PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Evaluating the role of paternal factors in etiology and prognosis of recurrent pregnancy loss: study protocol for a hospital-based multicentre case-control study and cohort study (REMI III project)
AUTHORS	du Fossé, Nadia; van der Hoorn, Marie-Louise; Eikmans, Michael; Heidt, Sebastiaan; le Cessie, S; Mulders, Annemarie; van Lith, Jan; Lashley, Eileen

VERSION 1 – REVIEW

REVIEWER	Michael Eisenberg Stanford University Department of Urology
REVIEW RETURNED	05-Aug-2019

GENERAL COMMENTS	The authors propose a case-control and cohort study to examine paternal factors in the etiology of RPL. As the authors note, the majority of RPL has an idiopathic etiology, and the authors are to be congratulated for attempting to examine novel paternal risk factors. The protocol appears well thought out. If successful, the work will be a major step forward for RPL. My only comment would be to expand the methodology for simultaneously including paternal and maternal variables that are highly correlated. For example, ages have a correlation coefficient of 0.7. As such, additional analyses (eg. age stratified) may be required.

REVIEWER	Henriette Svarre Nielsen Fertility Clinic and Recurrent Pregnancy Loss Unit Rigshospitalet, Copenhagen University Hospital, Denmark
REVIEW RETURNED	13-Aug-2019

GENERAL COMMENTS	This is a protocol for an important study that will focus on providing new data that can potentially benefit couples with RPL. The protocol is clearly presented included reference to earlier work in RPL, what is lacking and methods and recruiting Few comments 1) Miscarriage is a confirmed intrauterine loss in contrast to a pregnancy loss that also includes biochemical. I recommend to use this terminology through out the protocol when indeed it is a pregnancy loss and not miscarriage that is described 2) You include treated and untreated pregnancies of unknown location. In my understanding a treated pregnancy of unknow location is an ectopic pregnancy. It should be clear if ectopics are included in your RPL diagnosis.

REVIEWER	Ole Bjarne Christiansen Department of Obstetrics and Gynaecology, Aalborg University Hospital, Aalborg, Denmark

REVIEW RETURNED	18-Aug-2019

GENERAL COMMENTS	The protocol describes a very important study that aims to assess the contribution of male lifestyle factors and sperm-related factors to recurrent pregnancy loss (RPL). Overall, the planned study seems feasible but there are several points that need clarification. The study organization is poorly described in the text and the figure is much more informative, In the case-control part of the study, it must be more clearly described that only lifestyle and demographic parameters are compared between RPL patients and controls. In the cohort part of the study (that seems to include all patients included retrospectively and prospectively), it must be more clearly described how the statistical analyses are planned. With regard to the retrospectively included patients, the multivariate analysis can only include factors in the women that have been actually investigated and lifestyle and demographic factors in both partners. In the prospective part of the study, the same factors are probably included but in addition also data from the sperm investigations will be include as independent variables. It is well-described in the protocol how the prospectively included patients will be monitored but what about the retrospectively included patients? Are/were the pregnancy outcomes in these patients monitored in the same way (annual questionnaires)? The sample-size calculation is not optimal. It is based on figures from the study by Zhao et al. reporting a 34% rate of increased DNA fragmentation rate in patients with miscarriage after IVF and 19% in controls. Would it not be better for sample-size calculations to use figures on RPL patients from the meta-analysis by McQueen et al and Tan et al. ? There is no thoughts about how the analysis of the semen cytokines will be done. What is the hypothesis? Is storage of the semen at -20oC good enough when DNA analysis and especially cytokine analyses is planned at later time point? I would prefer that the authors use the term “risk factors” instead of “causes” with regard to phospholipid antibodies, thyroid antibodies uterine anomalies etc. There are indeed no or very few proven causes for RPL but many risk factors. According to the ESHRE RPL guideline, heriditary thrombophilias and homocysteinaemina are not associated with RPL and it is not recommended to screen for them. What is “instable endocrine disorders”?

VERSION 1 – AUTHOR RESPONSE

Reviewer: 1

Reviewer Name: Michael Eisenberg
Institution and Country: Stanford University
Department of Urology
Please state any competing interests or state ‘None declared’: None

Please leave your comments for the authors below
The authors propose a case-control and cohort study to examine paternal factors in the etiology of RPL. As the authors note, the majority of RPL has an idiopathic etiology, and the authors are to be congratulated for attempting to examine novel paternal risk factors. The protocol appears well thought out. If successful, the work will be a major step forward for RPL. My only comment would be

to expand the methodology for simultaneously including paternal and maternal variables that are highly correlated. For example, ages have a correlation coefficient of 0.7. As such, additional analyses (eg. age stratified) may be required.

> *Thank you for your comment. We absolutely agree that it is necessary to adjust for paternal and maternal variables that are highly correlated in the analyses. As stated in the section 'control of bias' we will perform stratified analyses and use regression models including both maternal and paternal factors to deal with this. We have added an example to this section about maternal age being an important confounder for the effect of paternal age on RPL. We have also made an addition to the section 'statistial analysis plan', to make more clear that we will perform stratified analyses and will include both paternal and maternal variables in the multivariate logistic regression.*

Reviewer: 2

Reviewer Name: Henriette Svarre Nielsen
Institution and Country: Fertility Clinic and Recurrent Pregnancy Loss Unit Rigshospitalet, Copenhagen University Hospital, Denmark
Please state any competing interests or state 'None declared': None declared

Please leave your comments for the authors below
This is a protocol for an important study that will focus on providing new data that can potentially benefit couples with RPL. The protocol is clearly presented included reference to earlier work in RPL, what is lacking and methods and recruiting Few comments
1) Miscarriage is a confirmed intrauterine loss in contrast to a pregnancy loss that also includes biochemical. I recommend to use this terminology through out the protocol when indeed it is a pregnancy loss and not miscarriage that is described

> *Thank you for your comments. We have revised the terminology in the manuscript and the terms 'pregnancy loss' and 'miscarriage' are consequently used in the right contexts now. We have also complemented a sentence to the first paragraph of the introduction to point out the difference between both terms.*

2) You include treated and untreated pregnancies of unknown location. In my understanding a treated pregnancy of unknow location is an ectopic pregnancy. It should be clear if ectopics are included in your RPL diagnosis.

> *According to the ESHRE RPL guideline we include non-visualized pregnancy losses, including biochemical and/or resolved and treated pregnancies of unknown location. A treated pregnancy of unknown location (PUL) is defined as those women who are treated medically without confirmation of the location of the gestation by TVS, laparoscopy or uterine evacuation, while an ectopic pregnancy is the ultrasonic or surgical visualization of a pregnancy outside of the endometrial cavity (Kolte et al., 2015, DOI: 10.1093/humrep/deu299, Barnhart et al. 2011, DOI: 10.1016/j.fertnstert.2010.09.006). If identified as such, we do not include ectopic pregnancies in our definition of RPL, in accordance to the ESHRE guideline. To make this more clear, we have complemented a sentence about ectopic (and molar) pregnancies to the section "eligibility criteria".*

Reviewer: 3

Reviewer Name: Ole Bjarne Christiansen
Institution and Country: Department of Obstetrics and Gynaecology, Aalborg University Hospital, Aalborg, Denmark
Please state any competing interests or state 'None declared': None declared

Please leave your comments for the authors below
The protocol describes a very important study that aims to assess the contribution of male lifestyle factors and sperm-related factors to recurrent pregnancy loss (RPL).
Overall, the planned study seems feasible but there are several points that need clarification.
The study organization is poorly described in the text and the figure is much more informative,

In the case-control part of the study, it must be more clearly described that only lifestyle and demographic parameters are compared between RPL patients and controls.

> *Thank you for your comments. We tried to improve the description of the study organization in the text. We apologize that it was not clear in our previous version of the manuscript that in the case-control part of the study also cytokine levels and DNA fragmentation will be measured (and not only lifestyle and demographic factors). We added a sentence to the first paragraph of 'collection and analysis of samples' to clarify this. Samples will only be collected from prospectively included cases and controls, so these comparisons can only be done in these prospective 'subgroups' of cases and controls. This is also shown in the figure by the blue box entitled 'Blood-Semen'. This box applies to both cases and controls. The couples that participate as cases in the case-control study are also part of the cohort study of RPL patients (as stated at the end of the first paragraph of 'study population and recruitment').*

In the cohort part of the study (that seems to include all patients included retrospectively and prospectively), it must be more clearly described how the statistical analyses are planned. With regard to the retrospectively included patients, the multivariate analysis can only include factors in the women that have been actually investigated and lifestyle and demographic factors in both partners.

> *During consultation at the recurrent pregnancy loss clinics we collect lifestyle and demographic data of both partners in a semi-standardized way (using a template in the electronic medical records; we complemented this to the section 'collection of clinical characteristics'). This procedure has been the same for many years. Hence, we are confident that data of both retrospective and prospective patients will be collected in a similar way and this also guarantees that the retrospective data is rather complete. However, for some factors we started registering more recent (for instance paternal physical exercise), so these factors will be missing in part of the retrospectively included patients. Regarding the statistical analysis, we will cope with missing values by performing multiple imputations, as also stated in the section 'statistical analysis plan'*

In the prospective part of the study, the same factors are probably included but in addition also data from the sperm investigations will be include as independent variables.

> *We indeed include the same factors in the analyses of the prospective part of the cohort study with in addition blood and sperm variables. We clarified this in the section 'statistical analysis plan'.*

It is well-described in the protocol how the prospectively included patients will be monitored but what about the retrospectively included patients? Are/were the pregnancy outcomes in these patients monitored in the same way (annual questionnaires)?

> *In the cohort study we aim to complete a five-year follow-up of all participating RPL couples, starting from the intake consultation. When couples are retrospectively included and (part of) their follow-up period is missing in their medical records, for instance when they did not visit our clinics anymore, we will send them a questionnaire to ask for pregnancy outcomes during that missing time period. We complemented this to the section 'collection of clinical charcteristics'. When the five-year follow-up period has not finished by now, they will also receive an annual questionnaire until the end of the five years; this is the same questionnaire as used for prospectively included patients.*

The sample-size calculation is not optimal. It is based on figures from the study by Zhao et al. reporting a 34% rate of increased DNA fragmentation rate in patients with miscarriage after IVF and 19% in controls. Would it not be better for sample-size calculations to use figures on RPL patients from the meta-analysis by McQueen et al and Tan et al. ?

> *We understand this comment about the sample size calculation. We are aware of the studies of McQueen et al. and Tan et al., that were published just after we initially performed our sample size calculation. Based on these new studies, we recalculated our sample size. The meta-analyses of McQueen and Tan both report a mean difference in sperm DNA*

fragmentation between RPL and control patients of ~11% (SD 6%). When this mean difference and standard deviation are used for sample size calculation, the estimated sample size would be only 5 in each study arm (alpha = 0.05, power = 80%) (http://statulator.com/SampleSize/ss2M.html). Since we are not exclusively interested in mean sperm DNA fragmentation level, but use this as a proxy for other lifestyle and demographic factors of interest (smoking, paternal age etc.), such a small sample size is not appropriate for this study. In addition, we do not only study the role of the semen parameters in terms of etiology, but we will investigate their prognostic capacities for future pregnancy outcomes as well. Also for this purpose a more extensive sample size is required. For these reasons, we think it is most convenient to stick to our initial sample size calculation.

There is no thoughts about how the analysis of the semen cytokines will be done. What is the hypothesis?

We hypothesize that imbalances in seminal immunomodulatory factors may contribute to the development of RPL. As stated in the Introduction, some small previous studies suggest correlations between levels of several pro- and anti-inflammatory cytokine in seminal plasma and reproductive outcome. A panel of cytokines was selected (based on previous literature) and will be analyzed by Bio-Plex Luminex™ system assay. In our study we aim for a correlation between various factors and RPL and these factors may serve as a starting point for future research to establish an etiological relation. In addition, we will assess the potential of paternal cytokine levels as 'biomarkers' in RPL couples, in terms of their predictive value for future reproductive outcomes.

Is storage of the semen at -20oC good enough when DNA analysis and especially cytokine analyses is planned at later time point?

Previous cytokine analyses in seminal plasma have been performed in our lab (Meuleman et al. The immunomodulating effect of seminal plasma on T cells. DOI: 10.1016/j.jri.2015.01.012). For these experiments, seminal plasma was also stored at -20ºC, and we decided to follow the same procedure in this study. Regarding sperm DNA fragmentation analysis, we found several studies that described storage of semen at -20ºC until analysis in their methods (Brahem et al. 2011, DOI: 10.1016/j.urology.2011.05.049, Zidi Jrah et al. 2016, DOI: 10.1016/j.fertnstert.2015.09.041). We also conducted a pilot study to test different freeze and thaw conditions and storage at -20ºC did not significantly influence the results (unpublished data).

I would prefer that the authors use the term "risk factors" instead of "causes" with regard to phospholipid antibodies, thyroid antibodies uterine anomalies etc. There are indeed no or very few proven causes for RPL but many risk factors.

Thank you for your comment. We agree with you that factors such as antiphospholipid antibodies, thyroid antibodies and uterine anomalies are associated with RPL but not proven causative. Therefore it is indeed be more appropriate to use the term 'risk factor'. We adjusted this in the new version of the manuscript.

According to the ESHRE RPL guideline, heriditary thrombophilias and homocysteinaemina are not associated with RPL and it is not recommended to screen for them. What is "instable endocrine disorders"?

Thank you for drawing attention to this. The information in the manuscript about hereditary thrombophilias and homocysteinaemia was outdated and incorrect. In our centres we do not screen for hyperhomocysteinaemia anymore. For hereditary thrombophilias we only screen in the context of research. We removed hereditary thrombophilia and hyperhomocysteinaemia from the known risk factors in the sections 'introduction' and We replaced 'instable endocrine disorders' for thyroid autoimmunity.

VERSION 2 – REVIEW

REVIEWER	Michael Eisenberg Stanford University USA
REVIEW RETURNED	11-Sep-2019

GENERAL COMMENTS	The authors have suitably revised the protocol.

REVIEWER	Henriette Svarre Nielsen RIgshospitalet, Denmark
REVIEW RETURNED	09-Sep-2019

GENERAL COMMENTS	No further comments.

REVIEWER	Ole Bjarne Christiansen Department of Obstetrics and Gynaecology, Aalborg University Hospital, Aalborg, Denmark
REVIEW RETURNED	13-Oct-2019

GENERAL COMMENTS	The manuscript has now be sufficiently revised to allow publication.