Author's response to reviews

Title: Prevalence and distribution of high-risk Human papilloma virus (HPV) types in invasive squamous cell carcinoma of the cervix and in normal women in Andhra Pradesh, India

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AUTHORS RESPONSE TO REVIEWS:

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TITLE: Prevalence and distribution of high-risk Human papilloma virus (HPV) types in invasive squamous cell carcinoma of the cervix and in normal women in Andhra Pradesh,

Answers:

All the queries raised by the three reviewers have been answered point-wise and the suggested changes made in the manuscript are noted in the parenthesis.

Reviewer 1: Gary M Clifford:

1) The sentence “It is interesting to note that HPV73…..” doesn’t really belong in the results. This is further confused by the fact that HPV 73 is reported as MM9 (in the table).

Ans: This sentence has now been deleted and in the table MM9 is replaced by HPV73. Corrections made on page 9, line 9 and in Table 1.

2) The prevalence of HPV found in this Indian community was not discussed in comparison with other cited studies among rural Indian women. Is the prevalence of high-risk HPV similar in these different populations? Is the age specific pattern similar?

Ans: Our HPV prevalence (10.4%) is very similar to two large population-based studies of largely cytologically normal women. Sankarnarayanan, et al. reported 10.3% high risk HPV prevalence as detected using hc2 testing in Osmanabad District in West India [15], and somewhat lower prevalence estimates by hc2 in a separate multicentric study in Mumbai (6.3%), Trivandrum (4.8%), and two cities in Kolkata (7.8 and 5.2%, respectively) [24]. Franceschi et al [14] report a similar high risk prevalence from Dindigul District in South India (9.6%) using consensus primer PCR methods. None of these studies, including ours, found an association of HPV prevalence with age. Our study and that of Sankarnarayanan restricted enrollment to women over age 30 years, which may represent the age-associated plateau found in other reports [25]. However the study from Dindigul District sampled a large number of women under 25 years and found no increase in HPV prevalence among the younger women. The lack of an age association with HPV prevalence in India is yet unexplained. The same has now been included in the discussion (Page 13, line 18)
3) Also are the authors able to estimate the participation rate of women from the community? This would help the understanding of the studies representativeness.

**Ans:** Of the 489 women who were eligible, 190 (38.9%) consented to participate and were enrolled. Participation rates were significantly higher among women age 30 – 50 years (57%) relative to women over 50 years (12%). This has now been included in the Methods and Results section (Page 6, line 8 and, Page 9, line 12).

4) Declaration of Competing Interest:
   Included in the text (page 7, line 18) and also stated separately (page 16, line 17).

**Reviewer 2: Elizabeth Unger**

1. The results include a lot of points that are more appropriate for discussion. The discussion and introduction overlap a little. Both the discussion and introduction should be more focused on this study and shortened.

   **Ans:** As suggested by the reviewer the Introduction has been shortened and the description given in the results have been omitted and explained in the discussion portion. The discussion has been modified to avoid the overlap.

2. Unless the hc2 method differs from the manufacturer’s protocol, the details should not be provided. Similarly the details of the line blot assay are described in other papers, so only changes should be noted after referencing method.

   **Ans:** The details have been omitted for the hc2 and line blot assay, however the procedural details on DNA extraction methodology has been given as it was asked by Reviewer #3.

3. Figure 1 is not necessary.

   **Ans:** This has now been deleted

4. MM9 in Table 1 should be replaced with HPV 73 as used in text. In Table 3, the Patient ID column should be omitted. The RLU values should rounded to one decimal point.

   **Ans:** MM9 has been replaced by HPV 73 in the table and also in the text (Page 9, line 9). Patient column ID has been omitted and the RLU values have been rounded to one decimal in the table.
5. The testing strategy needs to be clarified. In the text it is stated that PCR was performed only if hc2 was positive, however from Table 3 it appears that PCR was done for both physician collected and self-collected samples in participants with positive hc2 result in either sample.

**Ans:** HPV genotyping was done in women who tested hc2 positive in either method of collection. It has now been clarified in the text (Page 7, line 16 and Page 9, line 19).

6. In methods it is stated that Digene sampling brush was placed in Digene STM, and then sample aliquotted. How were cell dislodged from brush, and was brush removed? The usual hc2 protocol calls for denaturation of samples prior to removing aliquots.

The STM tubes are vortexed to dislodge the cellular sample, and then aliquoted out without removing the brush (page 6, line 15). The aliquots were removed prior to denaturation so that the remaining STM could be used for DNA extraction for future PCR analyses. We have experience to suggest that denatured STM is unstable for use in PCR after even short term storage (P Gravitt, personal communication).

7. What method was used for extraction of tissue DNA? How was sample disrupted, how much was extracted and what was final volume of extract. What volume was put into the PCR?

The cervical punch biopsy (approximately 50mg) was pulverized using liquid nitrogen and suspended in 1000µl of digestion buffer (100mM NaCl, 10mM Tris-Cl pH 8, 25mM EDTA, 0.5% SDS and 0.2mg/ml proteinase K) and extracted using standard phenol:chloroform method [Ref 16] and resuspended in 100µl of TE. 5µl of the DNA was then used to carry out PCR in a final volume 95µl. These details have now been provided in the methodology section under the HPV-DNA testing section (Page 7).

8. The method states that 70 microliters of each STM was extracted, but does not state the final volume of extract. The 10 microliters of extract was put into what volume PCR?

70µl of the STM sample was digested at 65°C in digestion buffer (20mM Tris-HCl,1mM EDTA pH 8.5) containing 200 µg/ml Proteinase K and 0.1% of Tween 20 for 1 hour. Following heat inactivation of the Proteinase K (95°C for 10 minutes), the DNA sample is precipitated with ethanol and ammonium acetate. The precipitated DNA is suspended in a final volume of 35µl TE buffer. For PCR 5µl of the DNA sample is amplified using a cocktail of the biotinylated PGMY 09/11 and beta globin primers in a final volume of 95µl (Page 7, HPV-DNA testing by the Roche PCR Line blot assay in the methods section).
9. The authors attribute hc2 positive/PCR negative samples to low “viral titers” in the sample. The term titer should probably be avoided in this context; viral load is preferable. The authors conclude that viral load is low because the RLU values are low. It is equally possible that the low RLU are because of non-specific background problems and false positive hc2 results cannot be excluded. The authors should modify their interpretation of these discrepant test results.

“Viral titre” has now been replaced with “viral load” (page 14, line 8). Discrepancy in HC2 positive samples with low RLU/Co which were negative on PCR could be due to (a) very low viral load, or (b) indicative of a false positive result. The suggested modification has now been made in the discussion (page 14, line 10)

Reviewer 3: Elizabeth Maloney

1. METHODS. Collection of Specimens. The authors should specify if tissue biopsy specimens were collected from all women undergoing surgery at the regional MNJ Cancer Hospital, or if some women refused to participate or had inadequate specimens for testing. Along those same lines, do the 42 histopathologically proven cases of squamous cell carcinomas represent all identified cases, or a subset of all such cases who agreed to participate.

Ans: Women were consecutively recruited at the time of their visit to the cancer clinic at the MNJ Cancer hospital. A total of 45 tissue biopsy specimens were collected between 2002-2003 from women attending the cancer. Following the cervical punch biopsy a small piece of tissue was sent for histopathology and the rest of the specimen was snap frozen in liquid nitrogen and stored at -70°C. Out of the total 45 women who had given consent, DNA was extracted from forty-two histopathologically proven cases of squamous cell carcinomas. For three of the cases the histopathology was not available. This has now been revised in the methods section (page 5, under the subheading of collection of cancer specimens).

2. METHODS. Collection of Specimens. The authors refer the readers to a submitted manuscript for more details on the community-based cervical cancer screening pilot study. The authors should provide adequate detail about study design and subject selection in the community study. How were study participants selected? What was the participation rate among the eligible target population? Over what period of time was this study conducted?
**Ans:** The details on the community screening study have been described in the methodology section (Page 6, line 4). For the CATCH pilot study women who were in the age group of 30 years and above were enrolled from a single village of Pudur during the period July – Oct 2003. Consent was taken from women who had not undergone hysterectomy and were not pregnant. A total of 657 age-eligible women were recruited to participate. Of the 489 women who were eligible, 190 (38.9%) consented to participate and were enrolled. Women aged 30-45 were twice as likely to participate (60.4%) relative to the next age group (45-55; 29.8% enrolled). Of the 190 women, HPV analysis was done for 185 women.

3. **METHODS.** The authors omitted a section for Statistical Methods. Acknowledging that this paper is predominantly a descriptive study, a kappa statistic was used and it should be described in the methods section. Additionally, the authors provide a table of HPV DNA prevalence by age group. It would be of interest to conduct a statistical test to see if prevalence varies significantly by age, since the authors state that it does not but do not include a p-value to support that statement. Authors should state what software was used to compute frequencies and kappa statistic.

**Ans:** The details of the statistical method used are as follows. Agreement between self- and clinician-collected samples was calculated using kappa statistics to provide estimates beyond chance agreement. To test for differences in HPV prevalence by age, we calculated Pearson’s chi-square. All frequencies, kappa estimates, and chi-square tests were computed using Stata/SE version 9.0 (Stata Corp., College Station, TX). The same has now been included under Methods section (page 8, line 10).

**RESULTS.** The prevalence of major high-risk HPV types detected in the cervical cancers are not consistently reported in the Abstract, Results and Tables. The biggest discrepancy is between the Abstract and the Results. The Tables should report prevalence carried out to one decimal point as reported elsewhere in the manuscript.

**Ans:** The discrepancy appearing in the abstract has now been corrected (page 2, line 21). However it has to be noted that while the table gives a break up of single and multiple infections, the values in the Result-section are indicative of the overall prevalence (combining the single and multiple infection) there by making it appear a discrepant. The RLUs have been given to one decimal point in the Table 3.

**RESULTS.** The authors should describe the demographic distributions of women participants in the community sample within the text of the Results section. Were these women of similar socioeconomic status as the women participants in the cervical cancer study?
Ans: The women participants in the cervical cancer study were mostly from low-socioeconomic status (1000-1500 Rs where mostly men are earning and most of these women are housewives), based on general estimates from the cancer hospital. However, specific details regarding SES factors were not collected from individual women participating in this study (Page 8, line 21). The median household income reported by the women in the community study was 1500 Rs, which is similar to the general characteristics of the population served at the regional cancer hospital. This too has been included now in the results.

CONSENT: A separate section has been included in the Methodology Section (Page 6, line 18)