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**Reviewer A**

The authors test 2955 sperm samples of 564 males. There is no information about the fact whether those males had experienced COVID infection before being vaccinated. Therefore no reliable conclusions can be drawn from this series. It would be interesting to compare samples before and after vaccination of the same males.

**Reply 1:** Thank you for your thoughtful comments. We are so sorry that we didn't explain the situation of the COVID-19 infection in the study. We have excluded the participants who were ever infected with COVID-19. In fact, in our study, the sperm donor candidates had been conducted an inquiry whether ever infected with COVID-19 when they were the first consultation and they finished the sperm donation by online questionnaires. And, we agree with you that it would be interesting to compare samples before and after vaccination of the same males. In the table 2, <sup>b</sup> P values were obtained from pair-wise t test based on 305 semen samples from 71 individuals who provided semen samples before and after receiving COVID-19 vaccine, and the results showed no statistical difference for any sperm quality parameter, when compared before and after vaccination of the same males.

**Changes in the text:** The sentence that "And the sperm donor candidates, who have been ever infected with COVID-19, were excluded in the study" in the Materials and Methods (see Page 5, line 8-9).

There is 4 groups with predicted different reaction to vaccination:

- 1/ Never had covid-19 and have sample before and after vaccination
- 2/ Had had covid-19 before vaccination and has sample before and after vaccination
- 3/ No covid-19 infection before vaccination, but had infection after vaccination (en een staal voor en na vaccinatie)
- 4/ Had covid-19 infection before AND after vaccination (2 x covid-19)

**Reply 2:** These are very thoughtful comments. Thank you for your suggestion. Yes, in theory there are 4 groups with predicted different reaction to vaccination. However, in fact, because we exclude the participants with ever COVID-19 infection

to avoid the influence of confounding factors. So that, in the study, the population were not infected with COVID-19, because of the tight prevention and control system involving all sectors of society and rapid rollout of COVID-19 vaccination.

**Changes in the text:** The sentence that “And the sperm donor candidates, who have been ever infected with COVID-19, were excluded in the study” in the Materials and Methods (see Page 5, line 8-9).

Also, different vaccine types can not be pooled into one category because it was shown that patients after Covid-19 infection produce both protective (RDB/sspige IgG) and harmful (Nucleocapside IgG) antibodies (cfr Depuydt eBioMedicine 2023) and the different vaccines trigger both or only RBD IgG. (PiCoVacc = inactivated vaccine will produce both antibodies against RBD and N, while mRNA vaccine probably only targets RBD/spike. What immune response the protein triggers is unclear.

**Reply 3:** Thank you for your suggestion. We agree with you that different vaccine types can not be pooled into one category. But in the study, the different types of vaccines were not analyzed and discussed, because about 90% participants were vaccinated with inactivated vaccine, and over 94% received two shots.

**Changes in the text:** Taking the above factors into account, no changes have been made in the article.

Also, the time frame after vaccination/covid-19 is important when statistical analyses are performed because most sperm parameter fluctuations are temporal (sperm concentration dip after 43 days).

**Reply 4:** This is a very good point, thank you. We entirely agree with you on that the time frame after vaccination/covid-19 is important. So, as you can see, subgroup analysis of semen quality parameters from semen samples 60-90 days after vaccination was conducted in the Table S2 and Table S3, and the results were similar to the larger study population.

**Changes in the text:** Taking the above factors into account, no changes have been made in the article.

Timing vs detection of SARS-CoV-2 RNA in semen, (line 4-5 page 3), also refer to Donders et al 2022 en Depuydt et al 2023.

**Reply 5:** Thank you for your advice. The recent studies from the teams of Donders

and Depuydt are important, so they should be added in the paper.

**Changes in the text:** “Early studies on COVID-19’s impact on male reproductive system reported inconsistent findings. Some scientific evidence has shown that COVID-19 infection can adversely affect testes (He et al., 2021; Patel et al., 2021), but other studies suggest that SARS-CoV-2 genetic material was rarely found in semen samples, with no observed viral transmission during sexual contact or assisted reproductive techniques (Holtmann, et al., 2020; Guo et al., 2021).”

Revises into: “Early studies on COVID-19’s impact on male reproductive system reported inconsistent findings. Some scientific evidence has shown that COVID-19 infection can adversely affect testes and sperm quality (He et al., 2021; Patel et al., 2021; Depuydt et al., 2023), but other studies suggest that SARS-CoV-2 genetic material was rarely found in semen samples, with no observed viral transmission during sexual contact or assisted reproductive techniques (Holtmann, et al., 2020; Guo et al., 2021; Donders et al., 2022).” (see line 5-8 page 3)

You expect changes in sperm quality parameters associated with vaccination, or having had covid pre or post vaccination (cfr HPV vaccination in male and improved fertility)

**Reply 6:** Thank you for your suggestion. Because we exclude the participants with ever COVID-19 infection to avoid the influence of confounding factors. Therefore, in the study, the population were not infected with COVID-19, the change of sperm parameters from the persons having had covid pre or post vaccination would not be discussed. And in our another study, we will discuss the question in the paper titled “Sperm Quality and Sexual Function After the First COVID-19 Infection During the Omicron Surge: An Observational Study in Southwest China”.

**Changes in the text:** Taking the above factors into account, no changes have been made in the article.

Reference: Carlsen, E., et al., History of febrile illness and variation in semen quality.

Hum Reprod, 2003. 18(10): p. 2089-92.

Fever (1 20 p9), fever was not correlated with impaired sperm quality (Donders et al

2022).

**Reply 7:** Thank you for your suggestion. The study from the team Donders showed that the fever was not correlated with impaired sperm quality (Donders et al 2022). However, fever is widely reported as one of the most common clinical manifestations of COVID-19, and the results of a study by Carlsen et al., who verified that a history of febrile illness adversely affected semen quality (sperm concentration, morphology, and motility), which was also dependent on the fever duration. And there is still no consistent conclusion.

**Changes in the text:** Taking the above factors into account, no changes have been made in the article.

We know that DNA damage matters for fertility, why was this not measured ?

**Reply 8:** Thank you for your suggestion. We quite agree with you that DNA damage matters for fertility. But in the study, the sperm DNA damage of 2955 semen samples was not analyzed and discussed, because of restrictions on fees and conditions in our Sperm Bank. And, we add the reason in the limitation of the paper.

**Changes in the text:** “And because of the limitation of the cost and conditions, more sperm parameters of the all samples, such as the sperm DNA damage, are not analysed in the study” was added in the limitation. (see line 14-16 page 10)

How many pregnancies in each group ?

**Reply 9:** Thank you for your suggestion. Since our study population was sperm donor candidates, their mean age is about 25 years old, and more than 86% of them are single and never married. On the other hand, as a sperm donor, it will take a long time more than years, when getting the feedback of the fertility outcome of their semen samples when provided for women, especially in the Sperm Bank, which have plenty of semen samples for providing. Therefore, in the study, data of pregnancies in each group was not included.

**Changes in the text:** Taking the above factors into account, no changes have been made in the article.

## Reviewer B

This work is of high quality and deserves to be published with minor modifications.

**Reply 1:** Thank you so much for your comments and reviewing the manuscript so carefully. And we will revise our paper according to your thoughtful suggestions. Thanks again for giving us an opportunity to further improve our paper.

Page 3 Line 4: words are pasted in

“can adversely affects sperm quality”

**Reply 2:** Thank you for your careful review. And the revised sentence now reads “can adversely affect testes and sperm quality...”

**Changes in the text:** “Early studies on COVID-19’s impact on male reproductive system reported inconsistent findings. Some scientific evidence has shown that COVID-19 infection can adversely affect testes (He et al., 2021; Patel et al., 2021), but other studies suggest that SARS-CoV-2 genetic material was rarely found in semen samples, with no observed viral transmission during sexual contact or assisted reproductive techniques (Holtmann, et al., 2020; Guo et al., 2021).” Revises into: “Early studies on COVID-19’s impact on male reproductive system reported inconsistent findings. Some scientific evidence has shown that COVID-19 infection can adversely affect testes and sperm quality (He et al., 2021; Patel et al., 2021; Depuydt et al., 2023), but other studies suggest that SARS-CoV-2 genetic material was rarely found in semen samples, with no observed viral transmission during sexual contact or assisted reproductive techniques (Holtmann, et al., 2020; Guo et al., 2021; Donders et al., 2022).” (see line 5-8 page 3)

line 23 24 page 3

"Excellent efficacy" must be tempered by recent results on circulating variants (e.g. Nabin K Shrestha and others, Effectiveness of the Coronavirus Disease 2019 Bivalent Vaccine, Open Forum Infectious Diseases, Volume 10, Issue 6, June 2023, ofad209, <https://doi.org/10.1093/ofid/ofad209> and vaccinated people remain highly infectious (<https://doi.org/10.1038/s41591-022-02138-x>

While efficacy may have been appreciable at the start of the epidemic, it has become

low or non-existent since the appearance of Omicron (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10393554/>).

On the other hand, the benefit-risk ratio is not necessarily favorable for the vaccine in young, healthy men (Fraiman J, Erviti J, Jones M, Greenland S, Whelan P, Kaplan RM, Doshi P. Serious adverse events of special interest following mRNA COVID-19 vaccination in randomized trials in adults. *Vaccine*. 2022 Sep 22;40(40):5798-5805. doi: 10.1016/j.vaccine.2022.08.036. Epub 2022 Aug 31. PMID: 36055877; PMCID: PMC9428332) in particular because of the risk of myocarditis (COVID-19 Vaccine safety updates Advisory Committee on Immunization Practices (ACIP)

June 23, 2021 Tom Shimabukuro, <https://www.cdc.gov/vaccines/acip/meetings/downloads/slides-2021-06/03-COVID-Shimabukuro-508.pdf> )

and

Wong HL, Hu M, Zhou CK, Lloyd PC, Amend KL, Beachler DC, Secora A, McMahon-Walraven CN, Lu Y, Wu Y, Ogilvie RP, Reich C, Djibo DA, Wan Z, Seeger JD, Akhtar S, Jiao Y, Chhillarige Y, Do R, Hornberger J, Obidi J, Forshee R, Shoaibi A, Anderson SA. Risk of myocarditis and pericarditis after the COVID-19 mRNA vaccination in the USA : a cohort study in claims databases. *Lancet*. 2022 Jun 11;399(10342):2191-2199. doi : 10.1016/S0140-6736(22)00791-7. PMID : 35691322 ; PMCID : PMC9183215.

**Reply 3:** Thank you for your thoughtful suggestions. And we totally agree with you that different types of COVID-19 vaccines in the different studies, effectiveness or efficacy is different, especially with the change of different variants of the coronavirus. And the words "Excellent efficacy" was not appropriate. And we modified it into "relatively excellent efficacy"

**Changes in the text:** "To date, the majority of COVID-19 vaccines demonstrate excellent efficacy based on published data of phase III clinical trials, and the risk of adverse events is acceptable (Cai et al., 2021; Sharif et al., 2021)" was revised into "To date, the majority of COVID-19 vaccines demonstrate relatively excellent efficacy based on published data of phase III clinical trials, and the risk of adverse events is acceptable (Cai et al., 2021; Sharif et al., 2021)." (see line 23-24page 3)

Page 4

Line 17 : No ADE detected in animal tests

Preclinical animal tests did not show ADE, but this may be due to the limited duration of observation, as ADE has been observed in clinical cases.

SRIDHAR P, SINGH A, SALOMON N, J. STEIGER D. VACCINE-INDUCED ANTIBODY DEPENDENT ENHANCEMENT IN COVID-19. *Chest*. 2022 Oct;162(4):A646–7. doi: 10.1016/j.chest.2022.08.506. Epub 2022 Oct 10. PMID: PMC9548747.

Bando, T, Takei, R, Mutoh, Y, Sasano, H, Yamano, Y, Yokoyama, T, et al. Two cases of acute respiratory failure following SARS-CoV-2 vaccination in post-COVID-19 pneumonia. *Respirology Case Reports*. 2022; 10:e0995. <https://doi.org/10.1002/rcr2.995>

Hirschbühl K, Schaller T, Märkl B, Claus R, Sipos E, Rentschler L, Maccagno A, Grosser B, Kling E, Neidig M, Kröncke T, Spring O, Braun G, Bösmüller H, Seidl M, Esposito I, Pablik J, Hilsenbeck J, Boor P, Beer M, Dintner S, Wylezich C. High viral loads: what drives fatal cases of COVID-19 in vaccinees? - an autopsy study. *Mod Pathol*. 2022 Aug;35(8):1013-1021. doi: 10.1038/s41379-022-01069-9. Epub 2022 Apr 1. PMID: 35365771; PMID: PMC8974809.

**Reply 4:** Thank you for your careful and professional corrections. And we agree on your corrections. And, the sentence was added, “while ADE has been observed in clinical cases (Hirschbühl et al., 2022; Bando et al., 2022)”.

**Changes in the text:** “while ADE has been observed in clinical cases (Hirschbühl et al., 2022; Bando et al., 2022)” was added. (see line 19-20 page 4)

line20: The safety of Covid vaccines should not be taken for granted given the numerous adverse events reported

(Gubernot D, Jazwa A, Niu M, Baumblatt J, Gee J, Moro P, Duffy J, Harrington T, McNeil MM, Broder K, Su J, Kamidani S, Olson CK, Panagiotakopoulos L, Shimabukuro T, Forshee R, Anderson S, Bennett S. U.S. Population-Based background incidence rates of medical conditions for use in safety assessment of COVID-19 vaccines. *Vaccine*. 2021 Jun 23;39(28):3666-3677. doi: 10.1016/j.vaccine.2021.05.016. Epub 2021 May 14. PMID: 34088506; PMID: PMC8118666.)

in particular the myocarditis in young men discussed here

(Diaz GA, Parsons GT, Gering SK, Meier AR, Hutchinson IV, Robicsek A. Myocarditis and Pericarditis After Vaccination for COVID-19. *JAMA*. 2021;326(12):1210–1212. doi:10.1001/jama.2021.13443)

**Reply 5:** Thank you for your careful and professional suggestions. We entirely agree

that the safety of Covid vaccines should not be taken for granted given the numerous adverse events reported. Based on it, we were focused on the potential impact of such vaccines on human fertility and sperm quality. And most of studies in a nationwide mass vaccination setting, the COVID-19 vaccine was not associated with an elevated risk of most of the adverse events examined (Barda et al., 2021; Cai et al., 2021).

**Changes in the text:** Taking the above factors into account, no changes have been made in the article.

**Reference:**

Barda N, Dagan N, Ben-Shlomo Y, et al. Safety of the BNT162b2 mRNA Covid-19 Vaccine in a Nationwide Setting. *N Engl J Med.* 2021;385(12):1078-1090. doi:10.1056/NEJMoa2110475

Cai C, Peng Y, Shen E, et al. A comprehensive analysis of the efficacy and safety of COVID-19 vaccines. *Mol Ther.* 2021;29(9):2794-2805. doi:10.1016/j.ymthe.2021.08.001

Line 21:

Specify that mRNA is distributed in testicles (see discussion)

**Reply 6:** Thank you for your careful and professional corrections. We revised that “Previous studies found that SARS-CoV-2 mRNA could be detected in the semen, testis, and prostatic fluid of infected males (He et al., 2021; Holtmann et al., 2020).”

**Changes in the text:** We revised that “Previous studies found that SARS-CoV-2 mRNA could be detected in the semen, testis, and prostatic fluid of infected males (He et al., 2021; Holtmann et al., 2020).” (see line 17 page 9)

page 7: line 18

Why were semen samples taken only between 60 and 90 days after vaccination? Were sperm collected within 2 months of the vaccinations? Or was it a precondition not to do so?

Were the sperms collected within 2 months analyzed?

**Reply 7:** Thank you for your professional comments. Our population were sperm donor candidates, and most of them provided more than two semen samples within three months. And only 71 persons of 564, who provided semen samples between 60 and 90 days after vaccination (N=71, n=133), is a subgroup for analysis to assess long-term impact of COVID-19 vaccination. Due to the actual work and volunteers'

wishes, we did not mandate the time for volunteers to collect semen after vaccination. And we were so sorry that we did not analyze the sperms collected within 2 months, because most of their semen samples were provided within 2 months after vaccination. Moreover, only a part of semen samples provided between 60 and 90 days after vaccination (N=71, n=133), were a subgroup, as you can see in Table S2 and Table S3; and the results of the subgroup were similar to the larger study population (Supplemental Table S2) and the overall sperm quality was similar to that of the larger sample (Supplemental Table S3).

**Changes in the text:** Taking the above factors into account, no changes have been made in the article.

Page 9 line 15

Covid infection's damage to sperm is not obvious Zeginiadou T, Symeonidis EN, Symeonidis A, Vakalopoulos I. SARS-CoV-2 infection (COVID-19) and male fertility: Something we should be worried about? *Urologia*. 2023 Jun 5:3915603231175941. doi: 10.1177/03915603231175941. Epub ahead of print. PMID: 37278004; PMCID: PMC10247691.

**Reply 8:** Thank you for your careful and professional corrections. Of course, some studies showed that COVID-19 infection's damage to sperm is not obvious. But others' recent results indicated a significant decrease in sperm quality or semen parameters after SARS-CoV-2 infection in comparison to their respective values before the infection (Cakir et al., 2023; Ali et al., 2023; Shi et al., 2023).

**Changes in the text:** Taking the above factors into account, no changes have been made in the article.

**Reference:**

Cakir C, Kuspinar G, Kurt G, et al. Comparison of semen parameters in the same patients before and after diagnosis of COVID-19. *J Med Virol*. 2023;95(9):e29094. doi:10.1002/jmv.29094

Ali AM, Abdlwahid RF, Ali KM, Mahmood KI, Rashid PMA, Rostam HM. The influence of SARS-CoV-2 on male reproduction and men's health [published online ahead of print, 2023 Sep 19]. *Eur J Clin Invest*. 2023;e14097. doi:10.1111/eci.14097

Shi S, Hu H, Wang J, Huang X, Li J, Li D. Evaluation of semen DNA integrity and related parameters with COVID-19 infection: a prospective cohort study. *Virol J*. 2023;20(1):218. Published 2023 Sep 28. doi:10.1186/s12985-023-02192-y

line21: the claim that Covid vaccines have no effect on female fertility needs to be tempered (see discussion of effects on menstrual cycles and mRNA accumulation in the ovaries).

Official statistics in several countries show a decline in the birth rate, which has been increasing since 2022 and which was not present in 2021 following the Covid pandemic of the pre-vaccine era.

UKHSA September 2022

[https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment\\_data/file/1101870/vaccine-surveillance-report-week-35.pdf](https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/1101870/vaccine-surveillance-report-week-35.pdf)

Australia: Bureau of Health Information <https://www.bhi.nsw.gov.au/data-portal>

France INSEE (National Institute of Statistics <https://www.insee.fr/fr/statistiques/7670212#onglet-2>

**Reply 9:** Thank you for your careful and professional corrections. We agree with you that the claim that COVID-19 vaccines have no effect on female fertility needs to be tempered. We have revised that “Moreover, mounting evidence suggests that the COVID-19 vaccination seemingly does not have significant effect on female reproductive health either.”

**Changes in the text:** “Moreover, mounting evidence suggests that the COVID-19 vaccination does not have significant effect on female reproductive health either” was revised into “Moreover, mounting evidence suggests that the COVID-19 vaccination seemingly does not have significant effect on female reproductive health either”. (see line 21 page 9)

## DISCUSSION

Virus enters the cell through spike binding to ACE2 , ACE2 expression is higher in testes than in other organs

Zeginiadou T, Symeonidis EN, Symeonidis A, Vakalopoulos I. SARS-CoV-2 infection (COVID-19) and male fertility: Something we should be worried about? Urologia. 2023 Jun 5:3915603231175941. doi: 10.1177/03915603231175941. Epub ahead of print. PMID: 37278004; PMCID: PMC10247691.

Vaccines are all based on the spike or the inactivated virus carrying the spike. This

vaccine spike is also able to bind to ACE2. Although the testes have more ACE2 receptor than the ovaries, vaccine LNPs concentrate in the ovaries and much less in the testes, although they are found for a few hours in these organs [https://pubmed.ncbi.nlm.nih.gov/37445690/], Pfizer: https://www.tga.gov.au/sites/default/files/foi-2389-06.pdf

and Moderna :ModernaTX, Inc. 2.4 Nonclinical Overview https://foiaproject.org/case\_detail/?title=on&style=foia&case\_id=34594

https://www.judicialwatch.org/documents/jw-v-hhs-biodistribution-prod-4-02418-pgs-295-302/ and (https://www.judicialwatch.org/wp-content/uploads/2022/12/JW-v-HHS-Biodistribution-Prod-4-02418-pgs-671-701.pdf ModernaTX, Inc. 2.4 Nonclinical Overview mRNA-1273 and https://www.judicialwatch.org/wp-content/uploads/2022/12/JW-v-HHS-Biodistribution-Prod-4-02418-pgs-370-649.pdf

NON-GLP FINAL REPORT AMENDMENT NO. 01 Test Facility Study No. 5002121 ]

Extensive research has been carried out on the effect of SARS-CoV-2 viral infection on spermatogenesis, but unfortunately this is not the case for the effect of vaccination, whereas such research is required for GTPs, the category to which anti-Covid mRNAs belong [https://pubmed.ncbi.nlm.nih.gov/37445690/].

The presence of the virus in semen is highly exceptional, according to several studies, most of which conclude that it is absent.

Female vaccination has been associated with menstrual cycle disturbances (BMJMED 2022;1:e000297. doi:10.1136/ bmjmed-2022-000297

Kristine Blix et al. Unexpected vaginal bleeding and COVID-19 vaccination in nonmenstruating women. *Sci. Adv.* 9, eadg1391 (2023). DOI:10.1126/sciadv.adg1391 that may be explained by LNP concentration in the ovaries or by vaccine-induced inflammation. (Nazir M, Asghar S, Rathore MA, Shahzad A, Shahid A, Ashraf Khan A, Malik A, Fakhar T, Kausar H, Malik J. Menstrual abnormalities after COVID-19 vaccines: A systematic review. *Vacunas.* 2022 Sep-Dec;23:S77-S87. doi: 10.1016/j.vacun.2022.07.001. Epub 2022 Jul 19. PMID: 35873308; PMCID: PMC9294036.)

**Reply 10:** Thank you very much for your so professional suggestion on discussion.

We entirely agree with you and your suggestions.

**Changes in the text:** Taking the above factors into account, no changes have been made in the article.

Limitations

As with other studies on the same subject, this is an observational study only of subjects who presented for sperm donation after vaccination, and not of all subjects who donated sperm before and after vaccination. It may be that some patients who suffered an adverse reaction to the vaccine did not return to donate, and it is possible that sperm quality was impaired only in such individuals.

**Reply 11:** This is a very good point, thank you. Based on your suggestion, the sentence: “Moreover, not of all subjects who donated sperm before and after vaccination, which is another limitation” was added.

**Changes in the text:** The sentence: “Moreover, not of all subjects who donated sperm before and after vaccination, which is another limitation” was added. (see line 14-15 page 10)

### **Reviewer C**

I found this paper interesting, mainly due to the fact of different attitude towards open population wide screening versus individual variability (pairwise) statistical analysis. It can be treated as a warning to all the analyzed phenomena after Covid-19 vaccination so far when non critical statistical analysis has been performed. However, authors do not explain why three interesting sperm motility parameters as VLC, VAP, VSL (of unknown value for conception) remained statistically sound (Discussion).

**Reply 1:** Thank you for your thoughtful and professional suggestion. Firstly, we have revised VLC to VCL. We are so sorry for the mistake. The sperm motility parameters such as VCL, VAP, VSL, which were thought as more objective indicators by using the Makler chamber and Computer Assisted Sperm Analysis (CASA) system. Their definitions were showed in the guidelines of WHO (World Health Organization, 2010). Secondly, sperm motility parameters were thought to be more sensitive for a single sperm and more objective as motility parameters (Yang et al., 2021). In the discussion, we did not discuss the values of them, there were few studies focused on the influence of SARS-CoV-2 vaccination on male reproduction and sperm quality discussed the sperm motility parameters. On the other hand, pair-wise comparison based on the 305 samples from the 71 individuals who donated before and after vaccination showed no statistical difference for any sperm quality parameter, including sperm motility parameters (Table 2).

**Changes in the text:** Taking the above factors into account, no changes have been made in the article.

Reference:

World Health Organization, 2010. WHO Laboratory Manual for the Examination and Processing of Human Semen. 5th ed. Geneva: World Health Organization, 100.

Yang T, Deng L, Sun B, et al. Semen quality and windows of susceptibility: A case study during COVID-19 outbreak in China. Environ Res. 2021;197:111085. doi:10.1016/j.envres.2021.111085

Minor issues:

VCL versus VLC (term) has been mixed up throughout the paper. This must be unified. In Results- we do not have clear population percentages mounting up to 100%. From the text we remain with 89.7% plus 1.77% plus 1.24%. Lacking part of population sample should be explained.

**Reply 2:** Thank you for your careful and professional corrections. Firstly, we have revised VLC to VCL. We are so sorry for the mistake. Secondly, from the text we remain with 89.7% plus 1.77% plus 1.24%, and lacking part of population were 41(7.27%) persons who did not know or remember the type of vaccine, as you can see in the Table 1.

**Changes in the text:** Taking the above factors into account, no changes have been made in the article.

Authors must point out (Discussion) that routine observation of semen parameters does not preclude harmful influence of vaccination towards conception - motility has been observed as the most often affected but it seems to be the one of the most sensitive parameters fluctuating with environmental changes, and this is only the small part of andrological analysis. These two facts must be discussed as the part of limitation to conclusions.

**Reply 3:** Thank you for your careful and professional suggestions. We entirely agree that that routine observation of semen parameters does not preclude harmful influence of vaccination towards conception, and sperm motility often might be affected by fluctuating with environmental changes. And our model was adjusted for time between vaccination and semen sample collection, age, BMI, education level, ethnicity, abstinence duration (days), drinking status (yes/no), smoking status

(yes/no), 90-day average concentrations of air pollutants (PM2.5, PM10, O3, SO2, NO2 and CO), 90-day average weather parameters (temperature and relative humidity) and vaccine manufacturer. It was not analyzed in others' previous studies.

**Changes in the text:** The sentence, "Further studies are needed to determine the effects of COVID-19 vaccination on male reproductive health because results from such studies could help to optimize the decision-making process and management for individuals of reproductive age" have revised into "Further studies are needed to determine the effects of COVID-19 vaccination on male reproductive health and conception, because results from such studies could help to optimize the decision-making process and management for individuals of reproductive age". (see line 5 page 10)

#### **Reviewer D**

This study was valuable as evidence regarding vaccination is not affect to male fertility potential. I hope this study will help to perform vaccination for younger age. I have no comment anymore. Thank you for your sophisticated study.

**Reply 1:** Thank you so much for your thoughtful comments. Thanks again for giving us an opportunity to further improve our paper.

#### **Reviewer E**

The study by Yang et al, investigated the impact of COVID-19 vaccine on semen quality. To this aim, the Authors retrospectively compared conventional semen parameters and kinematic sperm variables in semen donors recruited before and after vaccination, using a mixed model. The study is of interest, however, a rough problem in the statistical approach is present. In Table 3, legend it is reported that the primary dependent variable was whether the semen sample was collected before or after vaccination. Conversely, in this study the dependent variables are all those listed in the first column of the table. Belonging to the group before or after vaccination is an independent covariate. Beside this flaw, many important data are lacking. Indeed, they have 71 men who performed semen analysis before and after vaccine shot. This group appears to me very interesting and has to be studied deeper.

**Reply 1:** Thank you for your careful and professional suggestions. Your suggestion helps us to clearly present the results, thank you. Table 3 is summary of regression results from a mix-

effects model, where repeated data from the same individuals (same IDs) were included in the regression. To control for clustering effect, the model has both the fixed effects (the covariates, or independent variables listed in the left most column) and the random effect (by ID). In this model, an individual could have samples taken prior to vaccination and after vaccination (repeated samples) and we did not put "belonging to the group before or after vaccination" as an independent variable. This response is based on how we understood the reviewer's question. We would love to hear if the reviewer has further question regarding this table or this analysis.

And pair-wise comparison based on the 305 samples from the 71 individuals who donated before and after vaccination showed no statistical difference for any sperm quality parameter, including sperm motility parameters (Table 2). Previously, we felt that the presentation of this part of data alone seemed a little repetitive. If it is necessary, the detail information from 71 men who performed semen analysis before and after vaccine shot could be showed in Table S5 of the paper.

**Changes in the text:** If it is necessary, the detail information from 71 men who performed semen analysis before and after vaccine shot could be showed in Table S5 of the paper.

Table S5. Summary of Semen Quality Parameters of 305 semen samples from 71 individuals who provided semen samples before and after receiving COVID-19 vaccination

Semen Quality Parameters	N=71, n=305 Mean (SD)	Pre- vaccination Mean (SD)	Post- vaccination Mean (SD)	P value
Volume (mL)	4.44 (1.31)	4.46 (1.31)	4.40 (1.32)	0.676
Sperm Concentration (mil/mL)	113 (50.61)	120 (49.7)	121 (54.8)	0.940
Total sperm count (mil)	509 (267.64)	502 (260)	514 (278)	0.676
Total forward sperm (mil)	346 (206.56)	348 (201)	336(216)	0.267
Progressive Rate (%)	67.89 (8.93)	68.14 (7.90)	67.49 (9.52)	0.617
VCL (µm/s)	53.87 (11.8)	53.5 (11.9)	55.2 (11.8)	0.342
VAP (µm/s)	38.67 (8.80)	39.1 (8.81)	39.0 (8.79)	0.970
VSL (µm/s)	30.13 (8.10)	30.5(7.88)	30.2 (8.15)	0.772
WOB (%)	0.72 (0.04)	0.72 (0.05)	0.71 (0.04)	0.032
STR (%)	0.59 (0.08)	0.59(0.07)	0.58 (0.09)	0.351
LIN (%)	0.56 (0.07)	0.56 (0.07)	0.55 (0.08)	0.109
ALH (µm/s)	3.97 (0.75)	3.99 (0.79)	3.97 (0.72)	0.870
BCF (%)	12.41 (2.08)	12.66(1.98)	12.3 (2.25)	0.190

Introduction:

I would not say that COVID 19 are completely safe as we don't know anything about possible long term effects. In addition, their effect on reproduction is also poorly defined: the reason for which you conducted your study. The web link doesn't work (line 31, page 3), please check

Page 4: Indicate the three types of chinese vaccines with an acronyms: would help to read the paper

**Reply 2:** Thank you for your careful and professional corrections. We entirely agree with you. According to your suggestions, we have modified the expression in the introduction: “Early studies have established that SARS-CoV-2 can impact on male reproductive system”. And the web link have been changed into [https://www.asrm.org/practice-guidance/covid-19-resources/?\\_ga=2.29493116.278323476.1698131285-1001808178.1656556236&\\_gl=1\\*\\_qetixa\\*\\_ga\\*MTAwMTgwODE3OC4xNjU2NTU2MjM2\\*\\_ga\\_T403PGFCFZ\\*MTY5ODEzMTI4NS4xNS4xLjE2OTgxMzE1NDc1uNDYuMC4w](https://www.asrm.org/practice-guidance/covid-19-resources/?_ga=2.29493116.278323476.1698131285-1001808178.1656556236&_gl=1*_qetixa*_ga*MTAwMTgwODE3OC4xNjU2NTU2MjM2*_ga_T403PGFCFZ*MTY5ODEzMTI4NS4xNS4xLjE2OTgxMzE1NDc1uNDYuMC4w). The mainly three Chinese COVID-19 vaccines are inactive vaccines, mRNA vaccine, recombinant protein vaccines, which would be added to read the paper easily.

**Changes in the text:** “Early studies on COVID-19’s impact on male reproductive system reported inconsistent findings” is revised into “Early studies have established that SARS-CoV-2 can impact on male reproductive system”. (see line 4- 5 page 3)  
“In China, three types of vaccines, received emergency-use approval (Baraniuk, 2021) based on their high efficacies and safety levels” is revised into “Vaccination has evolved into a routine and highly effective method for illness prevention during the COVID-19 pandemic. In China, three types of COVID-19 vaccines, which are mRNA vaccine, recombinant protein vaccines respectively, received emergency-use approval (Baraniuk, 2021) based on their high efficacies and safety levels” (see line 11-12 page 4)

Please specify which protein is contained in CHO cells (line 14, page 4) and for which protein, did mRNA contained in the second vaccine code.

**Reply 3:** Thank you for your careful and professional corrections. As for the recombinant protein vaccines, the principle is to recombine the S protein receptor binding region (RBD) gene of the SARS-CoV-2 into the gene of Chinese hamster ovary (CHO) cells, express it in vitro to form RBD dimer, and add aluminum hydroxide adjuvant to improve immunogenicity.

**Changes in the text:** “The third type, a recombinant new coronavirus vaccine (CHO Cells), is produced by Zifivax using a protein to trigger an immune response (Su et al., 2021)” was revised into “The third type, a recombinant new coronavirus vaccine (CHO Cells, which are stably expressing SARS-CoV-2 spike protein), is produced by Zifivax using a protein to trigger an immune response (Su et al., 2021).” (line 19-21 page 4)

Please specify if the text (lines 18-24, page 4) refers to all types of vaccine or to the Chinese types (the first, the second and the CHO cells vaccine)

**Reply 4:** Thank you for your careful and professional corrections. In the sentence: “As of November 28, 2022, more than 3.44 billion vaccine doses had been provided freely for all people in China. China’s State Council stated that more than 1.34 billion people, or 92.54% of national population, had received two vaccine doses”, “vaccine doses” and “two vaccine doses” refer to all types of vaccine or to the Chinese types, from the Chinese Official data.

Statistical analysis:

In Table 3, legend it is reported that the primary dependent variable was whether the semen sample was collected before or after vaccination. Conversely, in this study the dependent variables are all those listed in the first column of the table. Belonging to the group before or after vaccination is an independent covariate.

**Reply 5:** Thank you for your careful and professional suggestions. Table 3 is summary of regression results from a mix-effects model, where repeated data from the same individuals (same IDs) were included in the regression. To control for clustering effect, the model has both the fixed effects (the covariates, or independent variables listed in the left most column) and the random effect (by ID). In this model, an individual could have samples taken prior to vaccination and after vaccination

(repeated samples) and we did not put "belonging to the group before or after vaccination" as an independent variable. This response is based on how we understood the reviewer's question. We would love to hear if the reviewer has further question regarding this table or this analysis. This question was answered in the reply 1.

Page 7, line 6. The type of linear model has to be specified

The adjusting model has to consider as covariate also the number of received vaccine doses. The Authors analysed a subgroup made by semen samples collected between 60 and 90 days after the first vaccination shot (If I correctly understand sample collected within 60 days from vaccination were excluded). Why did you choose this period? Why not 90 days? (Similarly to pollutant exposure)

**Reply 6:** Thank you for your careful and professional corrections. The linear model was generalized linear model. In the part of statistical analysis, we were mainly discussed the data of semen quality between before and after the first vaccination shot, so in the adjusting model, we did not consider the number of received vaccine doses but vaccine manufacturer as covariate. And as for the reason of the time of between 60 and 90 days after the first vaccination shot, because on the one hand, the time of spermatogenic cycle of the human was from 64 days to 90 days according to different studies [1-6], and we thought the period of time between 60 and 90 days was proper to represent an approximate spermatogenic cycle. On the other hand, if we only limit the time at a certain point in time, the number of samples that fit the time criteria was smaller. Based on the reasons, we had chosen this period.

Reference:

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3. Gilbert SF. Developmental Biology. 6th edition. Sunderland (MA): Sinauer Associates; 2000. Spermatogenesis. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK10095/>
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5. Clermont Y. (1972). Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiol. Rev.* , 52, 198-236. PMID: 4621362 DOI.
6. Qiu Y, Yang T, Seyler BC, et al. Ambient air pollution and male fecundity: A retrospective analysis of longitudinal data from a Chinese human sperm bank (2013-2018). *Environ Res.* 2020;186:109528. doi:10.1016/j.envres.2020.109528

Results:

In Table 1, it would be important to report also the percentage of men performing semen analysis within 90 days from vaccine shot, distinguishing between those who performed semen analysis within 90 day from the first, the second and the third vaccine shot

**Reply 7:** Thank you for your careful and professional corrections. The percentage of men performing semen analysis within 90 days from the first and the second vaccine shot were 59% and 72%, respectively.

Pag 8, line 10 and following. It is not clear whether the 71 men were those who donated before and after vaccination or those who underwent semen analysis between 60 and 90 days after the first vaccination shot (as suggested by table S2)?

**Reply 8:** Thank you for your careful and professional suggestions. I was so sorry that we confused you with the time of the 71 men who provided semen samples before and after vaccination respectively. Their time of donating semen samples was not limited between 60 and 90 days after the first vaccination shot.

Table 2. Please report also the number of samples and men corresponding to the analyses before and after first vaccine shot. It would be important to report also values before and after vaccine in the 71 subjects performing semen analysis before and after the first vaccine shot: they are more interesting than values of all semen samples. In these 71 men, report also the mean time between vaccination and semen analysis.

Finally, the same data should be shown before any vaccine shot and after two doses of vaccine (both global data and those corresponding to men performing semen analysis

Semen Quality Parameters	N=564, n=2955	Pre-vaccination	Post-vaccination	P value
	Mean (SD)	N=347, n=869 Mean (SD)	N=277, n=942 Mean (SD)	
Volume (mL)	4.06 (1.47)	4.05 (1.40)	4.07 (1.60)	0.8847
Sperm Concentration (mil/mL)	127.76 (70.61)	128 (72.4)	128 (69.4)	0.9873
Total sperm count (mil)	493.29 (277.64)	492 (267)	500 (300)	0.6999
Total forward sperm (mil)	344.43 (206.56)	347 (203)	341 (216)	0.2326
Progressive Rate (%)	68.95 (9.93)	69.70 (9.88)	67.40 (9.89)	<0.001
VCL ( $\mu\text{m/s}$ ) <sup>a</sup>	49.29 (11.11)	47.4 (10.3)	53.2 (11.8)	<0.001
VAP ( $\mu\text{m/s}$ ) <sup>a</sup>	34.67 (8.13)	33.4 (7.67)	37.4 (8.39)	<0.001
VSL ( $\mu\text{m/s}$ ) <sup>a</sup>	26.18 (7.30)	24.9 (6.84)	28.8 (7.50)	<0.001
WOB (%) <sup>a</sup>	0.69 (0.07)	0.68 (0.07)	0.70 (0.06)	<0.001
STR (%) <sup>a</sup>	0.60 (0.08)	0.60 (0.08)	0.59 (0.09)	<0.001
LIN (%) <sup>a</sup>	0.51 (0.09)	0.49 (0.09)	0.54 (0.08)	<0.001
ALH ( $\mu\text{m/s}$ ) <sup>a</sup>	4.36 (1.00)	4.59 (1.00)	3.90 (0.81)	<0.001
BCF (%)	11.64 (2.16)	11.3 (2.03)	12.3 (2.28)	<0.001

before and after two doses)

**Reply 9:** Thank you for your careful and professional corrections. We have added the number of samples and men corresponding to the analyses before and after first vaccine shot in the Table 2, Table S5, Table S6. The mean time between vaccination and semen analysis was 50 days. And according to your suggestions, we have added the Table S6, which showed the global data should be shown before any vaccine shot and after two doses of vaccine. But The samples from men performing semen analysis before and after two doses were so limited, and the sample size is small. So that we didn't show this data separately.

**Changes in the text:** The modification was in the Table 2, Table S5, Table S6. And the Table S6 was added for “Summary of Semen Quality Parameters Before and After Second Vaccine Dose”.

**Table S6.** Summary of Semen Quality Parameters Before and After Second Vaccine Dose.

I'm perplexed on discussion. If I correctly interpreted the Authors found no effect of

vaccination on conventional semen parameters, but a subtler effect on some kinematic variables. In the discussion, the Authors should attempt an explanation about such result and not to repeat that SARS-CoV-2 infection does have an effect on semen quality (already reported in the introduction).

**Reply 10:** Thank you for your careful and professional suggestions. Yes, our results have showed that vaccination did have no effect of on conventional semen parameters, but a subtler effect on some kinematic variables (Table 2). However, pair-wise comparison based on the 305 samples from the 71 individuals who donated before and after vaccination showed no statistical difference for any sperm quality parameter, including some kinematic variables (Table S5). We also confused with it, and we also attempted an explanation about such result. On the one hand, we guessed that sperm motility parameters, such as VCL, VAP, VSL, ALH, were more sensitive parameters by using Computer Assisted Sperm Analysis (CASA) system. Moreover, the sperm motility parameters were improved after post-vaccination, and it might be a stimulation response for COVID-19 vaccine. In the discussion, we did not discuss the values of them, there were few studies focused on the influence of SARS-CoV-2 vaccination on male reproduction and sperm quality discussed the sperm motility parameters.