Author's response to reviews

Title: Human papillomavirus is found in peripheral blood CD20+ and CD56+ cells during HPV-16 semen infection.

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Author's response to reviews: see over
Reviewer #1

1. **Title** – insert “proteins” after “papillomavirus”; insert “and semen” after “blood”.
   - Corrections have been made accordingly.

2. **Methods** – Patients - lines 8-10 and 14-15 – please clarify how men were divided into “HPV-DNA positive” and control groups. Did men have to be HPV-DNA positive in semen (or sperm?) by FISH, AND be HPV-16 positive on whole semen to be included in the “HPV-DNA positive” group? Which genito-urinary sites were screened to determine controls were truly HPV-DNA negative? If group selection was determined by HPV16-DNA in sperm and/or HPV16 in non-sperm cells in semen, then p-values should not be applied to the last two columns of Table 1.
   - We apologize for the lack of clarity in this methods paragraph. Indeed, patients were enrolled on the basis of the detection of HPV-16 in genito-urinary tract. More precisely, semen samples from patients were formerly assessed for the presence of HPV-DNA on sperm and/or non-spermatozoal cells by FISH analysis. Successively, patients underwent HPV genotyping, performed by INNO LiPA analysis, on uretral/coronal sulcus brushing specimens and whole semen samples. Positivity to the sole HPV-16 allowed the enrollment as patients. On the other side, control samples resulted negative for any type of HPV in genito-urinary tract as defined above. Corrections have been performed accordingly in the text.

3. **Methods** – FISH for HPV-DNA – were positive controls done?
   - In agreement with Reviewer's criticism, we added positive controls of FISH staining for HPV-DNA, performed in HPV-infected Caski cells, in Supplemental Material (Figure S1A).

4. **Methods** – Immunofluorescence – were positive controls done? Given the emphasis placed in this paper on HPV16 proteins in CD20+ and CD56+ cells, positive controls for all leukocyte markers used should be shown.
   - In agreement with Reviewer's criticism, we added positive controls as Supplemental Material. In particular, control of immunofluorescence staining for HPV-E6/-L1 proteins, was performed on HPV-infected Caski cells (Figure S1A). Controls for the panel of leukocyte markers employed, was performed on isolated peripheral blood mononuclear cells (Figure S1B). The text has been updated accordingly.

5. **Figure 1a I and II** – please show corresponding light microscopy or phase contrast images to accompany the FISH images, to illustrate the morphology of the cells described in lines 3-6 under Results.
   - We agree with the Reviewers to add bright-field images to illustrate the morphology of the cells described in the results session. Bright field image of non-spermatozoal cells have been accordingly added as inserts within the corresponding immunofluorescence pictures in figure 1 aI and aII.

6. **Results** – Analysis of semen round cells – lines 24-27 – are CD4 and/or CD8 T-cells found in semen? Of semen round cells, what proportions are CD4/CD8/CD20/CD56 positive (regardless of HPV positivity)? What percentage of CD20+ or CD56+ semen round cells express HPV16 E6 or L1 in the HPV16-infected group?
   - We agree with the interest of the Reviewer about the phenotype panel of semen leukocyte. Unfortunately, due to the procedures of sample enrichment in semen round cells, we were not able to perform a precise assessment of semen leukocyte sub-populations that actually goes beyond the aims of this study. Nevertheless, in the attempt to answer to the criticism arisen by the Reviewer, about the 80% of semen CD56+/CD20+-leukocytes displayed HPV16 E6 or L1. This datum has been added to the results session accordingly.

7. **Results** – Analysis of peripheral blood leukocytes – what percentage of CD20+ or CD56+ PBMCs express HPV16 E6 or L1 in the 4 men who are PBMC FISH+?
   - Indeed, the percentage of CD20+ or CD56+ cells displaying HPV proteins in patients 6, 8, 10 and 14 were
respectively: 0.7%, 0.3% 1.9% and 0.8%. However, given the very low number of both subjects enrolled and HPV-positive cells within each sample, any statistical statement would be not conclusive. Thus, in order to not affect the clarity of the manuscript and unless expressly required by the Reviewer, we would not add these data to the text.

9. Conclusions – line 25-27 – please reference statement regarding NK and iNKT cells being the main innate cells responsible for clearance of HPV infection (otherwise remove).


10. Conclusions – could the authors expand and further emphasise that the detection of HPV16 proteins in these cells do not necessarily indicate productive infection. Therefore please remove the second last sentence of the abstract conclusion.

- According to the Reviewer’s suggestion, we removed the sentences of the abstract conclusion.

Minor Essential Revisions

1. Table 2 – remove unnecessary abbreviations from the legend.

- In agreement with the Reviewer’s suggestion, we removed unnecessary from the legend.

2. Conclusions – line 17 (second last page) & 22 (last page) – change ‘rise’ to ‘raise’.

- We corrected accordingly

Discretionary Revisions

1. Conclusions – lines 8-11 (last page) – an alternative explanation for these findings is that semen immune cells can become unproductively infected with HPV16 when trafficking through the seminal compartment in HPV16-infected men, and these cells may traffic back into circulation and be detected at low frequency in peripheral blood

- We added the sentence accordingly
Reviewer #2

1. In the background, it should be made clear that few, if any, of the previous studies on HPV DNA in blood have shown HPV inside blood cells; most or all of the studies have merely shown the present of HPV DNA in blood, or on the outside surface of cells.
- In agreement with the Reviewer’s concern, we added the statement “However, the vast majority of these studies did not show HPV inside blood cells. Most or all of the studies merely focused on the expression of HPV DNA in blood, or on the outside surface of cells” in the background session.

2. In the absence of evidence of HPV DNA in the nucleus, or mRNA expression, the presence of infection cannot be proven. Please adjust the descriptions of your findings to say "detection", "positivity" etc as appropriate, rather than infection unless there is enough evidence to prove infection.
- We agree with the Reviewer’s point of view. Accordingly, dealing with our findings in semen and peripheral blood leukocytes, we changed “infection” with more cautious terms such as “detection” or “positivity”.

3. Results Analysis of semen round cells Line 23: Please state whether these cells are CD45 positive or negative.
Remove the phrase "not myeloid mononuclear leukocytes (17)."
- We agree with the Reviewer’s concern and, as also suggested by the previous Referee, we corrected the sentence accordingly.

4. Background Line 29: It should be made clear that the metastatic cells referenced are not blood cells, as is inferred from previous sentences.
- We apologize for the lack of clarity. We actually meant that in previous studies, authors concluded that HPV detected in blood cells of cancer patients was not, or not-only, subtended by metastatic cells released at a later stage of disease but was also associated to blood mononuclear cells. We corrected the background session accordingly.

5. Results page 8 Analysis of peripheral blood leukocytes: The absence of HPV DNA in the nucleus of these cells would indicate that they are not infected; please replace the word infected in lines 1 and 5.
- In agreement with the Reviewer’s concern, we corrected accordingly.

6. Conclusions Page 9 Line 9: The authors have not proven that the CD56+ cells are NK cells, as a number of different immune cells express CD56. Either provide more evidence of the identity of these cells or amend the statement.
- Indeed, other CD56+ populations than natural killer-cells are variably represented among immune cells [Hu D, et al. Identification of cytolytic CD161- CD56+ regulatory CD8 T cells in human peripheral blood. PLoS One. 2013;8(3):e59545. doi: 10.1371/journal.pone.0059545]. Unfortunately, given the large paucity of HPV-positive leukocytes found in semen and peripheral blood, we were unable to better characterize this cell population. Thus, in agreement with the Reviewer’s concern, we amended HPV-DNA+/CD56+ cells into “Natural killer-like cells”

7. Conclusions Page 9 Line 18 and 24: Reference 29 is used inappropriately as there is no mention of HPV in the cited article. Please amend to the correct reference or omit.
- In agreement with the Reviewer’s concern, we corrected the reference accordingly.

8. Conclusions Page 9 Line 25: Replace “understood to be the primary HPV receptor” with “theorised to potentially be the primary HPV receptor” and cite appropriate reference(s). A secondary receptor is thought to also be necessary for HPV infection; the authors should comment on this in the context of their results.

9. Conclusions Page 9 Line 26: Heparan-sulfate proteoglycans are expressed by many cell types and the attachment of HPV to HSG’s is non-specific. Therefore this statement does not lend weight to the authors' argument and is misleading. Please amend.
- In agreement with the Reviewer’s suggestion, we amended the two sessions and added a specific comment on α6-integrin or annexin A2 heterotetramer as putative secondary receptor involved in HPV entry.
10. What were the specific CD receptor expression patterns of HPV DNA or HPV protein-positive cells? CD4+/-, CD8+/-, CD20+/-, CD45+/-, CD56+/-? Please make clear so that the reader can decide for themselves which cell types these were.
- We apologize for the lack of clarity. As suggested by the Reviewer we modified the result session in order to be more didactic without emphasize the reader’s conclusion.

Minor Essential Revisions
11. Background Line 6: HPV has been associated with most male *anal* cancers.
- We corrected accordingly

12. Background Line 9: replace "sperm parameters" with "sperm motility"
- We corrected accordingly

- We corrected accordingly.

14. Background Line 24-25: The study in reference 12 used Bovine Papillomavirus, therefore on Line 24 use of "HPV-DNA" is not appropriate and should instead say "papillomavirus DNA".
- We corrected accordingly

15. Results Analysis of semen round cells Line 10-11: Please state cell counts as x 10^3 rather than x 10^6.
- We apologize for the misprint. The table has been corrected accordingly.

16. Results Analysis of semen round cells Line 26: Cell surface markers should be described as being found "on" x% of cells rather than "in" x% of cells; please adjust elsewhere as necessary.
- We corrected accordingly
Reviewer #3

In this context, a recent paper on detection of BPV DNA and transcripts in blood cells a few days after experimental inoculation of horses with BPV1 may be mentioned in the discussion (Hartl et al., 2012; JGV). Roperto et al. also identified subsets of bovine blood cells that are permissive for BPV infection, i.e. T and B lymphocytes. Please compare these findings to yours and discuss this.

- We thank the Reviewer for the positive comments and for the useful suggestions. On this regard, in order to maintain the overall clarity of the manuscript, the reference dealing with experimental infection of BPV in animal model [Hartl B, 2011], together with other existing data on this field [Roperto et al, 2011], has been added and commented in the background session.

Regarding MHC I downregulation: E5 is equally involved in it - please change the respective sentence accordingly and add the appropriate reference (you may cite e.g. Ashrafi GH, Brown DR, Fife KH, Campo MS. Virus Res. 2006 Sep;120(1-2):208-11.)

- We changed the sentence and added the reference accordingly