replace the term “scar” for “epithelialized lesion”.

In the section of methods [pages 5 (lines 21-23) and 6 (lines 1-2)] the inclusion criteria was
rewrite: “An ESH case was defined as a patient with a positive parasitological exam for
Leishmania spp. and epithelialized lesion(s) without crusts in the period between the laboratory
investigation for the disease diagnosis and the expected date for the start of the specific treatment
[12]. The morphology of included lesions were ulcer/ulceration, papule, nodule or plaque [12].
The lesion should be present in a current context of illness.” We added a reference* that explains
the nomenclatures used for lesion morphology.

Comment 8:

Methods: Line 15 - Patients were divided in ESH and CL patients with typical CL requiring treatment. This goes back to the point raised earlier: it would be more accurate to classify patients as ESH and non-ESH. Otherwise, it seems that CL treatment is optional.

Answer 8: We took note of the reviewer suggestion and renamed the groups into early spontaneous healing (ESH) and non-early spontaneous healing (NESH) in the whole manuscript (including figures and tables), as explained in comment 2.

Comment 9:

Methods: Line 18 - again, how long between a positive exam and the expected date for the start of specific treatment?

Answer 9: As explained in the comment 6, patients were examined at the first medical appointment when clinical data and laboratory tests were performed for diagnostic purposes. The median time between the first appointment and the return to the health service was 29 days. In this second appointment, the ESH group showed clear signs of progression to cure, such as epithelialization, reduction of lesion diameter or less infiltration, leading to the attending physician to follow up the case without treatment in the pre-established clinical and laboratory care routine. If there were no clinical signs of lesion healing the treatment was instituted and the patient was followed up in the same way as the ESH group. To remedy the problem, we added the following statement to the results session (page 8, lines 18-19): “The median time between the first and the second appointment was 29 days in the whole studied cohort.”

Comment 10:

Page 6 - please include the PCR assay used (Reference), please explain what is immunohistochemistry with fragments of cutaneous lesion. Is that a tissue section from a biopsy? Were the tests performed on biopsy samples IFA, imprint, PCR? From which sample were Leishmania parasites cultivated?

Answer 10: We thank the reviewer for the observation. A reference was included for the PCR technique (reference number 13-page 6, line 14). Two changes were made on methods to clearly state the nature of the samples: “indirect immunofluorescence assay (IFA) and/or immunoenzymatic assay (ELISA) in blood samples; scarification with smears obtained from the cutaneous lesions to enable direct visualization of the parasites; culture for Leishmania sp. isolation, imprint, histopathology, polymerase chain reaction (PCR) [13] and anti-Leishmania immunohistochemistry (IHC) in fragments of the lesions obtained through biopsy procedure”
The samples were obtained at the first appointment. After the sample uptake, the diagnosis was confirmed by parasitological isolation that included direct examination (material collected by scarification of cutaneous lesion or imprint of a lesion fragment obtained through biopsy procedure), histopathological examination, immunohistochemistry and isolation of the parasite in culture from a lesion fragment (from samples obtained through skin biopsy procedure). These procedures were part of the diagnostic routine.

Comment 11:

4) Results: Authors state that 445 patients were evaluated (Page 8) but in 9 cases, scars were observed in the initial clinical examination. Were these scars from a previous CL so these patients had a scar and an ongoing active lesion? 432 patients were treated for ACL while 13 showed spontaneous healing. In these 13, were the 9 presenting a scar included? This is shown in Table 1. I disagree that an individual presenting a scar at the time of enrollment (not in the inclusion criteria) can be considered an ESH case, especially because this patient was not evaluated at the time of lesion appearance. A ESH case is a case that had a lesion that healed before the onset of treatment (accoding to authors) and presence of a scar is history of previous CL. Also, in the CL patients, 5 had scars. Were these previous CL as well? Also, in the ESH, how is it that 7 had ulcer and 6 did not whereas 4 had scar and 9 did not? What is the clinical sign if not the presence of an ulcer? A papule?

Answer 11: We are grateful to reviewer for pointing this out. What we previously named as “scar” is not a previous atrophic lesion, but a non-ulcerated lesion still showing clear signs of inflammatory activity (erythema and infiltration for example) in the context of the current history of disease, at the first physical examination of the patient. We emphasize that all patients were examined in the first medical appointment and their lesions, even if not-ulcerated, were biopsied and analyzed. If within the period between the first appointment and the patient's return to check the results (on average 3-4 weeks) the lesion showed signs of progression to healing (fast-resolving lesion), such as reduction of its diameter, of the erythema and / or of the infiltration, the patient was not treated and was followed-up with clinical and laboratory exams, as well as considered as an ESH case. We also emphasize that only patients with parasitological confirmation of leishmaniasis were included in ESH and non-ESH groups. To clarify the misunderstanding, we replaced the term “scar” for “epithelialized lesion”.

Considering the whole cohort, we observed 9 patients with “epithelialized lesion” at the first medical appointment. In the NESH group, 5 presented “epithelialized lesion” at this first attendance (but the assistant doctor opted to treat because there were no signs to progression to definite healing). In the ESH group, 4 patients presented “epithelialized lesion”; they were not
treated due to the clear progression to definite healing. The clinical presentation of ESH group was: 7 ulcers, 4 epithelialized lesions and 2 non-ulcerated plaque lesions, totaling the 13 cases. To the better understanding of this issue, we added a footnote to the Table 1 for the lesions in the ESH group that were neither ulcers nor epithelialized: **Two of the ESH patients had plaque lesions. We added this explanation to the results section, pages 8 (lines 20-24) and 9 (lines1-2).

Comment 12:

5) Table 1: One individual in the ESH group had 2 or more lesions. Did both heal spontaneously and at the same time? Or both developing at the same "rate"?

Answer 12: We thank the reviewer for this observation. Both lesions resolved in the same time in this case of ESH. We added this information in the results session page 9, lines 9-10.

Comment 13:

Table 1: Why is it that for the 13 ESH cases, results were not shown for IFA, culture, ELISA, imprint and PCR?

Answer 13: We are grateful to reviewer for pointing this out. Unfortunately in some cases the complete laboratory exams panel was not carried out, and there were some missing data.

We can observe from Table 1 that out of a total of 13 patients from the ESH group: only 9 performed IFA, 10 performed culture, 10 performed ELISA, 9 performed imprint and 8 performed PCR. However, we must emphasize that the whole cohort had nevertheless parasitological confirmation of cutaneous leishmaniasis by at least one method. Unfortunately because it was a retrospective cohort, some limitations are found.

Comment 14:

Table 2: Please indicate which tests were positive for ESH and CL.

Answer 14: We would like to thank the reviewer for the observation. This information is contained in Table 1 (pages 24-26) that addresses the clinical and laboratory profile of the whole studied cohort.
Comment 15:

The authors could try to evaluate the agreement of the techniques used, especially given the diversity of reports in the literature regarding efficacy. This would also strengthen the assumptions regarding the "lower number of parasites" in ESH cases.

Answer 15: We thank the reviewer for the comment. When analyzing only cases with parasitological confirmation (positive in the culture, imprint, PCR, histopathological and / or immunohistochemical exams) between the ESH and NESH groups among the positivity of different laboratory tests performed, we can prove, with statistical evidence, the data previously discussed. There were statistically significant differences between the NESH and ESH groups regarding the IFA (p = 0.004), ELISA (p = 0.002), MST (p = 0.05) and culture (p = 0.001). In the ESH group we observed differences between the parasitological confirmed cases and the positivity for the PCR test (p = 0.002). These tests also suggest the presence of lower parasitic load in the ESH group and strengthen the findings obtained in tables 1 and 2. The recommendation for using the PCR technique, as a routine, in patients with clinical evidence of ESH may also be considered as an unfolding of these complementary analyzes. We added the following results on page 10 (lines 5-7): “Considering the concordance concerning the positivity in each test, there were statistically significant differences between the NESH and ESH groups regarding the IFA (p = 0.004), ELISA (p = 0.002), MST (p = 0.05) and culture (p = 0.001) (data not shown).”

Comment 16:

Also, if ESH cases had only a scar and not an active ulcer (if correctly understood), where were samples obtained for culture, PCR, imprint, etc.

Answer 16: As explained before, we really regret our misuse of the term “scar”. What we previously named as “scar” is not a previous atrophic lesion, but a non-ulcerated lesion still showing signs of inflammatory activity (erythema and induration for example) in a context of current history of disease, at the first physical examination of the patient. We emphasize that all patients were examined in the first consultation and their lesions, even if not-ulcerated, were biopsied and analyzed. If in the period between the first consultation and the patient's return to check the results (on average 3-4 weeks) the lesion showed signs of progression to healing (fast-resolving lesion), such as reduction of its diameter, erythema and induration, the patient was followed-up with clinical and laboratories exams, as well as consider an ESH case. We also emphasize that only patients with parasitological confirmation of leishmaniasis were included in ESH and non-ESH groups. To clarify the misunderstanding, we replace the term “scar” for “epithelialized lesion”. The active lesion samples obtained through biopsy in the first medical visit served as substrate for the accomplishment of histopathological, immunohistochemistry,
PCR and culture tests. We tried to clarify this doubt by adding a sentence in the session of methods [pages 5 (lines 21-23) and 6 (lines 1-2)].

Comment 17:
Please check if Fig 1 was mentioned.

Answer 17: We are grateful to the reviewer for pointing this out. Figure 1 is mentioned on section of methods, page 5, line 20.

Comment 18:
6) Discussion Page 12 - Line 10 - "Therefore we may question the need to propose specific treatment is cases with evidence to ESH".

I think this is a tough point to make. This implies a lag time between diagnosis and onset of treatment and long enough for lesions to show signs of healing. This certainly varies in different clinical settings and certainly involves some kind of risk, just as using antimonials do.

Answer 18: We thank the reviewer for this observation. In fact, in cases of cutaneous leishmaniasis with early spontaneous resolution the process of epithelialization/ healing is fast, coinciding with the time necessary for the establishment of diagnosis (on average 3-4 weeks in the studied cohort). The major risk of the lack of treatment would be the development of mucosal lesions. This possibility is not frequent in Brazil, accounting for less than 5% of the American tegumentary leishmaniasis in our country. We emphasize that the risk of the development of mucosal lesions in our patients is somewhat reduced by the careful follow-up of the patient with an ear-nose-throat specialist up to 5 years. In early spontaneous healing cases, the assistant doctors evaluated that the risk involving treatment with pentavalent antimonials (morbimortality) outweighed the risk of non-treatment complications. We added these statements in the discussion section, page 13 (lines 20-24).
Comment 1: It is, however, required that the authors clarify and explicitly state a definition of how they considered a lesion to be healed.

Answer 1: We appreciate the reviewer's concern and emphasize that the cure parameters are clinical, based on the experience of the specialists at our reference center. We define a lesion as healed when a complete epithelialization (with no crusts) occurs, with total regression of infiltration and of erythema. In the section of methods [pages 5 (lines 21-23) and 6 (lines 1-2)] the inclusion criteria was rewrite: “An ESH case was defined as a patient with a positive parasitological exam for Leishmania spp. and epithelialized lesion(s) without crusts in the period between the laboratory investigation for the disease diagnosis and the expected date for the start of the specific treatment [12]. The morphology of lesions included ulcer/ulceration, papule, nodule or plaque [12]. The lesion should be present in a current context of illness.” We added a reference* that explains the nomenclatures used for lesion morphology.

Comment 2: In the results section (page 8, para 4), time to heal is listed as 35 and 77 days. This conflicts with the date from lower limbs (page 9, para 2), where 237 and 201 days are listed. An explanation for this discrepancy is required.

Answer 2: We thank the reviewer for this observation. Considering all the studied lesions, regardless of their location, it was observed that in the ESH group the average of healing time was 35 days whereas in the non-ESH group (NESH) the healing time was 77 days. Concerning the NESH group, the mean time to cure the lesions in lower limbs was higher than lesions in other locations, as discussed on page 14, second paragraph: “Some authors demonstrated that ACL lesions located in the lower limbs needed more time to heal [41]”. To make it clearer, we added “In the NESH group” in page 9, line 20.

Thank you again for your suggestions and your time,

With kind regards,

Fatima Conceição Silva