



April 14, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 2429-review.doc).

Title: Semen lactoferrin promotes CCL20 production by epithelial cells: involvement in HIV transmission

Author: Alan Grupioni Lourenço, Marilena Chinali Komesu, Alcyone Artioli Machado, Silvana Maria Quintana, Thomas Bourlet, Bruno Pozzetto, Olivier Delézay

Name of Journal: *World Journal of Virology*

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The manuscript has been improved according to the suggestions of reviewers:

1 Format has been updated.

2 Revision has been made according to the suggestions of the reviewer.

REFeree 00053556 (changes according to this referee have been enlightened in yellow in the marked manuscript)

Comments to the Editor:

Thanks for inviting me to review the review article entitled " Semen lactoferrin promotes CCL20 production by epithelial cells: involvement in HIV transmission ". Minor Comment: ? The editing of the manuscript needs minor revision. ? Language level: B. Minor revision is needed.

R: ok

1. TITLE Reflect the major content of the article.

R: ok

2. ABSTRACT fulfill the journal requirements however, the following points are better to be considered: o aim is better to be started with an action verb,

R: the first sentence of the abstract was modified accordingly to the referee's remark (see page 3 of the new manuscript).

3. HIV? / HIV+: when mentioned for the first time are better to be fully written; HIV seronegative (HIV-) / HIV seropositive (HIV+).

R: the change was done (see page 3 of the new manuscript).

4. No need to mention the ethical approval in the abstract section.

R: ok

5. The technique used in measurement of HIV viral load has to be mentioned.

R: the name of the kit was added (see page 3 of the new manuscript).

6. No need to specify the statistical tests used to evaluate results in the abstract section.

R: All the references to statistical tests were withdrawn from the abstract (see pages 3 and 4 of the new manuscript).

7. INTRODUCTION Well written and the systematic searches through the relative databases were well established. However, the last sentence in the last paragraph concerning the aim of the work needs to be revised and clearly written: "It showed that seminal plasma is able to promote the production of CCL20 by HEC-1A cells and that this secretion is correlated to the amount of lactoferrin present in the specimen."

R: The last sentence of the Introduction section has been amended (see page 6 of the new manuscript).

8. MATERIALS AND METHODS: This section is well covered and statistical analyses were appropriate; however, the following points are better to be considered: Seminal plasmas samples "HIV- men were tested for the absence of other infectious agents." Authors have to specify these agents.

R: The tested agents of IST have been listed (see page 7 of the new manuscript).

8. Cell culture: o The concentration of antibiotic-antimycotic solution has to be mentioned.

R: This concentration has been added (see page 7 of the new manuscript).

9. 10% of fetal bovine serum (FBS) is needed for cell growth until confluence, however, for stimulation experiments, much less concentration is needed and this has to be revised.

R: The referee is right. The concentration of FBS used for maintaining the cells was 2%. The change was done (see page 7 of the new manuscript).

10. Using cell density of 200000 cells/well needs revision or justification as the optimum conc. for cultured until confluence is usually of 100000 cells/well.

R: The referee is right again. The right concentration of cells per well was 100000 instead of 200000. The change was done (see page 7 of the new manuscript).

11. Viral load in seminal plasma. The used technique needs more details in order to be reproducible.

R: More details were given in this paragraph (see page 8 of the new manuscript).

12. Measurement of total protein in seminal plasma The Bradford technique lacks its reference.

R: The reference was added (n°11 in the new version). Accordingly, all the further references were renumbered.

13. RESULTS: o Subheadings are well maintained and results were well organized. o The concentration of CCL20 in the supernatants of HEC-1A cells incubated with seminal plasma specimens from 12 HIV- subjects as well as 22 HIV+ subjects were measured, however in results section as well as in figure 1A, the concentration of CCL20 from 12 HIV- subjects only was mentioned, while that of HIV+ subjects were expressed in term of relative CCL20 index only.

R: The referee is right. A homogenisation of the results was performed and all the data were expressed in terms CCL20 index. Figure 1 was modified accordingly: specimens from HIV- and HIV+ patients were presented on the same Figure (new Figure 1) together with the positive control. The abstract (page 3), the Results section (page 10) and the caption of Figure 1 (page 20) were amended accordingly.

15. Furthermore, this index was mentioned as the mean stimulation of CCL20 and this needs revision and correction.

R: The text was changed (see page 10 of the new manuscript).

16 Figure 3: X & Y axes have to be identified.

R: Legends for axes have been added to Figure 3.

17. DISCUSSION: It is well organized and an overall theoretical analysis is incompletely given, where, the following items are better to be considered; o Third paragraph: SIV: simian immunodeficiency virus (SIV) o Incomplete analysis of all results where, correlation between viral load in seminal plasma from HIV+ subjects and its ability to stimulate the production of CCL20 by HEC-1A cells were not well covered in this section, where, the discussion is only about the role of seminal plasma lactoferrin in increasing the production of CCL20 by HEC-1A cells.

R: The abbreviation of SIV has been explicated. Concerning the second point, a whole paragraph and an additional reference (n°24 from 2014) were added to answer to the excellent remark of the referee (see 2nd paragraph of page 14 of the new manuscript).

18. REFERENCES: Finally relevant but insufficient references were cited, especially the most current literatures (3/22 references only were cited from publications \geq 2008). The journal style for writing this section is well maintained.

R: Some of the references were updated (No 4, 8, 13, 14 and 24). However, it is sometimes more relevant to use an original reference than another more recent commenting the first one.

REFEREE 00731613 (changes according to this referee have been enlightened in blue in the marked manuscript)

The authors need to do the following modifications and submit further consideration.

1) Write the abbreviations in expanded form when used for the first time in the manuscript. Further, usage of abbreviations is acceptable.

R: The text was revised accordingly (see pages 5, 6, 8, 13 of the new manuscript).

2) The role of human IL-1beta as a positive control needs to be elaborated for better understanding.

R: Two references (No 7 and 10) were used to validate this choice.

3) Mention the sample size in the methodology sections.

R: This mention was added in the first sentence of the M & M section (page 7 of the new manuscript).

4) Elaborate inclusion and exclusion criteria more clearly.

R: This was done in page 7 of the new manuscript.

5) It is necessary to mention in the manuscript on whether aseptic precautions were followed in collecting semen samples and how were they stored?

R: This was done in page 7 of the new manuscript.

6) I suggest the authors highlight the clinical significance of their study findings. Overall, the study has significant findings and the manuscript is well written.

R: This was done in the last three lines of the Discussion section (see page 14 of the new manuscript).

REFEREE 00202286 (changes according to this referee have been enlightened in green in the marked manuscript)

1. In this paper, the authors suggest that semen lactoferrin promotes CCL20 production by endocervical epithelial cells and propose some relationship with heterosexual HIV-1 transmission. The experiments appear to have been carefully planned and performed, with appropriate controls. The results have been adequately interpreted. The statistical tests have been well chosen. The paper is well written.

R: Thanks a lot.

Abstract The Abstract appears to be a bit long. “HIV?” is not “HIV-“. The authors should decide what term they will further on use in the paper. There are some instances where both terms are used. It should be checked all along the MS.

R: The abstract was shortened and the abbreviation for HIV- was homogenized.

Results section First paragraph: What is the CCL20 concentration obtained in the supernatant of HIV+ seminal plasma treated cells? And if not available, why was it not done or expressed?

R: As already mentioned in the answer to point 13 of the first referee, the first paragraph of the Results section was rewritten together with Figure 1.

Some abbreviations should be spelled out: i.e., LCs, SIV, to increase the general readership. There are few typos. This reviewer corrected some of them. The edited text is attached.

R: LCs had been explicated in the Introduction section. The other abbreviations were carefully checked. Concerning the edited text from the reviewer, I only noted a correction concerning the transitive use of the verb “to correlate”. This was modified all along the manuscript.

REFeree 00503963 (changes according to this referee have been enlightened in magenta in the marked manuscript)

The current article described the seminal plasma/ lactoferrin affects the CCL20 production by HEC-1 cells. It is an interesting and important topic. My comments are as below:

1. Page 7, cell culture, I doubt that if too many/crowded cells at a density of 200000 cells per well in 96-well culture plates.

R: Actually, the right concentration is 100000 (see point 10 of the first referee).

2. Page 8, Measurement of total protein in seminal plasma, “Bradfort” should be “Bradford”, please check which one is correct?

R: Bradford is correct. The text was revised accordingly (see page 9 of the new manuscript).

3. Fig. 1A, why not include seminal plasma specimens from HIV+ subjects?

R: This remark meets those of two previous referees. Figure 1 was modified accordingly together with the Results section and the abstract (see answer to point 13 of the first referee).

4. About the role of lactoferrin, why not fractionate the seminal plasma from HIV+ sample simultaneously?

R: The amount of specimens was not enough to perform this experiment with HIV+ samples. This precision was given in pages 13-14 of the new manuscript.

5. Fig. 3B, why fractions with the higher lactoferrin revealed in discontinuous pattern (fractionations 1, 3-5, 7-9, 10-13)?

R: Possible answers to this question were given in the first paragraph of page 14 of the new manuscript (Discussion section).

6. In Fig. 4, 5 samples revealed a detectable viral load, however, 17 samples with undetectable viral load. How define HIV+ in the current study?

R: “HIV+” means “seropositive for HIV”. This point was recalled in page 11 when Figure 4 is presented.

REFEREE 00504484

Lourenço et al. describe the effect of seminal lactoferrin on the induction of chemokine ligand 20 (CCL20) by the cell line HEC-1A. The results are straight

forward and they do support the conclusion that seminal lactoferrin increases the production of CCL20 in their cultures, but this is not new. The statement that this induction is higher by the semen of HIV+ patients is not clearly supported by their results, even if some marginal statistical difference can be claimed between HIV- and HIV+ seminal plasma. In conclusion, I think that this paper does not add significant new information to the field.

R: This statement is not in agreement with the advice of the other referees. This study is a modest contribution to the understanding of the effect of seminal plasma on the crossing of HIV through the female genital barrier.

Minor points: 1.- When HEC-1A cells are stimulated with seminal plasma, are they still maintained in medium supplemented with 10% fetal calf serum? I think that the stimulatory effect should be more evident in serum-free medium.

R: The referee is right. The correct concentration is 2% fetal bovine serum! (see point 9 of the first referee).

2.- Could the authors provide an explanation to the multiple peaks of lactoferrin observed in the chromatogram of Fig. 3? Please, add at least some comment in the Discussion.

R: See the answer to point 5 of the previous referee.

Fig.3: please indicate which parameter(s) is(are) shown in the ordinates axis.

R: See answer to point 16 of the first referee.

Thank you again for accepting to publish our manuscript in the *World Journal of Virology*.

Sincerely yours,

Bruno Pozzetto,

MD, PhD,

Professor of Medicine,

Director of the GIMAP group,

EA3064, Groupe Immunité des Muqueuses et Agents Pathogènes (GIMAP),
Faculté de Médecine Jacques Lisfranc, Université de Lyon, 42023 Saint-Etienne,
France.

bruno.pozzetto@univ-st-etienne.fr

Telephone: +33-477828434

Fax: +33-477828460