

## **Author's response to reviews**

**Title:** Human papillomavirus in semen and the risk for male infertility : a systematic review and meta-analysis

### **Authors:**

Zhangyan Lyu (lvzy2016@126.com)

Xiaoshuang Feng (fengxs2016@163.com)

Ni Li (nli@cicams.ac.cn)

Wei Zhao (zhaowei0714@nicemice.cn)

Luopei Wei (weiluopeiwl@163.com)

Yuheng Chen (yhchen@cicams.ac.cn)

Wenjing Yang (wenjing86824@126.com)

Hongxia Ma (hongxiama@njmu.edu.cn)

Bing Yao (yaobing@nju.edu.cn)

Kai Zhang (kai87968@sina.com)

Zhibin Hu (zhibin\_hu@njmu.edu.cn)

Hongbing Shen (hbshen@njmu.edu.cn)

Dong Hang (hangdong@njmu.edu.cn)

Min Dai (daimin2002@hotmail.com)

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### **Author's response to reviews:**

Dear Editor,

Thank you very much for your decision letter. We deeply appreciate the reviewers' valuable comments. The manuscript has been revised accordingly, with our point-by-point responses attached below. We thank you again and hope that the revised version will meet with approval.

Yours sincerely,

Dong Hang, Ph.D.

Comments from Martha Davila-Rodriguez (Reviewer 1):

It is a well-directed manuscript, conclusions with a good contribution to the topic.

1. It is necessary:

- to mention characteristics considered to define an infertile man.

Response: Thanks for the reviewer's valuable advice. We have added the definition of male infertility in the method section.

“Male infertility is defined based on the following criteria: at least 1 year of unprotected sexual intercourse without conception, and/or abnormal semen analysis referring to the standards in the guidelines of the WHO Laboratory Manual for the Examination and Processing of Human Semen (5th edition) [12], i.e. volume (ml) 1.5 (1.4-1.7), sperm number (10<sup>6</sup> per ejaculate) 39 (33-46), sperm concentration (10<sup>6</sup>/ml) 15 (12-16), total motility (PR + NR) 40 (38-42), progressive motility (PR, %) 32 (31-34), vitality (live spermatozoa, %) 58 (55-63), morphology (normal forms, %) 4 (3.0 -4.0), and so on.” (Line 127 - 134)

2. It is necessary:

- to discuss the results of reference No. 49 with those obtained in Cortés-Gutiérrez EI, et al. The presence of human papillomavirus in semen does not affect the integrity of sperm DNA. *Andrologia* 2017.

Response: We have added the discussion on the reference 49, according to the reviewer's comment.

“Finally, HPV might affect the integrity of sperm DNA. Connelly et al. reported that sperm cells transfected with exogenous HPV E6/E7 DNA had higher percentages of breakages characteristic of apoptosis compared to the uninfected controls [53]. However, in vivo evidences by Cortes et al. failed to observe the increased DNA fragmentation in semen containing HPV [54]. Further studies with large sample size and rigorous design are necessary to confirm whether HPV-positive spermatozoa are more susceptible to DNA damage.” (Line 329 - 335)

Comments from Christophe Depuydt (Reviewer 2):

1. A similar systematic review and meta-analysis has been done before (PMID: 24365799), the incidence and clearance of genital human papillomavirus infection in men has been described in 2011 (PMID: 21367446). Selecting slightly different studies resulted in similar results.

Response: Thanks for the reviewer’s valuable comment.

Laprise et al. (PMID: 24365799) identified 27 studies published by June 2013, and reported HPV DNA prevalence in 4029 semen samples varying from 0 to 100%. The pooled prevalence of 7 studies focusing on populations seeking fertility evaluation/treatment (not all infertile) was 16%, compared to 10% in general populations.

Giuliano et al. (PMID: 21367446) analyzed the type-specific HPV infection incidence and clearance of coronal groove, glans, axis and scrotum, but did not include the HPV infection rate of sperm.

In our study, a total of 31 studies published by December 2016 were included, providing a renewed overview on HPV virion prevalence in 5194 semen samples. In addition, we analyzed the type-specific HPV virion prevalence of 24 HPV genotypes in semen. By pooling the results of case-control studies which included infertile male patients as the case group and confirmed fertile males as the control group, we further explored the strength of association between HPV virions and male infertility. We believe that this is an in-depth and expanded study compared with previous studies.

2. HPV is an organism with 2 phases, the virion and the infected cell. Although it is easy to understand that viruses are organisms that pass in their ontogenetic cycle through two distinct phenotypic phases, the dissociation between viruses and virions or infection and disease remains difficult for clinicians and HPV detection (PMID: 28210481). A good example of confusing virus and virion is HPV DNA measurement. Both the infectious HPV virions (L1/L2 protein capsid with inside circular HPV DNA) as the infected cells (both dividing and non-dividing cells) contain HPV DNA. Infectious HPV virions can only be assembled in non-dividing cells. Since sperm and seminal plasma does normally not contain dividing cells the viral HPV DNA measured originates from HPV virions (infectious = never cancer). HPV virions can cause temporarily subfertility through binding the equatorial region of the sperm head and directly affect the spermatozoon's quality, but spermatozoa can also transfer infectious HPV virions into the oocyte interfering with cell division. Women with cervical cancer caused by HPV, have the HPV DNA located in the nucleus of the (clonal) dividing cells. This HPV DNA can therefore not be transferred to spermatozoa or oocytes and these women can become pregnant.

Lines 85-89: the 2 HPV induced pathways seems to have been mixed up.

Response: Thanks for the reviewer's insightful comments. We agree that HPV is an organism with two phases including the virion and the infected cell. As the reviewer said, HPV virions can induce two different pathways, namely the infectious virion producing pathway and the clonal transforming pathway. Indeed, the clonal transforming that causes cancers has been well acknowledged, whereas HPV virion induced health consequences such as idiopathic subfertility is greatly underestimated. We have revised the statements in the background section accordingly.

“In fact, HPV is an organism with two phases, the virion and the infected cell. HPV virions can induce the infectious virion producing pathway and the clonal transforming pathway, and infectious virions are only assembled in non-dividing cells [6]. Although the clonal transforming that causes cancers has been well acknowledged, HPV virion related health consequences are greatly underestimated.” (Line 87 - 92)

3. Lines 89-91: HPV is not a good terminology and is confusing. The virions can go wherever the Brownian movement brings them. The virions can normally only infect dividing cells, which is needed to start their lifecycle.

Response: We have revised the sentence and related descriptions accordingly.

“Notably, HPV virions can lie not only in the perianal region and external genitalia, including the penis foreskin, scrotum and glans penis, but also in the urethra, ductus deferens, epididymis, and testis [7].” (Line 92 - 94)

4. Line 92-93: HPV virions? This is also not the correct reference, different groups showed the presence of HPV virions on spermatozoa in 2011. The reference of Luttmer you refer to is not the correct one. Besides the study by Luttmer et al. is one of the studies without control group (bias) which as a consequence fail to show an effect of HPV presence in semen and impairment of semen quality.

Response: We have revised the description and reference (PMID: 21479232).

“Biological evidences indicate that HPV virions could localize at the equatorial region of sperm and affect sperm quality, thereby increasing the risk of male infertility [8].” (Line 95 - 97)

5. Line 94-100: the correct reference is (PMID: 24365799), and the inconsistency is due to the fact that HPV DNA measurement was confused/allocated to the HPV virus instead as to the HPV virions (PMID: 28210481).

Response: Thanks for the advice. We have added the reference (PMID: 24365799) in the background section.

“In addition, a previous systematic review on HPV prevalence in semen included 27 studies reporting HPV prevalence varying from 0 to 100% [9]. According to the meta-analysis of seven studies focusing on populations seeking fertility evaluation or treatment, the pooled prevalence was 16% (95%CI: 10-23%) [9]. However, the worldwide distribution of HPV types in semen has

not been systematically studied, and epidemiological evidence on the association between seminal HPV infection and infertility is inconsistent.” (Line 97 - 103)

6. Lines 120-126: It is incorrect to assume that healthy male volunteers are fertile men. It is also incorrect to assume that men seeking a fertility evaluation are infertile. It is also unfortunate that men who were diagnosed as fertile or infertile were excluded.

Response: Thanks for the comment.

By this meta-analysis, we provided estimates of HPV virion prevalence of semen in general population, including confirmed fertile men and healthy male volunteers. Meanwhile, we estimated HPV virion prevalence of semen in fertility clinic attendees, including confirmed infertile men and those who sought fertility evaluation or ART. We believe that these two groups could represent low-risk and high-risk infertility populations, respectively.

We acknowledge that, due to the incomplete data of published articles, we couldn't exclude infertile men from general population and exclude fertile people from the fertility clinic attendees, which may lead to potential selection bias. However, the pooled overall and type-specific HPV virion prevalence in semen were calculated separately in these two groups. When it comes to the calculation of ORs with 95% CIs, only case-control studies including infertile males as cases and confirmed fertile males as controls were utilized for meta-analysis, and four studies were eligible.

We have added the following statements in the method section.

“We believe that these two groups could represent low-risk and high-risk infertility populations, respectively. When evaluating the strength of association between HPV positivity and male infertile, only case-control studies including infertile males as cases and confirmed fertile males as controls were utilized for meta-analysis.” (Line 140 - 144)

7. Low and high risk relates to the risk of developing cancer. It is not the HPV induced transformation of clonal cell populations that affect fertility, that's the other pathway. Probably all HPV types, low, intermediate and high risk can bind the head of the spermatozoa and interfere with fertility.

Response: We agree that many HPV types can bind the spermatozoa's head and impair fertility. However, HPV infection might also exert its detrimental effects in other ways. For example, HPV-infected spermatozoa can transmit viral DNA to oocytes, which may be expressed in the developing blastocyst (PMID: 26609434). It is possible that gene expression of different HPV types may result in differential risk of miscarriage, by affecting trophoblastic apoptosis and endometrial implantation of trophoblastic cells in different degrees. In addition, HPV16/18 may cause spontaneous abortions before the 6th week of pregnancy due to their more oncogenic potential compared to other HPV types (PMID: 28210481). Thus, an HPV type specific effect on infertility cannot be ruled out. Here we explored the type distribution of HPV virions in semen, including high and low risk type classification, in order to lay the groundwork for elucidating the potential different effects of HPV types on infertility.

8. Lines 165-167: Men with infectious HPV virions.

Response: Thanks for the advice. We have revised the sentence.

“Finally, 738 men with infectious HPV virions among 5194 males from 16 countries were pooled into the meta-analysis.” (Line 183 - 185)

9. Lines 168-170: HPV virions (transient HPV infections) are limited in time. Fathering a child and HPV testing afterwards introduces a bias. Confirmed fertile should mean that the sperm/semen that was used to conceive was tested for HPV. Seeking fertility evaluation doesn't make someone infertile, and not all healthy male volunteers are fertile.

Response: We understand the reviewer's concern. As mentioned above, we provided estimates of HPV virion prevalence of semen in general population, including confirmed fertile men and healthy male volunteers, which could represent a low-risk infertility population. Meanwhile, we estimated HPV virion prevalence of semen in fertility clinic attendees, including confirmed infertile men and those seeking fertility evaluation or assisted reproductive techniques (ART), which could represent a high-risk infertility population. Assuming HPV virions act as a risk factor for infertility, we acknowledge that the analysis of our low-risk infertility population might overestimate HPV virion prevalence for true fertile males, and the high-risk infertility population might underestimate the prevalence for true infertile males, introducing a bias toward null assumption. However, we still found that the pooled prevalence in the high-risk population (20.4%, 95% CI = 16.2-24.6%) was higher than that in the low-risk population (11.4%, 95% CI = 7.8-15.0%) ( $P < 0.001$ ), supporting the potential role of HPV in infertility. In addition, to avoid such selection bias, the strength of association between HPV virions and male infertility was analyzed by pooling four case-control studies including confirmed infertile and fertile males.

We have added the discussion in the limitation section.

“Secondly, it is possible that the analysis of our low-risk infertility population overestimated HPV virion prevalence for fertile males, and the high-risk infertility population underestimated the prevalence for infertile males, introducing a bias toward null assumption. However, we still found that the pooled prevalence in the high-risk population was significantly higher than that in the low-risk population, supporting the role of HPV virions in male infertility.” (Line 339 - 345)

10. Lines 202-207: different sperm fractions have different HPV prevalence, it is not clear what is meant by in spermatozoa?

Response: Thanks for the reviewer's comment. According to the guidelines of the WHO Laboratory Manual for the Examination and Processing of Human Semen (5th edition), when the number of spermatozoa per high-power field (HPF) is low, the sample can be centrifuged and the seminal plasma being removed to do subsequent analysis. In addition to the fact that different sperm fractions have different HPV virion prevalence, centrifugation of semen samples may not pellet all spermatozoa (PMID: 15820801) and underestimate the concentration (PMID: 16598028). Therefore, we aimed to explore whether centrifugation has effect on HPV virions detection rate.

We have revised the “whole semen” to “non-centrifuged semen”, and revised the “spermatozoa” to “centrifuged semen” in the text and Table 2 accordingly.

“Semen specimens were either used directly or centrifuged to collect sperm for subsequent HPV analysis. Among general population, HPV virion prevalence detected in non-centrifuged semen (12.4%, 95% CI = 6.7-18.0%) was similar to that in centrifuged semen (10.9%, 95% CI = 5.4-16.3%,  $P = 0.346$ ). For fertility clinic attendees, the same situation was found in non-centrifuged semen (20.8%, 95% CI = 13.1-28.5%) and in centrifuged semen (18.0%, 95% CI = 12.8-23.3%;  $P = 0.201$ ).” (Line 221 - 227)

11. Lines 208-212: PCR versus hybridization for the detection of HPV DNA makes a big difference, since numerous inhibitors present in semen inhibits PCR but has less effect on ISH.

Response: PCR amplification has been considered as the most sensitive method for detection of HPV DNA, and is highly reproducible and easily monitored (PMID: 17760847), but it is unable to localize the virus to cells of interest (PMID: 17760847).

Compared with PCR, ISH could precisely locate targets which can be visualized in the context of well-preserved morphologic features (PMID: 21113720), however, it has poor sensitivity because of the limit DNA copies/cell and corresponding number of HPV genotypes detected (PMID: 17977987).

The present study identified 31 studies including 30 studies using PCR for HPV detection and only one study using ISH.

12. Lines 212-215: ?  $11 < 19$ ; text and numbers in table2 are opposite. By broad spectrum PCR you mean consensus PCR's for the detection of oncogenic (high risk HPV)? Consensus PCR's detects less HPV positives compared to type specific PCR's (PMID: 17760847).

Response: Thanks for the reviewer’s comment. The error has been corrected. We have changed the description of “broad spectrum PCR” to “consensus PCR”. We have cited this reference (PMID: 17760847).in the discussion to support our results.

“The analysis in fertility clinic attendees also showed relatively higher prevalence when using type-specific primers than using consensus primers (27.4%, 95% CI = 8.5-46.2%; 18.7%, 95% CI = 14.2-23.1%; P = 0.002, respectively) (Table 2).” (Line 233 - 236)

“Type-specific PCR method was commonly used to detect HPV DNA before consensus PCR which has been widely applied since 2000. However, evidence shows that the sensitivity and specificity of consensus PCR were lower than type-specific PCR [48].” (Line 289 - 292)

13. Lines 234-242: Please explain why you find a higher (not significant; CI 16.2 - 24.6%) HPV prevalence in fertility attendees compared to Laprise et al. 2014 (PMID: 24365799) who finds a HPV prevalence of 16% when focusing on infertile populations (CI 10-23%).

Response: Laprise et al reported that the pooled prevalence of seven studies focusing on infertile populations was 16% [95% CI: 10-23%]. Indeed, only 2 studies focused on infertile populations and 5 studies focused on fertility clinic attendees or male partners of couples undergoing in-vitro fertilization (IVF).

We updated the database to December 2016 that led to six additional studies for fertility clinic attendees, of which four studies reported HPV prevalence higher than 16%. Considering that the four studies have relatively large sample size of fertility clinic attendees, we believe that this makes the present result more reliable. Moreover, Laprise et al. excluded reports that targeted < 20 HPV genotypes, but we aimed to analyze the type-specific HPV virion prevalence of semen, and we made no such exclusion.

We have added the comparison in the discussion section.

“According to the meta-analysis by Laprise et al in 2014, the pooled prevalence of seven studies focusing on populations seeking fertility evaluation or treatment was 16% (95%CI: 10-23%) [9], lower than 20.4% (95% CI = 16.2-24.6%) in our present study. We included six additional studies published after 2014 for fertility clinic attendees, of which four studies reported HPV virion prevalence higher than 16% [26, 27, 29, 41]. Considering that the four studies have relatively large sample size, we believe that this makes the present estimate more reliable. Besides, Laprise et al. excluded reports that targeted < 20 HPV types, but we made no such exclusion and analyzed type-specific HPV virion prevalence in semen.” (Line 260 - 268)

14. Lines 246-253: Clonal HPV (not infectious) can cause cancer, and infectious HPV virions can cause temporal subfertility. HPV virions are the infectious messengers, especially in sperm/seminal plasma where no dividing (clonal) cells are present. It is not because HPV DNA was detected that this HPV DNA originates from a clonal HPV transformed cell population. Actually chances are high that the HPV DNA measured originated from virions (PMID: 26890987).

Response: Thanks for the comment. We agree that clonal HPV is mainly responsible for cancer initiation, and infectious HPV virions contribute to subfertility. HPV virions are only assembled in non-dividing cells, and sperm and seminal plasma does normally not contain dividing cells. Thus, HPV DNA measured in semen mostly originates from virions. We have added the descriptions in the discussion section.

“In fact, clonal HPV (not infectious) is mainly responsible for cancer initiation, and infectious HPV virions could contribute to subfertility. HPV virions are only assembled in non-dividing cells, and sperm and seminal plasma does normally not contain dividing cells. Thus, HPV DNA measured in semen mostly originates from virions [6].” (Line 275 - 279)

15. Lines 259-260: is incorrect (PMID: 23932300).

Response: Thanks for the advice. The error has been corrected.

“In addition, type-specific PCR targeting a specific region in the HPV genome (e.g. E6/E7) seems more suitable in cervical screening [49].” (Line 292 - 293)

16. Lines 262-264: the most important variation is the source of the measured HPV DNA. The virion iceberg makes it difficult to detect the sometimes very small amount of HPV DNA in cancer cells (only 1 HPV DNA copy per clonal cell).

Response: Thanks for the advice. We have revised the statements in the discussion section accordingly.

“The present study found that HPV virion prevalence in semen was higher in published studies using type-specific primers than in those using consensus primers. Thus, type-specific PCR may represent a better choice than consensus PCR to detect HPV virions in semen, especially when HPV DNA copy number is low in semen samples.” (Line 293 - 298)

17. Lines 266-272: Without proper control group/population it is indeed difficult to observe a consistent association (see PMID: 28210481 on p 219).

Response: Thanks for the advice. We have added the statement accordingly.

“Studies without proper control group are unable to determine the effect of HPV virions on male infertility [6]. Until now, only a limited number of case-control studies have been performed and their results are inconsistent. For example, a case-control study conducted in China showed that infertile males had significantly higher HPV prevalence in semen (17.4%) than fertile controls (6.7%) [42]; however, another study failed to confirm the association [44]. Relatively small sample size of individual studies may partly explain the inconsistent results. Therefore, we conducted a meta-analysis of published case-control studies to evaluate the association and its strength between HPV virions and male infertility.” (Line 299 - 307).

18. Lines 305-307: only three case control studies were included?

Response: In our study, a total of 31 studies including 8 case-control studies and 23 cross-sectional studies were included. However, when it comes to the calculation of ORs with 95% CIs, only four case-control studies which comprise confirmed infertile males as the case group and confirmed fertile males as the control group were eligible and included.

19. Please write in full before using the abbreviation: e.g. line 45 HPV, line 75 STI, line 209 ISH, line 284 ASAs

Response: Thanks for the advice. We have added the whole spell in the corresponding sections.

“Human papillomavirus (HPV)” (Line 45)

“sexually transmitted infections (STI)” (Line 76)

“assisted reproductive techniques (ART)” (Line 138)

“polymerase chain reaction (PCR)” (Line 151 - 152)

“in-situ hybridization (ISH)” (Line 229 - 230)

“anti-sperm antibodies (ASAs)” (Line 319)