Review, notes, comments, and feedback on Ref. No.: UKLFP/285147/2022-2:

Title of the PhD thesis: *Posttranslational modifications of nuclear and nonnuclear proteins in spermatozoa.*

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Supervision of: Prof. Jan Nevoral and Prof. Olga Garcia-Alvarez

Reviewer: Manuel Álvarez Rodríguez (BSc, PhD), as "Ramón y Cajal" senior researcher in cell and molecular biology of reproduction, at the Department of Animal Reproduction, INIA-CSIC, Madrid, Spain.

There is an increasing concern about infertility worldwide, is a real health problem associated with several accumulative factors like age, lifestyle, etc. Among the factors of study, epigenetics and, particularly, the study of post-traductional modifications in such specialized cells as sperm cells open a new window of research not only in basic science studies but also in the application of future use in ART procedures. The understanding of the natural phenomena in this PTM (eg. Sperm transit/maturation through the epididymis, sexual maturity of the male, etc.), as well as the changes "artificially" induced by *in vitro* gamete manipulation, including loos of motility and viability, premature capacitation, etc. highlight the utmost relevance of studies focus on elucidating and describing the mechanism behind these changes. These aspects and more are included and discussed in the current PhD thesis document.

The quality of this doctoral thesis is widely endorsed by two scientific articles published in two journals of proven relevance in Reproductive Biology, and one article under review. The methodology used is wide: flow cytometry, Western Blotting, nano-LC-MS, in situ detection of persulfidation, biotin switch, and pull-down assay, among other basic and advanced molecular techniques. The aims offer a solid basis to deal with a gap of knowledge that is resolved and discussed in a coherent manner at the end of reading the doctoral thesis document. The literature review covers most of the key points and background to address the necessity of design experiments to try to shed some light referred to the three sections inside the overview and own results section. The general conclusions and perspective adequately summarize the three main findings and suggestions to perform a follow-up of experiments in the future.

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Specific comments to the Appendix document (publications):

Appendix 1. *H3K4me2 accompanies chromatin immaturity in human spermatozoa: an epigenetic marker for sperm quality assessment*

A1.1. Strengths

· Epigenetics biomarkers have been lately of increasing interest among researchers in different research fields, including, of course, reproduction. Gametes, and embryos, carry epigenetic information that might be intergenerationally passed through in response to early events in reproduction. This paper highlights the relevance of H3K4me2 as such a type of biomarker.

A1.2. Questions/comments

· *The use of correlations in a heterogeneous population such as sperm cells did not allow to exactly elucidate if the sperm cell carrying a higher average cargo of H3K4me2 is the same that is less or more fragmented in terms of DNA integrity, etc. How do you consider that an experimental setup testing individually the sperm cells could be designed and performed?*

· *In tables 2, 3 and 4 you put together all the groups to perform the Spearman's rank correlation test, N, A, and OA, right? Did you consider performing the correlation analysis of the individual groups?*

Appendix 2. *Low doses of Bisphenol S affect post-translational modifications of sperm proteins in male mice.*

A2.1. Strengths

· Post-translational modifications on sperm samples, and in reproductive tissues are presented in here as a good testing model for low and very low exposition to BPS doses.

A2.2. Questions/comments

· *In figure 3 e and in figure 4 g, why did you not represent the rest of the group's BPS?*

· *Reference "17" allows you to estimate the average BPS intake, but is there a way of direct measurement of this parameter? Blood? Seminal plasma? Other bio-fluids?*

· *As you mention in the discussion section, there is a marked effect of the lower doses and the higher doses in sperm motility and histological variations, respectively. Do you have any proposed mechanism? Do you think that and effect of sub- and supra-physiological effects are happening? It means, two curves of effect in your results. What kind of additional experiments would you suggest proving that the systemic exposure to BPS is leading to the changes in reproduction?*

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· *If we pay attention to the list of candidate proteins in figure 4 e (acetylated) and 4 f (phosphorylated): can you design an experimental setup to individually asses one or the two post-translational changes, appart from densitometry included in figure g? E.g. rato acetylated/non-acetylated tubulin ratios. It could be possible to have an overall degradation of the non-acetylated tubulin that could mask the differences.*

Appendix 3. *Evidence of endogenously produced hydrogen sulfide (H2S) and persulfidation in male reproduction.*

A3.1. Strengths

· Hydrogen sulfide (H2S), a cytoprotective molecule turns out to be a good candidate to track changes in male reproduction at different levels, mainly focusing on the form of persulfidation in sperm physiology.

A3.2. Questions/comments

· In figure 3 c , according to the data that you represent, the adult values of CBS, CTH, and 3MPST in normalized to all the results in a way of establishing 100 % in the adults. Why did you choose to use this normalization and not a relative normalization to have a general idea of the standard deviation of the data?

· In future experiments, did you think about including other loading controls than H3? This fact should strengthen the overall results collected from your experimental designs.

· I wonder if you consider isolating different parts of the testis and even doing single-cell analysis of these tissues. Is there any possibility that sperm content in the testis (concentration, spermatogenesis wave, etc) also could modify your results?

· The use of a bulk, both ARN and protein analysis could mask some effects that are only happening at a local level. Such effects, through this individual mapping, I am sure are going to offer the Ph.D. candidate a brilliant future ahead, with multiple possibilities of research lines.

Conclusions:

The PhD thesis document establish a strong abilities and scientific methods in the PhD candidate. Finally, and considering all the aspects summarized in the present review document, **I strongly recommend the dissertation for defence.**

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In Madrid (Spain), June 3rd, 2022 **Manuel Álvarez-Rodríguez**

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