Author's response to reviews

Title: Determinants of Baseline Seroreactivity to Human Papillomavirus Type 16 in the Ludwig-McGill Cohort Study

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The Editor
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Ref: MS submission

Dear Madam or Sir,

I enclose herewith a revised version of an original manuscript entitled “Determinants of Seropositivity to Human Papillomavirus Type 16 in the Ludwig-McGill Cohort Study” by de Araujo-Souza, Ramanakumar, Candeias, Thomann, Trevisan, Franco and myself. Regarding the comments and questions addressed by the referees, I provide a list of answers below. We duly revised the manuscript in response to each and every comment. The revised text is marked in yellow highlight.

Sincerely yours,

[Signature]

Luisa Lina Villa, PhD
Responses to Dr Safaeian’s comments:

1) Abstract line 5 where they mention that natural HPV infection does not ‘necessarily’ result in protective antibody response. The appropriate word may be ‘always’ – there are many factors for example, the assay used to measure antibody levels, and their limit of detection.

Answer: We have edited the sentence, as suggested by the reviewer.

2) Abstract line 12 ‘diagnosed’ as the choice of word is not accurate. The serology test is not a diagnostic test.

Answer: We agree with reviewer. We have reworded accordingly.

3) Introduction, page 4, line 5, majority of sexuality active individuals are likely to be exposed to HPV ‘throughout’ life. It is at some point in their life, not necessarily throughout life.

Answer: We agree and have altered the sentence as suggested.

4) HPV serology: this paper is about correlates of HPV16 seropositivity. The authors need to describe what QC was included in the testing, and at the least report assay CV and ICC. The description of the cutoff was confusing, please clarify.

Answer: Details on the serological methods, choices of expressing seroreactivity, quality checks, and procedure to minimize errors were explained in a previous paper by our team (Ramanakumar AV et al., 2010), which was cited in the text and listed in the reference section. Since we conducted an extensive analysis and fully discussed QC characteristics of the ELISA assay in our previous paper, and owing to the word count limitation, we preferred not to repeat this material in the current paper. The focus of the present paper was not to validate a new assay; it was simply an application of a standard VLP-ELISA test; just the cutoff choice to discriminate between high (the top 20% of all subjects in terms of seroreactivity) from less than high (all other subjects) represented a new approach. We explained the cutpoint for defining this dichotomy for seroreactivity in the text and also as footnotes to the pertinent tables. In the absence of a clear definition of what is threshold of net absorbance values in ELISA that indicates presence of a humoral immune response against HPV16 we used this arbitrary definition, which is just as defensible as choosing the median seroreactivity to discriminate between low and high responders. We were careful not to use the terms ‘seropositive’ or ‘seronegative’ with this strategy; having preferred the more technically correct term ‘seroreactivity’. We revised the narrative in several places to reflect the latter point.

5) Statistical analysis: while one can deduce that the associations evaluated were all from the enrollment DNA and serology data, it would be helpful to clearly state whether the DNA and serology results and the factors evaluated were all from the
same enrollment  This is even more unclear when in the results they talk about numbers at V0 and follow-up visit.

Answer: We thank Dr Safaeian for prompting us to clarify the description of this methodological issue. We revised the text accordingly. HPV DNA and serology results at baseline are the focus of our study. In the interest of statistical precision for our analysis, we used the serological results from the second visit at 4 months if the women had missing serological results for the enrolment visit. The numbers in each visit are clearly indicated in the text now. Incidentally, a sensitivity analysis restricting the case series to only baseline serology/DNA (1745 cases, instead of 2049) produced similar results.

6) Results (page 7, line 26): please let the reader know # of missing samples that results were imputed for?

Answer: There were 1745 and 1656 subjects with valid ELISA results at visits 1 and 2, respectively. In all, there were 2049 women with serological results at either or both of these visit; 304 women had a serological result in the second but not in the first visit. These were the imputed cases. We have added this information to the text, as suggested.

7) Part of the 1st paragraph in the results could go in the methods section.

Answer: As suggested, we have moved the initial part of the paragraph to the methods section.

8) Comparisons to the Costa Rica trial – the relevant manuscript for comparison to this one is Seroprevalence and correlates of human papillomavirus 16/18 seropositivity among young women in Costa Rica, Coseo S et al, Sex Transm Dis. 2010 Nov;37(11):706-14.

Answer: We have corrected the reference in the discussion section and in the list at the end.

9) The discussion lacks acknowledgement of study limitations and strengths, and conclusions

Answer: We have expanded the discussion in several places to indicate the study limitations and underscore some of the features.
RESPONSES TO DR. SEPEHR TABRIZI COMMENTS:

Major compulsory Revision:

1) Title- Since this study has only included analysis of baseline seropositivity, it may be appropriate to change the title to: Determinants of baseline seropositivity…..

Answer: As suggested by Dr Tabrizi, we have changed the manuscript title.

2) Methods, serology- The cohort had multiple samples collected and it needs to be clarified in the methods that only first serum sample were utilized for the analysis in this study.

Answer: As indicated also in the response to Dr Safaeian’s comments, we have thoroughly revised this part of the methods section. We thank Dr Tabrizi also for pointed this out.

3) Source of VLP would need to be stated in the method. Similarly the detection of HPV on a single visit, i.e. baseline, would need to be stated more clearly.

We have used VLPs from baculovirus system, as it was previously described in lines 18-19, page 5 (HPV Serology section of Methods). However, in this revised version we have included a more detailed statement about preparation of VLPs. Regarding the information of HPV detection considered only at enrolment in this analysis, we have also included the information in the end of HPV DNA detection and typing section of Methods.

4) It is not clear which primers were used for PCR, i.e. MY09/11 or PGMY09/11. Were the samples analyzed for DNA dedicated for this test or were they taken in Preserv Cyt and then aliquot taken for DNA detection. In case of same sample being used for both cytology and DNA detection, need to state if an aliquot was taken prior to preparation of slides for cytology.

Answer: We used both PCRs in the cohort, for first few visits we used MY09/11 and later changed to other PCR protocol. We amended the text accordingly. Cervical sampling was done via an Accelon sampler (for conventional cytology, not liquid-based) as single collection for cytology and HPV DNA testing. In light of the above edits to clarify specimen collection, choice of specimen visits, and HPV DNA we believe that this point is now clear.

5) Viral load method- was GP5+/6+ used or GP5/6? How were cell numbers determined as results indicate HPV copy per cell? Last sentence of this method would need to be clarified
Answer: Viral load estimation was performed via a low stringency PCR reaction with GP5/6 primers (not GP5+/6+), which allowed the amplification of a series of host DNA fragments in addition to HPV L1 fragment. A host genome product was selected as an internal control and a ratio between HPV and control band was measured by densitometry and quantified (in copies per cell) by linear interpolation using a standard curve with known amounts of copies per cell, assuming a diploid genome. This standard was prepared with pre-selected amounts of a reference plasmid containing the entire HPV16 genome against a constant background of human DNA extracted from human breast tissue. All samples, controls and standards were analyzed in duplicate and viral load derived from the mean values. This method is described in detail in the two references we provided (Trevisan et al., 2013; Schlecht et al., 2003). Readers can find abundant detail in these two papers, one of which included information on validation of this method (Trevisan et al., 2013). We edited this section a little to provide more clarity regarding how we can derive the number of copies per cell.

6) Since only a single serum sample was analyzed for this manuscript, could the titre be correlated further to see how low and medium reactivity is correlated?

Answer: It was our intent to identify correlates of strong seroreactivity for women as they were enrolled into our cohort study; the definition of which is now clearly indicated in the Methods section. We intend to use more quantitative measures of serological response in a subsequent manuscript where we will be examining not only IgG but also IgM responses to HPV16.

7) Since information is potentially available for HPV genotype from all the samples collected, it would be important to look at persistent vs transient infection and how this is correlated.

Answer: As indicated above, we plan a future manuscript on repeated measurements of both HPV DNA and serology using more quantitative (titers) and qualitative (immunoglobulin type) expressions of serological response. These are outside of the scope of the present manuscript, which focuses on determinants of baseline seroreactivity.

8) Overall in the discussion, would need to state some of the challenges of interpretation of use of single serum and cervical sample in such analysis

We have added this information in the discussion.

Minor Essential Revisions

9) In paragraph for HPV detection method- “cell samples were submitted…” would need to be changed to “cells were extracted for DNA and DNA quality…”

Answer: Revised as indicated, keeping the context.
10) ref 15 in the text would need to be fixed.
Answer: We have corrected the reference in the text.

11) Viral load in Table 4 require clarification in the table headings.
Answer: We have corrected the Table 4 headings.