Seminal significance: the forgotten father in recurrent pregnancy loss

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CHAPTER 9

Summary and general discussion

Recurrent pregnancy loss (RPL) is a poorly understood condition that comes with many uncertainties, both for affected couples and healthcare providers. Important goals are to provide answers to these couples and to improve their pregnancy outcomes. To achieve this, we need a better understanding of contributing and predictive factors. Until now, the male role in RPL has been underexposed. In this thesis, we aimed to expand our knowledge regarding the 'forgotten father' in RPL. We have found strong clues that in RPL, male contribution really matters.

The main conclusions are that advanced paternal age and paternal smoking are associated with an increased risk of pregnancy loss, that inclusion of paternal factors into a prediction model improves the accuracy of predicting ongoing pregnancy after RPL, and that impaired immunomodulatory effects of seminal plasma may play a role in RPL. At the same time, our studies have led to new questions and uncovered new challenges, which are excellent opportunities for further research.

EPIDEMIOLOGICAL CLUES AND CHALLENGES

Aetiology: paternal age and paternal lifestyle factors

For many years the general public has been well-aware that increasing maternal age forms a strong risk factor for reproductive failure, including pregnancy loss.(1) Much less attention was given to possible consequences of men's age on pregnancy complications. **Chapter 2** shows a systematic review and meta-analysis of epidemiological studies investigating the association between paternal age and the risk of pregnancy loss. That a potential paternal age effect has not been a research topic of major interest, is reflected by the fact that only ten studies were retrieved that evaluated the association between paternal age and the risk of pregnancy loss. Still, by combining data of these ten studies we were able to find a significantly increased risk on pregnancy loss in case the father's age exceeds 40. For the age category 40-44 we found a pooled risk estimate of 1.23 (95% CI 1.06-1.43), which increased to 1.43 (95% CI 1.13-1.81) in the category ≥45 years of age (compared to the risk present in the reference group of men aged 25-29 years and adjusted for maternal age).

In **chapter 3** we aimed to provide an overview of available literature on paternal lifestyle factors in the preconception period and the risk of pregnancy loss. We focused on paternal smoking behaviour, alcohol consumption and BMI. A meta-analysis of data derived from eight different studies showed a significantly increased risk of pregnancy loss if men smoked more than ten cigarettes per day in the preconception period. Pooled risk estimates were 1.12 (1.09-1.16) for 11-20 cigarettes per day and 1.23 (95% CI 1.17-1.29) for ≥20 cigarettes per day (compared to the risk present in the reference group of non-smoking men and adjusted for maternal smoking status). It was not possible to find a conclusive answer regarding the association between preconception paternal alcohol consumption and the risk of pregnancy loss. Only five studies were available that were considerably heterogenous with respect to their definitions of alcohol consumption and meta-analysis could not be performed. Two out of these five studies reported increased risks of pregnancy loss in case of large quantities of paternal alcohol consumption, although their risk estimates did not reach statistical significance. Not a single study was retrieved that evaluated the link between paternal BMI and the risk of pregnancy loss. Alcohol consumption and BMI are paternal lifestyle factors that definitely deserve attention in future research.

A major challenge in observational clinical research is the inevitable existence of bias and confounding, which may adversely affect interpretation and validity of the results. (2) Critical appraisal of studies is therefore crucial and this formed the cornerstone of the two systematic reviews that we have conducted. We performed a thorough assessment of the risk of bias and confounding of all included studies. The confounding effect of

maternal characteristics certainly has to be taken into account in these studies. Maternal age and maternal lifestyle factors are strongly associated with their paternal equivalents, as well as with pregnancy outcome. If not adequately controlled for, this may lead to incorrect interpretation of paternal effects. In order to prevent such confounding to the greatest extent possible, we only included studies in our meta-analyses that adjusted for maternal age or maternal smoking (in **chapter 2 and 3**, respectively). Following our assessment, the majority of included studies used adequate methods for adjustment. On the other hand, as discussed in **chapter 2 and 3**, overadjustment for non-confounding variables including obstetric history should be avoided as this could bias the total effect estimate towards the null. Often, however, it is not straightforward to determine whether a variable is a potential confounder or not, the more because many causal relationships within this research area are yet to be established.

The critical appraisal of methodological aspects that we performed showed that different study designs have their own benefits and drawbacks with respect to the risk of bias. The included studies were generally of good quality, and their pooled results clearly indicate associations between the risk of miscarriage and paternal age and smoking, respectively. That these associations may involve a causal relationship becomes more likely based on the biological theories as discussed in **chapter 1** and also later in this chapter.

Nevertheless, still many questions remain unanswered. With regard to the risk of pregnancy loss associated with paternal smoking, the effect of the number of pack-years is unknown, as well as whether and how quickly the increased risk could disappear after smoking cessation. These issues were not addressed in any of the available studies. Furthermore, the studies only focused on cigarette smoking. A recent high-quality study showed that preconception male marijuana use ≥1 time/week is also associated with an increased risk of pregnancy loss (AHR 2.0, 95% 1.2-3.1), adjusted for male and female confounders.(3) Another point worth mentioning is that all existing studies, both on paternal age and lifestyle factors, were focused on single pregnancy loss. Most studies did include couples with RPL, but they formed a small proportion of the total numbers of participants and were not the main population of interest. Although it is likely that many risk factors for single pregnancy loss and RPL will overlap, it is desirable that studies specifically targeted at RPL couples will be conducted in the future.

The REMI III project: to evaluate the role of paternal factors in RPL

Chapter 4 shows the study protocol of the REMI III project: the first large multicentric study to investigate male contribution to RPL from both an epidemiological and immunological perspective. Part of the aims of the REMI III project have been achieved and the results are presented in this thesis, while other aims are the subject of ongoing

research. This is further elaborated on in the following paragraphs.

Prediction: taking both partners into account

A burning question of many RPL couples is related to their prognosis: what is the chance of a future successful pregnancy? In order to provide couples with well-founded information on their prospects, a prediction model can be helpful. The primary aim in prediction research is to predict a future outcome as accurate as possible, usually based on multiple variables (predictors). In prediction research, confounding is not an issue, as there is no single exposure of interest. Predictor variables do not necessarily need to have a causal relationship with the outcome. However, aetiological knowledge can still be applied in the selection of candidate predictors, as established causal risk factors for the outcome often have high predictive value.(4)

In today's clinical practice, two prediction models for couples with unexplained RPL are often used, as they are recommended by international clinical guidelines.(5-7) These models, however, were developed decades ago and neither performance measures nor validation procedures were described. In addition, they were based on only two predictors: the number of previous pregnancy losses and maternal age. In **chapter 5** we explored whether predicting the chance of ongoing pregnancy beyond 24 weeks of gestation could be improved by taking more candidate predictors into account, including paternal characteristics. As standards for prediction models have evolved considerably over time and the quality of reporting of methods and results is not up to these standards in many prediction articles, we closely followed the recommendations as published in the Transparent reporting of a multivariable prediction model for individual prognosis of diagnosis (TRIPOD) guideline.(8)

We found that prediction of subsequent ongoing pregnancy in couples with RPL improved after incorporating additional variables into the model (besides the number of previous pregnancy losses and maternal age), including paternal age, maternal and paternal BMI, maternal smoking status and previous IVF/ICSI treatment. The discriminative capacity of a prediction model, as expressed by the AUC, tells how much the model is capable of distinguishing between couples with and without the outcome. In this context, the AUC can be interpreted as the probability that a randomly selected couple with an ongoing pregnancy will have a higher predicted chance of ongoing pregnancy than a randomly selected couple without an ongoing pregnancy. An AUC of 0.5 indicates no discrimination and is comparable with tossing a coin, whereas an AUC of 1.0 indicates perfect discrimination between all couples with and without ongoing pregnancy. The AUC of our final model was 0.63, compared to an AUC of 0.57 for a model that only included the conventional predictors number of previous pregnancy losses and maternal age.

That the newly identified predictors, each having predictive value on top of the rest, also include male characteristics is an important finding for patients and clinicians that argues for a couple-focused instead of female-focused approach in RPL. However, our study also revealed challenges that need to be overcome in future research. These challenges include a need for higher model performance (which requires the identification of new predictors), predicting the most meaningful outcome for patients, and dealing with repeated predictions over time.

First, it needs to be stressed that although we showed improvement in predictive ability of the model by including extra predictors, an AUC of 0.63 still implies limited performance. More work needs to be done to improve the predictive potential of the model in order to be able to predict outcomes for couples with reasonable accuracy. We should strive to develop a model with an AUC value of at least 0.70, which is generally considered as acceptable discrimination. The performance of our model is in concordance with other prediction studies in reproductive medicine with live birth or ongoing pregnancy as outcome, which mostly report AUCs between 0.55-0.65.(9, 10) The question arises to what extent it is possible to develop a better model. The success of a pregnancy is determined by a multitude of clinical, biological, environmental and demographic factors. Our, as well as other studies, highlight the need for deeper biological insights into normal and abnormal pregnancy. The inclusion of promising biomarkers like the level of sperm DNA fragmentation could possibly increase performance of a prediction model. However, this is under the condition that new predictors can be measured easily and reliably, otherwise the clinical value of an extended model would still be limited. At the same time we should realise that pregnancy outcome is complex to predict. A healthy pregnancy is not a dichotomous phenomenon but can be considered as a stochastic process: it is impossible to guarantee that a couple will have a successful next pregnancy. Consequently, achieving a very high AUC (>0.80) for this outcome is unlikely to be feasible.(11)

Second, the goal of counselling couples with RPL is not per se to ensure that they will have a subsequent ongoing pregnancy, but rather that they will have a good chance of a live birth over some reasonable time period. In our study we pragmatically chose to use subsequent ongoing pregnancy as outcome (defined as a progression beyond 24 weeks of gestation in the first pregnancy after referral), because the long-term follow-up of pregnancies was not accurate enough. A model would have more clinical meaning as it would allow prediction of the chance of a live birth within a certain time frame, for instance within two or five years after referral. This requires a prospective follow-up study with adequate registration of couple's characteristics and pregnancy outcomes.

A third point to consider is that a model would ideally have the ability to accommodate the need for repeated predictions. All currently existing prediction models for RPL were

developed to use at the moment that a couple presents at a specialized RPL clinic. A drawback is that they cannot provide reliable predictions at later time points, when couples who had another pregnancy loss return to the clinic. Application of the model at later time points by simply updating the characteristics of the couple, i.e. more advanced ages, increased number of pregnancy losses etc., results in the calculation of erroneous estimates. It would lead to a systematic overestimation of predicted probabilities (i.e., too optimistic predictions) because RPL couples with an additional pregnancy loss belong to a selection of the population with a less favourable profile. To provide accurate repeated predictions, a dynamic prediction model is needed, for instance like the one presented by van Eekelen et al. for couples with unexplained subfertility.(12) Such a model can adapt to new information that is collected over time and correctly reassess chances.

BIOLOGICAL CLUES AND CHALLENGES

Seminal plasma: composition and immune regulatory effects

In **chapters 6 and 7** we investigated the role of seminal plasma in relation to RPL. Previous research already showed that seminal plasma is much more than just a transporter medium for the spermatozoa.(13-15) It contains a wide variety of signalling molecules, mainly cytokines but also some other important immunologically active factors like sHLA-G and PGE2. These molecules are able to interact with the maternal immune environment after entering the female reproductive tract. In healthy circumstances these seminal plasma factors are thought to help induce a state of active maternal immunotolerance towards the embryo. Disbalances in seminal plasma content may, however, play a role in the development of pathological conditions like pregnancy loss.

In **chapter 6** we performed a hierarchical cluster analysis on seminal plasma samples of men in couples with RPL. We identified two distinct seminal plasma expression profiles. One subgroup of RPL men had relatively high levels of pro-inflammatory cytokines in their seminal plasma including IL-6, IL-8, IL-12, IL-16, IL-18 and TNF-α. It has been postulated that a high pro-inflammatory seminal plasma profile may induce an inflammatory maternal immune response leading to pregnancy loss.(16) In our study, men with the pro-inflammatory seminal plasma expression profile were significantly older and had more unfavourable lifestyle characteristics in terms of cigarette smoking, alcohol consumption and overweight. Men belonging to the other RPL subgroup did not have a pro-inflammatory cytokine expression profile; their seminal plasma expression profile had more overlap with a control group consisting of men whose partners had healthy pregnancies. By performing cluster analysis we aimed to study seminal plasma expression profiles as a system instead of focussing on individual factors. This seems to be the appropriate method, as cytokines function in a network rather than acting in isolation. It enabled the identification of undefined patient subgroups that may share similar pathological mechanisms. In future, preferably larger sized studies, the identified patient clusters and the correlations found with age and lifestyle factors should be validated. A limitation of our study is that only one seminal plasma per patient was available. Collection of multiple seminal plasma samples over time would enable the investigation of possible fluctuations in seminal plasma content over time as well as potential effects of lifestyle modifications on the seminal plasma expression profile.

In **chapter 7** we studied interactions between seminal plasma and female immune cells. We used an in vitro model to assess the effects of seminal plasma on gene expression of female T cells and monocytes. These cells are thought to play a key role in attaining a state of maternal immunotolerance towards the embryo. Female T cells and monocytes obtained from an anonymous female blood donor were incubated with seminal plasma

of either men in couples with RPL (RPL males) or men whose partners had ongoing pregnancies (control males). The effect of seminal plasma stimulation was assessed by measuring changes in mRNA expression of important activation markers of T cells and monocytes. There were two key findings in this study.

First, we observed that seminal plasma has direct impact on female T cells and monocytes, compatible with a differentiation of these cells towards a more immune regulatory phenotype. After incubation with seminal plasma, mRNA expression of IL-10, CD25 and Foxp3 was significantly increased by T cells. This was in accordance with prior studies that showed similar effects of seminal plasma on T cells and monocytes.(17, 18)

Second, our study was the first to observe remarkable differences in the stimulatory capacity of seminal plasma of RPL males versus control males. Incubation with seminal plasma of RPL males led to significantly less mRNA expression of CD25 and IL-10 by T cells.. Expression of CD25 may be an indicator of the induction of a Tregs subset. Previous studies showed lower proportions of peripheral blood CD25+ cells in cases of unexplained (recurrent) pregnancy loss, compared to a control group with normal pregnancy.(19-22) IL-10 is an important immune regulatory factor that has consistently been linked to a suppressive immune response. On the other hand, we found mRNA expression of HLA-DR to be higher after stimulation with seminal plasma of RPL males compared to control males. An excess of HLA-DR+ cells has been associated with a reduced immune regulatory environment, which may lead to pregnancy failure.(23) The degree of expression of different T cell and monocyte markers was particularly correlated with the amounts of TGF-β and VEGF in the seminal plasma (positive correlations with IL-10 and CD25 and negative correlations with HLA-DR).

Altogether, the results presented in **chapters 6 and 7** suggest that the immune regulatory potential of seminal plasma may be impaired in cases of unexplained RPL. Immunomodulating properties of seminal plasma are related to concentrations of key signalling molecules present in the seminal plasma and those seem, in turn, to be associated with paternal age and lifestyle factors. Clearly, our studies were exploratory and mainly serve as a first indication that disturbances in seminal plasma priming may be involved in unexplained RPL. The study design of **chapter 7** only allowed for detection of initial changes in immune cell gene expression after 24 hours of incubation with seminal plasma. Future research should capture the interactions between seminal plasma and the maternal immune environment in greater detail, for instance by using a model that better mimics the implantation site, a longer period of culturing and more extensive monitoring and characterisation of cells.

Seminal plasma: influential but not essential

Although it has been established that seminal plasma deposition activates a series of adaptations in the female immune response and thereby contributes to an optimally suppressive environment, exposure to seminal plasma is not indispensable for the success of a pregnancy. This is demonstrated by the fact that women without a male partner can have effective IVF treatment. Thus, seminal plasma exposure is not an absolute prerequisite for pregnancy. A working hypothesis as proposed by Robertson et al., is that seminal plasma contributes to, but is not essential for the facilitation of maternal immune adaption to pregnancy.(15, 24) The hypothesis assumes three phases of activation and expansion of Treg cell populations in (pre)pregnancy. The first phase is characterised by systemic expansion of the Treg cell pool, directly caused by elevated circulating levels of estrogen at ovulation. Subsequently, in case of coitus, seminal plasma delivers paternal alloantigens and signalling molecules to the implantation site, which induces recruitment of tolerogenic dendritic cells. After these dendritic cells have phagocytosed spermatozoa and apoptotic male somatic cells, they drive the activation and expansion of Treg cells reactive with seminal plasma antigens, either by trafficking to draining lymph nodes or by interacting with locally present Treg cells. Next, in the event of conception and embryo implantation, alloantigens derived from apoptotic placental cells are cross-presented by maternal dendritic cells and ensure further expansion of clonal antigen-reactive Treg cells. If conception does not occur, it seems plausible that repeated seminal plasma exposure during subsequent cycles progressively boosts the Treg cell pool and increases the capacity of the maternal immune system to accept a future pregnancy.

Following this theory, it might be that in situations of absence of seminal plasma a relatively diminished Treg pool can be compensated by the response to alloantigens expressed by the gestational tissues after implantation. This could explain why pregnancy is indeed possible without female exposure to seminal plasma. However, in some instances of either total absence of seminal plasma or defective seminal plasma signalling, inappropriate immunity may occur. This may lead to compromised reproductive outcome. In pathologies of pregnancy, including recurrent pregnancy loss and preeclampsia, reduced Treg cell populations have been observed.(25, 26) These alterations may be linked to limited or defective seminal plasma priming. There is good evidence that prior exposure to the conceiving partner's semen in preconception cycles reduces the risk of gestational disorders. This is well illustrated in preeclampsia, which has a higher incidence in cases of limited semen contact.(15) The effects of seminal plasma exposure seem to be, at least partly, partner-specific, as multiparous women who conceive with a new partner have a higher risk of preeclampsia.(27, 28) Also studies showing that success rates of IVF treatment are significantly improved when women are exposed to seminal plasma around the time of embryo transfer fit with the

hypothesis that seminal plasma boosts an optimally suppressive environment, beneficial for pregnancy.(29-31) Consistent with these results is that the incidence of preeclampsia is relatively more increased when assisted pregnancies are conceived with donor sperm, and that this higher risk is alleviated in case of prior insemination cycles with sperm of the same donor.(32)

A growing body of evidence supports a contribution of seminal plasma to maternal immune adaptation to pregnancy and this raises the prospect of new therapeutic options in reproductive medicine. For instance, administration of specific seminal plasma factors or agents mimicking the effects of seminal plasma may promote the female suppressive immune response and improve pregnancy outcomes. For this to succeed, first more studies are required with the following aims (as mentioned in **chapters 6 and 7**):

- to characterise the complete panel of human seminal plasma signalling factors;
- to evaluate the intra-individual variability in seminal plasma expression profiles over time;
- to evaluate the inter-individual variability in seminal plasma expression profiles in different physiologic and pathophysiologic conditions;
- to evaluate the impact of exogenous factors on seminal plasma constituents;
- to comprehensively map interactions between seminal plasma and the maternal immune environment;
- to distinguish between general effects of seminal plasma constituents on maternal immune cells (for instance TGF-β) and specific effects triggered by deposition of seminal plasma paternal antigens.

Sperm DNA damage: how to measure and how to combat

We should not only focus on the role of the seminal plasma. Impaired DNA integrity of the spermatozoa seems to be another important clue in RPL. Previous studies showed substantial differences in levels of sperm DNA fragmentation between RPL cohorts and fertile control cohorts.(33, 34) Despite this discovery, many unknowns remain. Little is known about the exact pathophysiological pathways of which sperm DNA damage is part, nor about the best way to quantify the level of relevant damage and how to counter it.

One of the important steps yet to be taken is to unravel the relations between seminal plasma composition and sperm DNA integrity. As noted in chapter 6, indications exist that these elements mutually influence each other. It has been established that increased levels of sperm DNA fragmentation can be caused by excessive ROS in the seminal plasma. ROS can drive the production of cytokines and thereby influence seminal plasma composition.(35, 36) In turn, pro-inflammatory seminal plasma cytokines may stimulate generation of ROS.(37, 38)

In order to gain more insights into the complex interplay between seminal plasma factors and sperm DNA integrity, studies should be conducted that measure both at the same time. This has been one of the goals of the REMI III project, of which the study protocol was presented in **chapter 4**, and forms an important pillar of currently ongoing research. A complicating factor in sperm DNA fragmentation testing is that many different methods and protocols exist and it has not been established which test is most informative in which clinical scenario.(39) The most reliable tests for measuring sperm DNA fragmentation include the sperm chromatin structure (SCSA), Comet, sperm chromatin dispersion (SCD) and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) assays. Only the 2-dimensional Comet assay is able to distinguish between single-stranded and double-stranded DNA breaks, while the other tests determine the global sperm DNA fragmentation level without discriminating between the two forms. The exact mechanisms involved in RPL couples with high sperm DNA fragmentation are unknown, but studies have been suggesting that the presence of double-stranded DNA breaks is more lethal than single-stranded DNA breaks.(39-40) Double-stranded breaks are potentially more associated with RPL, while single-stranded DNA breaks are more often linked with infertility or a longer time to natural conception. Although sperm DNA fragmentation seems to be a very promising biomarker in the field of RPL, standardised protocols including guidelines for uniform processing and storage of semen, fixed periods of ejaculatory abstinence and validated assay cut-off points are needed.

It has been shown by us and other studies that both seminal plasma composition and sperm DNA integrity are related to male age and modifiable lifestyle risk factors. Whilst age is a factor that is inevitably beyond control, the influence of male lifestyle interventions should be a topic of future research on RPL. Clinical data on the effectiveness of smoking cessation and weight loss as interventions to reduce sperm DNA fragmentation are lacking, and these should be the first to focus on. Also the impact of other factors, for instance a sedentary lifestyle, dietary intake and use of medication, are worth investigating., Not only for sperm DNA damage, but also with regard to the seminal plasma expression profile, studies evaluating the impact of any lifestyle changes are currently non-existent.

Besides lifestyle modifications, a potential treatment to combat oxidative stress in the male germline might be antioxidant supplementation. Natural antioxidants like vitamin C, vitamin E, folic acid, carnitines, caretonids and micronutrients including iron, zinc and selenium have been shown to reduce levels of sperm DNA fragmentation both in vitro and in animal and human studies.(41, 42) In a Cochrane review focussing on subfertile men, low-quality evidence showed that antioxidants improved live birth rate after ART but not significantly decreased the risk of pregnancy loss.(43) The authors stated that there

is a need for more studies in order to make any conclusions on the effects of different types, dosages and combinations of antioxidants. The low costs and risks associated with antioxidant supplements are appealing to both patients and healthcare providers. However, there is currently no evidence that antioxidant therapy will have a positive effect on pregnancy outcome in couples with RPL.(5) Therefore, a well-designed placebo-controlled randomised clinical trial is needed to clarify the efficacy of antioxidants in this population. In this trial, couples with unexplained RPL should be included and men in the intervention arm should receive antioxidant supplementation for a period of at least six months. Semen samples should be collected at different time points and outcome measures must include both semen factors (sperm DNA fragmentation, antioxidant balance, seminal plasma expression profile) and pregnancy outcomes (of pregnancies conceived between randomisation and three months post-intervention). Other male lifestyle intervention studies could be designed in a similar way.

COUPLE-FOCUSED SUPPORTIVE CARE

As much as we are striving to unravel the pathogenesis of RPL and find new treatment strategies, as much effort must we make to provide appropriate supportive care to our patients. Especially since often no explanation can be found for RPL, adding a further emotional burden to affected couples, it is extra important to offer tailored psychological support. Prior studies evaluated women's perspectives on supportive care after RPL.(44, 45) In **chapter 8** we explored preferences for supportive care of both men and women affected by RPL. Using a questionnaire, we quantified preferences for three domains of supportive care: medical supportive care, soft skills and other types of supportive care (as established in the previous studies of Musters et al.(44, 45)).

For the medical domain, preferences of both genders were largely similar. They both desired to regularly see the same doctor during their consultations, to make a clear plan for the first trimester of a new pregnancy and to have frequent ultrasound examinations during early pregnancy. Women valued their doctor's soft skills more than men did; a significantly larger proportion of women indicated that they prefer a doctor that shows understanding and informs on wellbeing and emotional needs. Also noteworthy was that men expressed less need for support from their family and friends and their overall need for supportive care on a scale from 1-10 was significantly lower compared to that of women (6.8 in men versus 7.9 in women, P = 0.002).

Although the exact reasons for the differing preferences between men and women remain uncertain, some potential explanations can be put forward based on previous research. Multiple interview studies on experiences after pregnancy loss showed that men often take the 'supporter role' and try to be strong and positive for their partner.(46, 47) Compared to women, men are less inclined to disclose their feelings and seek support for themselves, even if they really need it.(46, 48) In line with this, it might be that in our study a social desirability bias was present. Furthermore, it is known that part of the men affected by pregnancy loss experience little support from family and friends, who tend to direct their support largely towards the female partner.(47-49) Also in healthcare settings where supportive care services are profoundly targeted at women, men may feel excluded from care.(49)

It seems that men affected by pregnancy loss may have different needs for supportive care than women. It is important that we try to meet men's needs, especially because studies have shown that they also experience high psychological burden after pregnancy loss.(48) In some cases this may even lead to harmful coping strategies including risk behaviours like substance abuse.(46, 48) In order to be able to offer more tailored supportive care, we should first investigate men's preferences in greater detail. An

important contribution is expected from Williams et al., who designed a currently ongoing study to explore the support requirements of men who experienced multiple pregnancy losses with a qualitative approach.(45) Results of interviews and focus group discussions will be used to inform the development of new interventions to support these men. Examples of a patient-driven initiatives in the Netherlands and England are the recently launched online platforms “The forgotten father” (in Dutch: “De vergeten vader”) and “Miscarriage for Men”.(51, 52) These forums, aiming to connect men affected by pregnancy loss, have attracted many members and received a lot of media attention. Consultation of members of such platforms is an excellent opportunity to enrich novel research plans and to take next steps towards supportive care that meets the needs of both partners affected by RPL.

CONCLUSION AND FUTURE PERSPECTIVES

In light of the results presented in this thesis, we can conclude that a female-focused approach in RPL is unjustified: the male partner urgently deserves our attention. We studied the male role in RPL from different perspectives. Both epidemiologic and biological findings indicate that the male plays a significantly larger role in aetiology and prognosis of RPL than previously thought.

With a frustrating, complicated and misunderstood condition as RPL, there may be a temptation to treat with unproven therapies for the sake of offering desperate couples something, rather than just providing supportive care. Additional pressure to offer therapies can be experienced by caregivers as policies regarding prescription of (experimental) treatments vary between countries, and even practices may differ between local clinics. Instead of offering experimental therapies (outside of clinical trials) with unknown benefits and harms, we should put our efforts in unravelling underlying disease pathways, generating the best possible evidence for targeted therapies and providing excellent patient counselling and supportive care.

Greater male involvement, both in research and in the clinic, could be the key to a long-desired breakthrough in RPL. It is presumable that, with relatively simple interventions focused on the male partner, we can considerably improve outcomes of at least part of the couples affected by RPL. There is sufficient scientific basis to start with male lifestyle intervention studies (e.g. smoking cessation, weight loss), which will do no harm and have the potential to be of great benefit. For all future studies within this field, we argue for a combination of epidemiologic and basic science approaches, as their joint contributions provide a real chance to accelerate the pace of discovering new answers. The link must always be made between the intervention, the composition of the semen (seminal plasma expression profile, level of sperm DNA damage) and clinical outcomes. In addition, we must fully commit to a better understanding of interactions between seminal plasma and the female reproductive tract immune environment. In order to proceed towards specific immune-targeted therapies, first more in vitro and in vivo studies are required, both in healthy and pathophysiologic conditions, to clarify which semen factors can really make the difference for a successful pregnancy and are potentially suitable to base therapies on. Insights from these studies may be valuable for other areas as well; a better understanding of immune modulation during pregnancy may also contribute to advances in organ transplant immunology, as it provides insights in determinants of (in)tolerance towards non-self antigens and may inspire strategies to inhibit transplant rejection.

Close collaboration between different disciplines lays the groundwork for true translational research that can change daily clinical practice. In addition, there are a number of other preconditions that we must meet if we want to make good progress for patients with RPL. One obstacle to overcome is the lack of consistency in used definitions for RPL, which complicates comparison between studies and pooling of results. This is why we should strive for international uniformity in the definition of (unexplained) RPL. Furthermore, joining forces at a national and international level would be beneficial for the research on RPL. Large prospective studies should be conducted that structurally collect clinical data and biological tissues of both partners in RPL couples. Setting up multicentric studies and sharing and combining data sources leads to larger datasets, representing an opportunity to apply more advanced data analysis techniques. However, this must still be done with caution since 'big data analysis' forms no solution for problems of missing observations, measurement errors and confounding, which may all lead to biased results and erroneous conclusions.(53)

Pregnancy loss has been a taboo subject for a long time. In recent years, several high-profile women publicly revealed their pregnancy losses and the ensuing media coverage has contributed to growing recognition and more open discussion. In addition to breaking with the taboo around pregnancy loss, it is about time to break with the misconception that RPL loss is unquestionably a condition of female origin. This thesis underlines that RPL can also be a result of paternal factors. This should be communicated to affected couples in the clinical setting as well as to the general public. It is high time to switch from a female-focused to a couple-focused approach in RPL.

REFERENCES

1. Heffner LJ. Advanced Maternal Age — How Old Is Too Old? N Engl J Med. 2004;351(19):1927-9.
2. Rothman KJ, Greenland S. Modern Epidemiology. Philadelphia, PA: Lippincott-Raven; 1998.
3. Harlow AF, Wesselink AK, Hatch EE, Rothman KJ, Wise LA. Male Preconception Marijuana Use and Spontaneous Abortion: A Prospective Cohort Study. Epidemiology. 2021;32(2):239-47.
4. van Diepen M, Ramspek CL, Jager KJ, Zoccali C, Dekker FW. Prediction versus aetiology: common pitfalls and how to avoid them. Nephrol Dial Transplant.. 2017;32(suppl_2):ii1-ii5.
5. Bender Atik R, Christiansen OB, Elson J, Kolte AM, Lewis S, Middeldorp S, et al. ESHRE guideline: recurrent pregnancy loss. Hum Reprod Open. 2018;2018(2).
6. Brigham SA, Conlon C, Farquharson RG. A longitudinal study of pregnancy outcome following idiopathic recurrent miscarriage. Hum Reprod. 1999;14(11):2868-71.
7. Lund M, Kamper-Jorgensen M, Nielsen HS, Lidegaard O, Andersen AM, Christiansen OB. Prognosis for live birth in women with recurrent miscarriage: what is the best measure of success? Obstet Gynecol. 2012;119(1):37-43.
8. Collins GS, Reitsma JB, Altman DG, Moons KG. Transparent Reporting of a multivariable prediction model for Individual Prognosis or Diagnosis (TRIPOD): the TRIPOD statement. Ann Intern Med. 2015;162(1):55-63.
9. Kolte AM, Westergaard D, Lidegaard Ø, Brunak S, Nielsen HS. Chance of live birth: a nationwide, registry-based cohort study. Hum Reprod. 2021.
10. Leushuis E, van der Steeg JW, Steures P, Bossuyt PM, Eijkemans MJ, van der Veen F, et al. Prediction models in reproductive medicine: a critical appraisal. Hum Reprod Update. 2009;15(5):537-52.
11. Coppus SFPJ, van der Veen F, Opmeer BC, Mol BWJ, Bossuyt PMM. Evaluating prediction models in reproductive medicine. Hum Reprod. 2009;24(8):1774-8.
12. van Eekelen R, Scholten I, Tjon-Kon-Fat RI, van der Steeg JW, Steures P, Hompes P, et al. Natural conception: repeated predictions over time. Hum Reprod. 2017;32(2):346-53.
13. Politch JA, Tucker L, Bowman FP, Anderson DJ. Concentrations and significance of cytokines and other immunologic factors in semen of healthy fertile men. Hum Reprod. 2007;22(11):2928-35.
14. Maegawa M, Kamada M, Irahara M, Yamamoto S, Yoshikawa S, Kasai Y, et al. A repertoire of cytokines in human seminal plasma. J Reprod Immunol. 2002;54(1):33-42.
15. Schjenken JE, Robertson SA. The Female Response to Seminal Fluid. Physiol Rev. 2020;100(3):1077-117.
16. Craenmehr MHC, Heidt S, Eikmans M, Claas FHJ. What is wrong with the regulatory T cells and foetomaternal tolerance in women with recurrent miscarriages? HLA. 2016;87(2):69-78.
17. Meuleman T, Snaterse G, van Beelen E, Anholts JD, Pilgram GS, van der Westerlaken LA, et al. The immunomodulating effect of seminal plasma on T cells. J Reprod Immunol. 2015;110:109-16.
18. Craenmehr MHC, van der Keur C, Anholts JDH, Kapsenberg JM, van der Westerlaken LA, van Kooten C, et al. Effect of seminal plasma on dendritic cell differentiation in vitro depends on the serum source in the culture medium. J Reprod Immunol. 2020;137:103076.
19. Sasaki Y, Sakai M, Miyazaki S, Higuma S, Shiozaki A, Saito S. Decidual and peripheral blood CD4+CD25+ regulatory T cells in early pregnancy subjects and spontaneous abortion cases. Mol Hum Reprod.

2004;10(5):347-53.

20. Yang H, Qiu L, Chen G, Ye Z, Lü C, Lin Q. Proportional change of CD4+CD25+ regulatory T cells in decidua and peripheral blood in unexplained recurrent spontaneous abortion patients. Fertil Steril. 2008;89(3):656-61.
21. Wang L, Yang Y, Liu F, Yang A, Xu Q, Wang Q, et al. Paternal smoking and spontaneous abortion: a population-based retrospective cohort study among non-smoking women aged 20-49 years in rural China. J Epidemiol Community Health. 2018;72(9):783-9.
22. Keller CC, Eikmans M, van der Hoorn MP, Lashley L. Recurrent miscarriages and the association with regulatory T cells; A systematic review. J Reprod Immunol. 2020;139:103105.
23. Kuon RJ, Schaumann J, Goeggl T, Strowitzki T, Sadeghi M, Opelz G, et al. Patients with idiopathic recurrent miscarriage show higher levels of DR+ activated T-cells that are less responsive to mitogens. J Reprod Immunol. 2015;112:82-7.
24. Robertson SA, Guerin LR, Moldenhauer LM, Hayball JD. Activating T regulatory cells for tolerance in early pregnancy- the contribution of seminal fluid. J Reprod Immunol. 2009;83(1-2):109-16.
25. Sasaki Y, Sakai M, Miyazaki S, Higuma S, Shiozaki A, Saito S. Decidual and peripheral blood CD4+CD25+ regulatory T cells in early pregnancy subjects and spontaneous abortion cases. Mol Hum Reprod. 2004;10(5):347-53.
26. Sasaki, Y. et al., Proportion of peripheral blood and decidual CD4(+)CD25(bright) regulatory T cells in pre-eclampsia. Clin Exp Immunol. 2007;149: 139–145.
27. Robillard, PY. et al., Paternity patterns and risk of preeclampsia in the last pregnancy in multiparae. J Reprod Immunol. 1993;24: 1–12.
28. Robillard PY, Périanin J, Janky E, Miri EH, Hulsey TC, Papiernik E. Association of pregnancy-induced hypertension with duration of sexual cohabitation before conception. Lancet. 1994;344: 973–975.
29. Tremellen KP, Valbuena D, Landeras J, Ballesteros A, Martinez J, Mendoza S, et al. The effect of intercourse on pregnancy rates during assisted human reproduction. Hum Reprod. 2000;15(12):2653-8.
30. Bellinge BS, Copeland CM, Thomas TD, Mazzucchelli RE, O'Neil G, Cohen MJ. The influence of patient insemination on the implantation rate in an in vitro fertilization and embryo transfer program. Fertil Steril. 1986;46(2):252-6.
31. Friedler S, Ben-Ami I, Gidoni Y, Strassburger D, Kasterstein E, Maslansky B, et al. Effect of seminal plasma application to the vaginal vault in in vitro fertilization or intracytoplasmic sperm injection treatment cycles-a double-blind, placebo-controlled, randomized study. J Assist Reprod Genet. 2013;30(7):907-11.
32. Kyrou, D. et al., Is the use of donor sperm associated with a higher incidence of preeclampsia in women who achieve pregnancy after intrauterine insemination? Fertil Steril. 2010;93: 1124–1127.
33. McQueen DB, Zhang J, Robins JC. Sperm DNA fragmentation and recurrent pregnancy loss: a systematic review and meta-analysis. Fertil Steril. 2019.
34. Tan J, Taskin O, Albert A, Bedaiwy MA. Association between sperm DNA fragmentation and idiopathic recurrent pregnancy loss: a systematic review and meta-analysis. Reprod Biomed Online. 2019;38(6):951-60.
35. Buch JP, Kolon TF, Maulik N, Kreutzer DL, Das DK. Cytokines stimulate lipid membrane peroxidation of human sperm. Fertil Steril. 1994;62(1):186-8.

36. Perdichizzi A, Nicoletti F, La Vignera S, Barone N, D'Agata R, Vicari E, et al. Effects of Tumour Necrosis Factor-α on Human Sperm Motility and Apoptosis. J Clin Immunol. 2007;27(2):152-62.
37. Rajasekaran M, Hellstrom WJ, Naz RK, Sikka SC. Oxidative stress and interleukins in seminal plasma during leukocytospermia. Fertil Steril. 1995;64(1):166-71.
38. Esteves SC, Santi D, Simoni M. An update on clinical and surgical interventions to reduce sperm DNA fragmentation in infertile men. Andrology. 2020;8(1):53-81.
39. Casanovas A, Ribas-Maynou J, Lara-Cerrillo S, Jimenez-Macedo AR, Hortal O, Benet J, et al. Double-stranded sperm DNA damage is a cause of delay in embryo development and can impair implantation rates. Fertil Steril. 2019;111(4):699-707.e1.
40. Ribas-Maynou J, Benet J. Single and Double Strand Sperm DNA Damage: Different Reproductive Effects on Male Fertility. Genes. 2019;10(2).
41. Gharagozloo P, Gutiérrez-Adán A, Champroux A, Noblanc A, Kocer A, Calle A, et al. A novel antioxidant formulation designed to treat male infertility associated with oxidative stress: promising preclinical evidence from animal models. Hum Reprod. 2016;31(2):252-62.
42. Zini A, San Gabriel M, Baazeem A. Antioxidants and sperm DNA damage: a clinical perspective. J Assist Reprod Genet. 2009;26(8):427-32.
43. Showell MG, Mackenzie-Proctor R, Brown J, Yazdani A, Stankiewicz MT, Hart RJ. Antioxidants for male subfertility. The Cochrane database of systematic reviews. 2014(12):Cd007411.
44. Musters AM, Taminiau-Bloem EF, van den Boogaard E, van der Veen F, Goddijn M. Supportive care for women with unexplained recurrent miscarriage: patients' perspectives. Hum Reprod. 2011;26(4):873-7.
45. Musters AM, Koot YE, van den Boogaard NM, Kaaijk E, Macklon NS, van der Veen F, et al. Supportive care for women with recurrent miscarriage: a survey to quantify women's preferences. Hum Reprod. 2013;28(2):398-405.
46. Due C, Chiarolli S, Riggs DW. The impact of pregnancy loss on men's health and wellbeing: a systematic review. BMC Pregnancy and Childbirth. 2017;17(1):380.
47. Obst KL, Due C. Australian men's experiences of support following pregnancy loss: A qualitative study. Midwifery. 2019;70:1-6.
48. Voss P, Schick M, Langer L, Ainsworth A, Ditzen B, Strowitzki T, et al. Recurrent pregnancy loss: a shared stressor---couple-orientated psychological research findings. Fertil Steril. 2020;114(6):1288-96.
49. Obst KL, Due C, Oxlad M, Middleton P. Men's grief following pregnancy loss and neonatal loss: a systematic review and emerging theoretical model. BMC Pregnancy and Childbirth. 2020;20(1):11.
50. Li DK, Ferber JR, Odouli R, Quesenberry C. Use of nonsteroidal antiinflammatory drugs during pregnancy and the risk of miscarriage. Am J Obstet Gynecol. 2018;219(3):275.e1-.e8.
51. De vergeten vader. The Netherlands, 2021 [Available from: https://www.devergetenvader.nl/].
52. Miscarriage for Men. England, 2021 [Available from: https://www.miscarriageformen.com/].
53. Obermeyer Z, Emanuel EJ. Predicting the Future - Big Data, Machine Learning, and Clinical Medicine. N Engl J Med. 2016;375(13):1216-9.