Seminal significance: the forgotten father in recurrent pregnancy loss

Fossé, N.A. du

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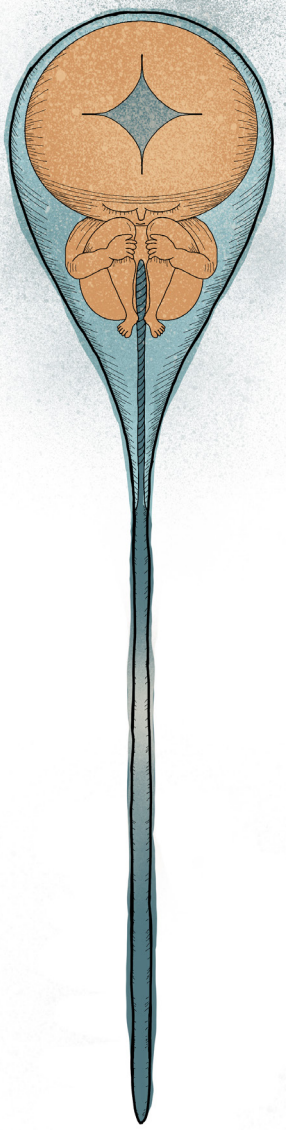

Nadia du Fossé

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The forgotten father in recurrent pregnancy loss

Nadia du Fossé

SEMINAL SIGNIFICANCE – THE FORGOTTEN FATHER IN RECURRENT PREGNANCY LOSS

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Un Philosophe est assez semblable a un homme qui marche dans un labyrinthe: Il va de conclusion en conclusion jusqu'a ce qu'il se trouve pris, & qu'il est oblige de rebrousser chemin, pour en chercher un autre qui soit meilleur, & qui le puisse mener è la verité.

A quote of Nicolaas Hartsoeker, addressed to René Descartes.
In: Eclaircissemens sur les conjectures physiques, Amsterdam, 1710.
Cited in original French spelling.

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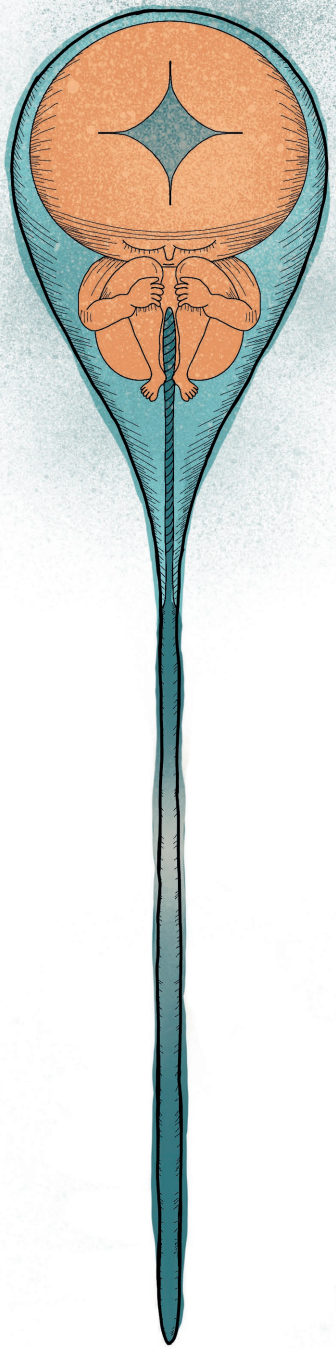


CHAPTER 1

Introduction

1 A TINY LITTLE MAN INSIDE THE SPERM HEAD

> *"These little animals, until then invisible, which must transform themselves into men, which swim in prodigious amounts in the liquor destined to carry them, which do not occur but among males, and which have the appearance of young frogs, with large heads and long tails and very vivid movements..."*

These words originate from Nicolaas Hartsoeker, a Dutch astronomer and natural philosopher, who studied at Leiden University in the 17th century.(1) During this time, many scientists were intrigued by the "question of generation": how do organisms reproduce and develop? Hartsoeker claimed to have discovered sperm with his self-built microscope. He produced the drawing of a tiny preformed human or 'homunculus', curled up inside the sperm head. In later years, his sketch (Figure 1) has become iconic of the theory of embryological development known as preformationism.(2) Spermist prefomationism was the idea that humans develop from a miniature version of themselves, which was entirely derived from the father and present in a sperm cell. Supporters of this theory held the belief that the sperm homunculus was placed inside the woman's uterus for growth into a child.

Figure 1. Homunculus in sperm
Pencil sketch by Nicolaas Hartsoeker, published as part of his paper Essai de Dioptrique in 1694(1)

THE FORGOTTEN FATHER

Since Hartsoeker first objectified spermatozoa through his microscope, knowledge about human reproduction has expanded enormously. However, many unanswered questions about pregnancy and associated complications remain. A striking contrast can be observed if one compares the view on human reproduction from Hartsoeker's time, when it was thought that the fetus originated entirely from the father, with that of the past century, when research in reproductive medicine was often focused on the woman. A reproductive disorder that is clearly illustrative of such a female-focused approach is recurrent pregnancy loss (RPL).

RPL means the spontaneous loss of two or more pregnancies in the period between conception and the moment that the fetus reaches viability (also commonly named miscarriages if gestational age is <16 weeks). As will be addressed in more detail later in this chapter, RPL is a poorly understood area in reproductive medicine. Even after comprehensive diagnostic investigations an underlying condition is found in fewer than 50% of cases.(4, 5) For couples with unexplained RPL, evidence-based therapies are currently non-existent. This contributes to the frustrating nature of RPL. Couples carry the burden of enduring uncertainty, while clinicians are unable to offer effective treatments with proven benefit. For a long time, the vast majority of studies in the field of RPL have focused on female factors. However, in order get more insight into the pathophysiology of RPL and to provide best possible care to affected couples, we should not forget the father.

In the studies that will be presented in this thesis, the male role in RPL was investigated. The aim of this first chapter is to introduce different aspects of RPL, to summarise what is currently known and to highlight existing knowledge gaps. Furthermore, biological hypotheses supporting male contribution to RPL are discussed.

1 RECURRENT PREGNANCY LOSS

Definition

Although 'miscarriage' is a term commonly used in the general population, the scientific definition of miscarriage is not straightforward and varies between countries and international research societies.(6) Generally, this term is linked to the loss of an intrauterine pregnancy before the fetus reaches viability, confirmed by ultrasound or histology. Depending on geographic locations, the gestational threshold for viability varies from 20 weeks to 28 weeks. In the Netherlands, as well as in the United Kingdom, the limit of viability is determined at 24 weeks and 0 days of gestation. The European Society of Human Reproduction and Embryology (ESHRE) advocated in their Early Pregnancy Consensus Statement the importance of consistent and generally accepted terminology.(7) They recommended to term a spontaneous demise of a pregnancy between the time of conception until 24 weeks of gestation as a pregnancy loss.

The term preferred by the ESHRE for encountering two or more pregnancy losses is recurrent pregnancy loss (RPL).(5) This term will be used throughout the rest of this thesis. On a similar note as for sporadic pregnancy loss, exact definitions of RPL differ between international guidelines.(8) The definition of RPL consists of four main elements: defining pregnancy (intrauterine, visualized or non-visualized, biochemical), defining the threshold of pregnancy viability, defining recurrence and deciding on whether pregnancy losses have to be consecutive. In this thesis, the definition of RPL as established in the most recent ESHRE guideline is maintained. The definition includes the loss of a minimum of two pregnancies, confirmed by at least serum or urine β-human chorionic gonadotropin (hCG). Non-visualized pregnancies are also included in the definition, but ectopic and molar pregnancies are not. The pregnancy losses do not have to be consecutive. Further, RPL can be differentiated into primary and secondary RPL.(5) Primary RPL is defined as RPL without a previous ongoing pregnancy (viable pregnancy) beyond 24 weeks of gestation. Secondary RPL is used for couples who suffer from RPL but have a history of at least one pregnancy progression beyond 24 weeks of gestation.

Prevalence

It is hard to provide a reliable estimate for the risk of (recurrent) pregnancy loss. As a result of variations in the definitions being used, both the numbers of women who experienced (recurrent) pregnancy loss (the numerator) and all women at risk of (recurrent) pregnancy loss (the denominator) are difficult to determine. Quenby et al. recently reviewed the currently available evidence and concluded that the pooled overall risk of pregnancy loss is 15.3% of all recognised pregnancies, based on data of nine large cohort studies.(6) For RPL, if defined as two or more losses, they reported a population prevalence of 2.6%. Real numbers are likely to be higher, as many cases,

especially of early pregnancy losses, go unreported.

Known risk factors

Multiple demographic, clinical, lifestyle and environmental risk factors for (recurrent) pregnancy loss have been identified throughout the years (Figure 2). These will be discussed in the next paragraphs:

Maternal age

A major risk factor for pregnancy loss, consistently found in many studies, is advanced maternal age. The association between maternal age and pregnancy loss can be attributed to a biological process. The risk of embryonic aneuploidy, particularly trisomy, rises with increasing maternal age. Women should be informed that the risk of pregnancy loss is lowest between 20 and 35 years , starts to increase after the age of 35 and sharply rises beyond 40 years.(6)

Maternal BMI

The association between maternal BMI and RPL was assessed in multiple studies. A systematic review showed a more than three times higher prevalence of RPL in women with obesity (BMI ≥30 kg/m^2) compared to women with a normal BMI (20-30 kg/m^2). (13) Another study reported a significantly increased risk of a subsequent pregnancy loss after previous RPL in obese women.(14) They found no increased risk for maternal overweight (BMI 25-30 kg/m^2). Also being significantly underweight (BMI <18.5 kg/m^2) was found to be associated with sporadic first trimester miscarriage.(15)

Maternal smoking

Cigarette smoking is another modifiable risk factor for pregnancy loss. Especially active smoking in the first trimester is shown to increase the risk of pregnancy loss.(6) The risk is dose-dependent and increases with the number of cigarettes smoked per day.

Maternal alcohol consumption

It has been known for a long time that alcohol consumption has a negative effect on pregnancy and fetal and neonatal outcomes. Multiple studies focusing on pregnancy loss have shown that maternal alcohol consumption during the first trimester is a risk factor for pregnancy loss, in a dose-dependent manner.(16, 17)

Other maternal lifestyle factors

High caffeine intake has been suggested as a risk factor RPL. While some studies reported an association(18, 19), other studies did not find any effect of caffeine when adjusting for nausea(15). It is thought that associations between caffeine and pregnancy are likely to be confounded, as nausea and vomiting are common symptoms in healthy pregnancy

and might reduce caffeine consumption.(20) Other factors that have been associated with pregnancy loss are night shift work and high stress.(6) For the latter, however, evidence for a causal relationship is lacking.(21)

Previous pregnancy losses

A major determinant of the risk of pregnancy loss is the number of previous pregnancy losses. This association has been consistently found in many studies. The risk is estimated to increase by a factor of 1.5 after one previous pregnancy loss to a factor of 4.5 after three or more pregnancy losses, compared to a reference group without previous pregnancy losses.(6). It is considered unlikely that this association represents a causative relationship; most probably the number of previous losses can be seen as a proxy for an underlying condition or unfavourable patient characteristics.

Environmental factors

Several studies investigated the effect of air pollution on the risk of pregnancy loss. Exposure to air pollution appears to significantly increase the risk of pregnancy loss, as shown by a large study in Beijing.(22) Also pesticides have been linked to (recurrent) pregnancy loss, based on an epidemiological study in South-Africa and a clinical study that found higher levels of serum organochlorine pesticides in women with RPL compared to controls.(23, 24)

Evidence-based diagnostic investigations and treatment options

Apart from the general risk factors previously mentioned, multiple conditions have been associated with RPL. Specialised RPL clinics often differ in the diagnostic tests and treatments they offer. The wide variation in clinical practice causes some couples to consult multiple clinics, both nationally and internationally.

In 2017, the available evidence on RPL investigations was reviewed by the ESHRE guideline development group.(5) They evaluated associations between diagnostic investigations and risk of pregnancy loss. If associations were identified, the probability of a causative or contributory relationship was assessed. In addition, the prognostic value of diagnostic test results and the evidence regarding treatment effects were reviewed. The tests (Figure 2) that were recommended by the ESHRE guideline development group include:

- the measurement of antiphospholipid antibodies (lupus anticoagulant and anticardiolipin antibodies) to screen for antiphospholipid syndrome;
- pelvic ultrasonography (preferably a three-dimensional transvaginal ultrasound) to identify potential uterine anomalies;
- thyroid function and presence of thyroid peroxidase (TPO) antibodies;
- parental karyotyping in selected couples, based on individual risk assessment.

To women with antiphospholipid syndrome a combination of low-dose aspirin and low molecular weight heparin can be offered, as this is associated with increased live birth rates after RPL, although the quality of evidence is low.(5, 9) Surgical treatment of uterine anomalies has been a subject of debate, however the recent TRUST trial did not reveal any improvement in reproductive outcome in women with a septate uterus that underwent septum resection.(10) Also for other uterine anomalies in women with RPL, surgical uterine reconstruction is not recommended.(5) In women with subclinical hypothyroidism levothyroxine therapy can decrease the risk of subsequent pregnancy loss, according to moderate-quality evidence.(5, 11) To couples with results of an abnormal parental karyotype, genetic counselling should be offered to discuss their prognosis and further diagnostic options. Based on the limited available evidence, there seems no benefit of preimplantation genetic testing in couples with RPL.(5)

Despite performing the diagnostics tests as described above, the aetiology of RPL remains unclear in the majority of patients. Nevertheless, also for those patients in whom no underlying condition could be diagnosed, various treatment options have been investigated in the past decades. The key interventions included insulin, human chorionic gonadotropin, immunomodulatory agents such as intravenous immunoglobulins and prednisone, micronutrient supplements and progestogens.(5, 12) Unfortunately, these medications seemed not to improve live birth rates after RPL. Based on the most recent review and meta-analysis, only micronized vaginal progesterone treatment might be considered for women with unexplained RPL who have a high number of previous pregnancy losses and first trimester blood loss, however this remains a topic of discussion.(12)

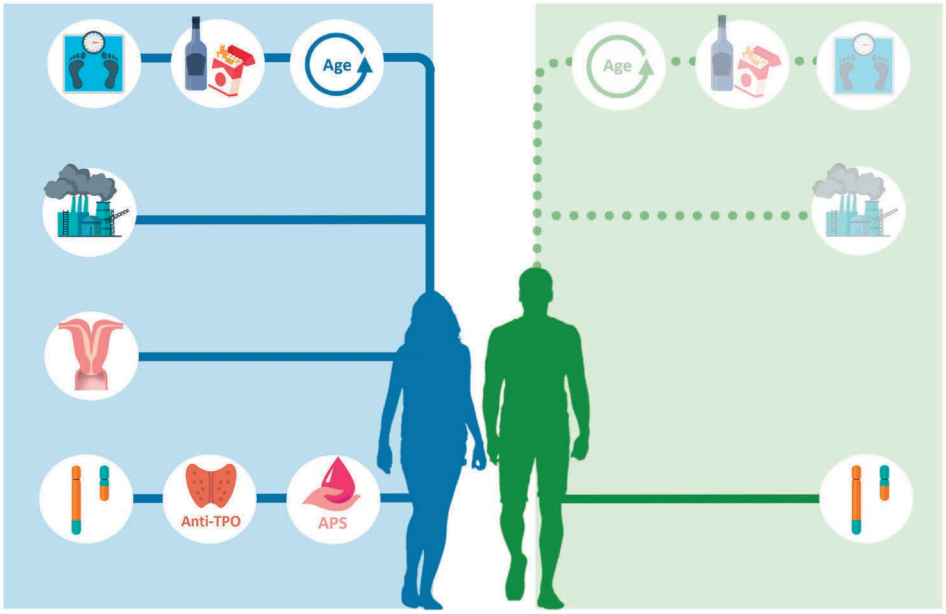


Figure 2. Known and potential risk factors for RPL

Key maternal risk factors: aging, cigarette smoking, alcohol consumption, body mass index, environmental factors, anti-thyroid peroxidase (TPO) antibodies, antiphospholipid syndrome (APS), uterus anomalies, balanced chromosomal translocations. *Paternal risk factor:* balanced chromosomal translocations. *(Selection of) potential paternal risk factors:* ageing, cigarette smoking, alcohol consumption, body mass index, environmental factors.

MALE CONTRIBUTION

The less than 50% of couples in whom an explanation for RPL can be found is a sobering statistic. As recently stated in the journal Fertility and Sterility, the search must go on for patients with RPL.(25) For a long time, the vast majority of studies have focused on female factors. In case of pregnancy achievement, the man's gametes were considered normal and later complications, including loss of the pregnancy, were attributed to female anomalies. Since the male partner contributes half the genetic material of the embryo, it seems reasonable to assume that his influence extends beyond just the conception. Therefore, a thorough evaluation of paternal factors seems an excellent opportunity to gain new insights in (recurrent) pregnancy loss. Although the male contribution has not been satisfactorily addressed so far, some studies exist that have already focused on this topic:

Paternal age

In contrast to the well-known impact of maternal age on the risk of pregnancy loss, the impact of increasing paternal age is less clear. In 2018, Oldereid et al. reviewed existing literature on the influence of paternal age on a wide range of perinatal and paediatric outcomes.(26) They found associations between advanced paternal age (starting around 40) and stillbirth, several birth defects as well as long term adverse outcomes in the offspring, including autism spectrum disorders. Although these results make it plausible that advanced paternal age may also contribute to pregnancy loss, this outcome was not assessed in this review. A number of studies were published investigating the relation between male age and pregnancy loss, with inconclusive results.(15, 27-29)

Paternal lifestyle and environmental factors

Knowledge on the impact of paternal lifestyle factors on pregnancy loss is very limited. Some studies assessed the relation between smoking and/or alcohol consumption and pregnancy loss. Results of these studies were ambiguous and no systematic reviews and meta-analyses exist.(15, 30-33) Another study found that the risk of RPL was significantly increased when paternal smoking, alcohol consumption and occupational exposure to environmental factors (including a wide range of exposures such as radiation, pesticides and heavy metals) were superimposed.(34)

Semen

The aforementioned studies were aimed at finding associations between paternal factors and pregnancy loss from an epidemiological point of view and most of these studies did not investigate potentially underlying biological mechanisms. However, in order to increase insight into the male contribution to RPL, besides epidemiological studies, it is essential to zoom in on the substance that actually forms the male contribution:

the semen. Semen comprises both spermatozoa (the mature sperm cell fraction) and seminal plasma (the acellular plasma fraction).

Spermatozoa

From entering puberty on, large numbers of spermatozoa are produced in the seminiferous tubules of the testis and the epididymis through a complex process or renewal, proliferation and differentiation, known as spermatogenesis. The entire process is estimated to occur approximately within 74 days and is classically divided into three phases(35, 36):

- a mitotic amplification phase (proliferation and maintenance of spermatogonia);
- a meiotic recombination phase (production of genetically diverse haploid gametes);
- a post-meiotic phase, known as spermiogenesis (re-packaging of the haploid paternal genome).

Mature spermatozoa are highly differentiated cells, consisting of a tail, a mid piece and a head, containing the nucleus with the paternal genetic material carried in the deoxyribonucleic acids (DNA) molecules. The main function of spermatozoa is to transfer the intact haploid paternal genome to the female reproductive tract. Protection of the DNA is crucial and this is safeguarded by the sperm-specific DNA packaging. In somatic cells, the DNA is wrapped around highly basic proteins named histone proteins, that are found in the cell nuclei. Histones act as “anchors” around which the DNA winds and forms units called nucleosomes. In turn, these nucleosomes are wrapped into fibers that form tightly packed chromatin. Without this way of packaging, unwound DNA chromosomes would be very long. Besides ensuring a compact DNA structure, histones protect the DNA from damage. Chromatin packing in mature spermatozoa differs from that in somatic cells. During the post-meiotic process of spermiogenesis, nuclear histones are replaced by protamines, which are smaller basic proteins. This facilitates even more compaction of the sperm nucleus, and consequently, of the sperm head. Condensation of the sperm chromatin is approximately seven times higher than in the nucleus of any somatic cells.(36) This chromatin reorganization is considered vital for the success of fertilization. The sperm head volume is directly related to the optimal velocity of the cell and nuclear compaction is important for protection of the paternal genome against chemical and physical modifications. These factors are both critical for the spermatozoa to safely move towards the oocyte in the female reproductive tract. Thorough protection of the sperm DNA is especially important as mature spermatozoa have limited DNA repair capacity; their translation and transcription activities are silenced in the later stages of spermatogenesis.(37)

Remarkably, several studies have shown that not all histones in mature sperm DNA are

replaced by protamines. It is estimated that around 7-15% of the sperm DNA, located in peripheral regions of the mature sperm nucleus, maintains the association with histones.(36, 38) The reason for this is not yet understood, however it is hypothesised that these persisting paternal histones might be critical for the early transcriptional reactivation of the paternal genome.(39) Regardless of the reason, the paternal DNA present in these less tightly packed regions seems more susceptible to damage.(36, 40)

Seminal plasma

The plasma fraction of semen is a combination of secretions from the male accessory sex glands, including the seminal vesicles, the prostate and the bulbourethral glands. The seminal plasma protects and nourishes the spermatozoa upon ejaculation and until subsequent fertilization. It needs to provide an optimal pH and viscosity to ensure sperm viability and motility. The majority of seminal plasma is produced by the seminal vesicles, which also provide fructose as an energy source for adenosine triphosphate (ATP) production. The remainder of the seminal plasma is secreted by the prostate and bulbourethral glands, in the form of mucus that serves as a lubricant for the passage of sperm through the male reproductive tract, as well as buffers which neutralise the acid milieu in the male urethra and vagina.(41)

Besides serving as a nutritive protective medium for spermatozoa, seminal plasma contains a wide variety of bioactive signalling molecules: cytokines, chemokines, prostaglandins and other immunological factors. These factors are produced by the Leydig and Sertoli cells, the seminal vesicles and glands, and leukocytes and other immune cells present in the male reproductive tract.(42)

In the seminal plasma of healthy fertile men, inflammatory factors, immune regulatory factors and growth factors can be detected.(43) Key inflammatory factors include interleukins (IL)-1α, IL-1β, IL-2, IL-6, IL-8, IL-12, IL-16, IL-18, tumour necrosis factor (TNF)-α and interferon (IFN)-γ. Immune regulatory agents abundantly present in the seminal plasma include transforming growth factor (TGF)-β in three isoforms (TGF-β1, TGF-β2 and TGF-β3) and prostaglandin E (PGE). Other factors with potentially regulatory roles include soluble human leukocyte antigen-G (sHLA-G) and sHLA class-I. In addition, seminal plasma contains growth factors such as VEGF, FGF, G-CSF and GM-CSF.(44) Previous research indicated that, after encountering female tissues, these immunologically active constituents of the seminal plasma affect the maternal immune system.(45) As a consequence, this may affect fertility and pregnancy outcome, which will be discussed in more detail later.

Associations between semen factors and RPL

Conventional semen analysis is performed following the World Health Organization

(WHO) guidelines, which provides reference values for the parameters volume, concentration and motility.(46) This analysis plays an important role in the evaluation of male infertility. Several studies have compared conventional sperm parameters between men in RPL couples and healthy fertile controls.(47-52) Based on these studies, no consistent association between these semen parameters and RPL was found.(5) Most studies showed no differences in sperm volume and concentration. Some studies reported a lower percentage of motile sperm or sperm with abnormal morphology, while other studies did not find any significant differences.

As a consequence of this lack of association between standard semen parameters and RPL, most recent studies addressing the male contribution to RPL have focused on other semen factors, mainly genetic defects. These genetic defects included chromatin integrity, Y chromosomal deletions, chromosomal anomalies and DNA damage.(53-56) Based on the available studies, anomalies and Y chromosomal deletions have no relation with RPL, while sperm DNA seems to be the most promising factor. Although some studies have been performed to explore the levels of seminal plasma cytokines and other immunological factors in fertile and infertile populations(43, 57, 58), no studies exist that evaluated the association between seminal plasma cytokine profiles and RPL.

Two biological theories regarding the potential male contribution to RPL exist that served as a foundation for studies presented in this thesis. The first theory is focused on DNA damage of the spermatozoa (Figure 3) and the second theory concerns impaired immunomodulation in the female reproductive tract due to disbalances in the seminal plasma (Figure 4). These theories will be further explained in the next sections.

Increased levels of sperm DNA fragmentation

Impaired DNA integrity does not necessarily prevent spermatozoa from successfully fertilizing an oocyte(59-61). If excessive sperm DNA damage is present, exceeding the repair capacity of the oocyte, this may cause complications after fertilization.(62) Two systematic reviews and meta-analyses compared the rates of sperm DNA fragmentation between male partners of women with RPL and a fertile control group.(63, 64) Findings of these reviews supported a significant association between sperm DNA fragmentation and RPL; both articles reported a pooled estimate of 12% higher sperm DNA fragmentation in the RPL group.

Sperm DNA damage can be induced by several mechanisms, during different stages of production and transport of the spermatozoa.(65) During the process of spermatogenesis, in part of the germ cells apoptosis (a genetically controlled programmed form of cell death) is induced, to limit the size of the germ cell population and to prevent defective germ cells from entering further stages of maturation. However, this screening

mechanism does not operate without errors and cells destined to be eliminated undergo only a partial maturational arrest and stills end up in the ejaculate, while their genomic integrity is affected. This is called abortive apoptosis. Another cause of sperm DNA damage is attributed to alterations in chromatin remodelling. And finally, DNA strand breaks may be induced by a rise in oxidative stress due to an abundance of reactive oxygen species (ROS).

ROS are highly reactive molecules containing oxygen that are important for physiologic cellular functioning such as destruction of infectious agents and intracellular signalling. (66) However, overproduction of ROS can cause damage. To maintain the right balance the human body has antioxidant defence mechanisms. When the production of ROS overwhelms these defence mechanisms, oxidative stress may occur (Figure 3).(67) ROS has the ability to directly disrupt sperm DNA integrity by attacking purine and pyrimidine bases of the deoxyribose backbone as well as by initiating apoptosis. Spermatozoa with oxidative DNA damage that achieve fertilisation may often lead to pregnancy loss in terms of embryo failure at the blastocyst stage or later during the early fetal stage. (68) However, there is still the potential of an ongoing pregnancy and previous studies suggested that damaged paternally derived DNA may also trigger the formation of *de novo* mutations and lead to more long term consequences in the offspring, including the initiation of genetic effects.(69-71)

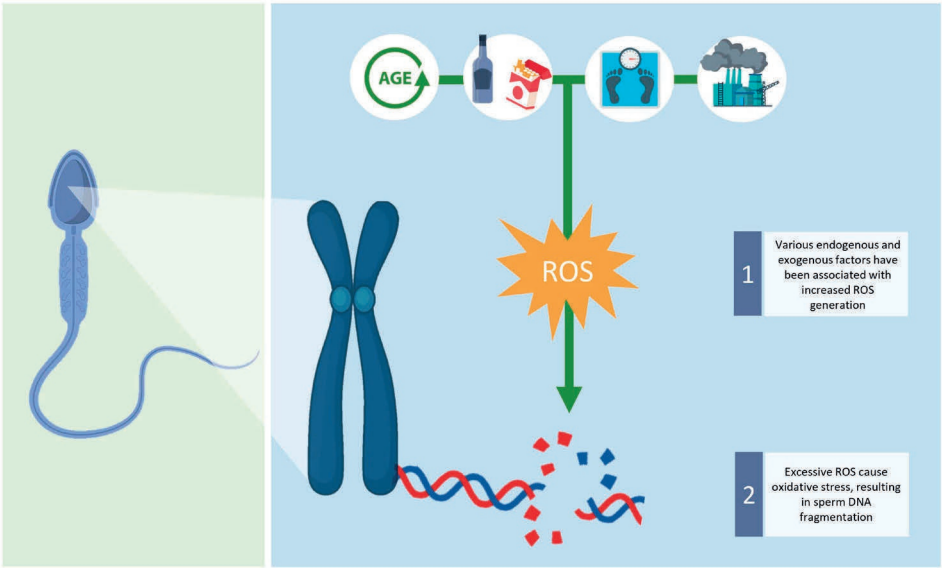


Figure 3. Sperm oxidative DNA damage
ROS = reactive oxygen species

Various known origins of sperm oxidative stress exist. Factors that have been associated with ROS generation and sperm oxidative stress include advanced age, obesity, dietary deficiencies, lack of exercise or extreme exercise, cigarette smoking, excessive consumption of alcohol or recreational drugs, infection, the presence of varicocele and exposure to chemotherapy, radiotherapy and several environmental pollutants.(68, 71)

Perturbations in seminal plasma-driven maternal immunoregulation

The phenomenon of a successful pregnancy is fascinating in many ways and certainly from an immunological point of view. To secure the maintenance of a pregnancy, the maternal immune system has to accept a semi-foreign body: the embryo. This requires an environment of active maternal immune tolerance. Although many studies have focused on the mechanisms underlying the so-called immunological paradox of pregnancy, it is still not fully understood how the semi-allogeneic embryo (and later in gestation: the fetus) escapes rejection by the maternal immune system. It has been hypothesized and supported by several previous animal studies and human *in vitro* studies that seminal plasma has the capacity to modulate the course of the maternal immune response and, as a consequence, has the potential to affect fertility and pregnancy outcome.(42, 44, 45) Therefore, perturbations in seminal plasma-induced maternal immunoregulation may lead to pregnancy loss.

The effects of seminal plasma exposure on female tissues are best described in mouse models. Hours after seminal plasma deposition, various immune cells including macrophages, dendritic cells and granulocytes are recruited into the endometrial stroma and lumen. Dendritic cells take up antigens present in the seminal plasma and transport these antigens to local lymph nodes.(72-74) This induces the activation and expansion of populations of regulatory T cells (Treg cells).(75) These specific Treg populations are reactive with seminal fluid major histocompatibility (MHC) antigens and known for their potent immunosuppressive competence.(76) After migrating into the endometrium, the Treg cells are thought to promote embryo implantation, as the embryo expresses the same paternally derived antigens as present in the seminal plasma.(74) If the Treg cells have sufficient suppressive capacity, they will support placentation and suppress the inflammation that would otherwise cause rejection of the semi-allogeneic embryo.(77)

As substantiated in previous research, seminal plasma does interact with human female tissues in a similar way (Figure 4). A study that analysed small cervical biopsies before and after unprotected coitus demonstrated an induction of a range of cytokines and chemokines that consequently lead to the infiltration of immune cells including macrophages, dendritic cells and T cells.(78) The response to seminal plasma has also been modelled in human *in vitro* experiments, which showed that ectocervical epithelial cells upregulated cytokine and chemokine expression after contact with seminal plasma.

(79) Other human *in vitro* studies showed that incubation of peripheral blood T cells and monocytes wit seminal plasma induced alterations in mRNA expression compatible with the induction of more tolerogenic phenotypes.(80, 81)

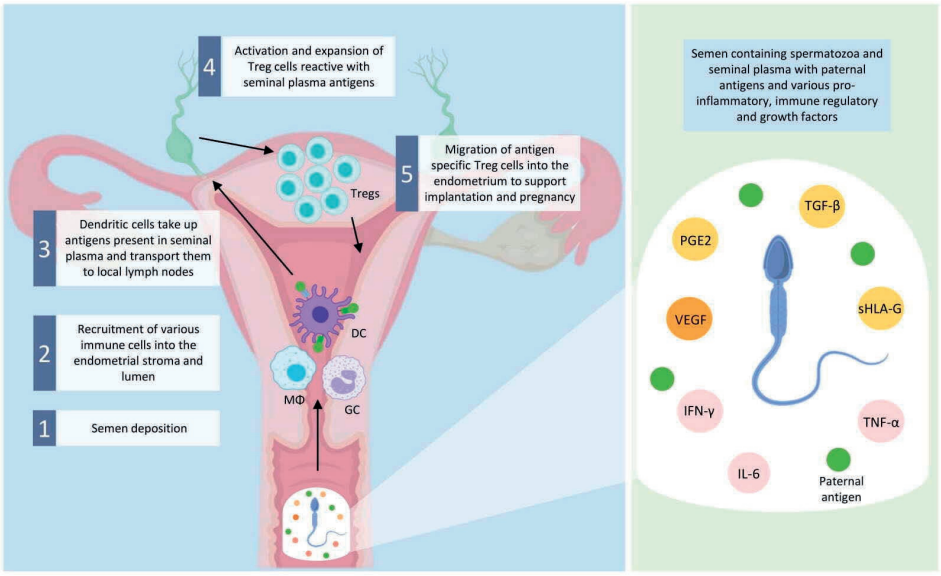


Figure 4. Maternal response to semen deposition

DC = dendritic cell; GC = granulosa cell; MΦ = macrophage; Tregs = regulatory T cells

To support a state of active maternal immunotolerance, it has been hypothesized that there needs to be an optimal balance between pro-inflammatory and immunoregulatory signalling molecules in the seminal plasma.(44) Pro-inflammatory factors present in the seminal plasma induce initial inflammatory effects resulting in the recruitment of lymphocytes and antigen presenting cells, needed to develop an immune response towards seminal plasma paternal antigens. In addition, pro-inflammatory markers including IFN-γ, TNF-α and IL-1β were shown to stimulate trophoblastic angiogenesis by regulating the release of VEGF.(82) On the other hand, a seminal plasma profile with excessively high inflammatory markers has been linked with infertility and pregnancy complications.(42, 44, 81) A principal regulatory trigger present in the seminal plasma is TGF-β. TGF-β has a potent effect on the proliferation and differentiation of various immune cells. Together with other tolerance-inducing seminal plasma agents including PGE2, sHLA-G and IL-10, TGF-β is considered essential in establishing a favourable maternal immune environment that supports pregnancy.(44, 45)

It is known that substantial variation in seminal plasma content may exist between individuals.(83) Some prior studies measured levels of cytokines, chemokines and other immunological factors in healthy fertile men, to provide reference values for studies in pathologic conditions.(43, 84) Very little is known about the link between seminal plasma expression profiles and pregnancy-related disorders including RPL. Furthermore, it remains to be explored whether differences in seminal plasma content are related to male lifestyle and environmental factors.

PSYCHOLOGICAL BURDEN AND SUPPORTIVE CARE

Pregnancy loss is a common complication of pregnancy and brings substantial disruption to the lives of many. It may lead to disorientation, depression and anxiety.(85) It is conceivable that these feelings may intensify in case of recurrence. Indeed, couples who encounter RPL may experience cumulative negative psychological effects, including feelings of grief and loss as well as fear of chronic pathology and never having a successful pregnancy.(86-89)

A Danish study showed that 8% of women with RPL scored moderate to severe depression rates, compared to 2% in a control group.(90) Although most studies investigating the psychological impact of RPL were restricted to women, Voss et al.(91) recently assessed psychological risks and coping strategies in both women with RPL and their male partners. They found that both men and women affected by RPL show high risks of developing anxiety and depression, although risks in women were significantly higher compared to men. For men, the most burdening issues included worries about their partner, the desire for a child and their job situation. While women have higher risks with regard to depression and anxiety, men showed higher scores of limited social support than their female partners.

In the qualitative study of Koert et al.(92) male partners of women who had experienced RPL indicated that they felt pressured to stay positive and to support their partner, while they were also grieving. Furthermore, this study highlighted a discrepancy between the couples' perceived needs and their experience of care after RPL. The couples desired sensitivity, empathy and recognition of their losses from the medical staff and they stressed that these desires were not always met during their experiences in hospital. Furthermore, the study participants believed that care for RPL should include psychological support, both in the period during and after a pregnancy loss and during a subsequent pregnancy. It should be mentioned however that the results of this study were based on a small, self-selected sample of couples with RPL.

Current international clinical guidelines recommend to organise care for couples with RPL in specialised outpatient clinics, where both investigations and possible treatments as supportive care should be offered. These clinics should provide a dedicated and focused service to couples affected by RPL, should take the psychological needs of the couples into account and offer them supportive care. A study of Musters et al.(93) explored what is actually perceived as supportive care by women with RPL. In their qualitative in-depth interviewing study they identified 20 supportive care options preferred by the women. In a subsequent questionnaire study by the same authors(94), women's preferences for the different supportive care options were quantified. Examples of the preferred

supportive care services were to make a plan with their doctor for their next pregnancy, frequently repeated ultrasound examinations during early pregnancy and continuity of care in terms of seeing the same doctor during different consultations. Moreover, they desired a doctor who is specialised in RPL and can provide reliable information but also has excellent communication and pays attention to psychological aspects and emotional needs.

Importantly, the preferences of the male partner were not taken into account in the above mentioned studies of Musters et al.(93, 94) It remains unclear whether the needs of men and women who faced RPL differ and also whether men feel sufficiently involved in RPL clinics. A previous systematic review on patient-centred early pregnancy care underscored that women and their partners undergoing (recurrent) pregnancy loss appreciate an individual approach.(95) However, the perspective of the male partner was examined in only three out of 27 studies included in this review. Since previous studies indicated that men do also suffer from RPL and they experience less (social) support compared to women, more research into support requirements of men confronted with RPL is warranted.

AIMS AND OUTLINE OF THIS THESIS

In order to improve care for couples with RPL, it is essential to increase knowledge on the risk factors and underlying pathophysiological mechanisms of this condition. This should lead to additional targets for diagnostic testing as well as novel therapeutic strategies. Besides a better understanding of the aetiology of RPL, it is of great importance to better estimate the prognosis of individual couples. This may provide an answer to a critical question of couples with RPL: what is the chance of a future successful pregnancy? In order to expand current knowledge, we must go off the beaten track of the studies that have been performed previously.

The aim of the research presented in this thesis is to gain more insight into the male contribution to RPL. We evaluate the relation between paternal factors and RPL both from an epidemiological and immunological point of view. In addition, we investigate the preferences for supportive care of both men and women affected by RPL.

- In **chapters 2 and 3**, the current literature regarding the link between paternal age and lifestyle factors and the risk of pregnancy loss is evaluated. In both chapters a systematic review with meta-analysis is presented.
- In **chapter 4,** a study protocol is presented to evaluate the role of paternal lifestyle and biological factors in the aetiology and prognosis of RPL, which involves both a case-control study and a cohort study (the REMI III project).
- In **chapter 5**, the results of a prediction study are shown. This study was performed to identify, besides maternal age and the number of previous pregnancy losses, additional characteristics of women and men affected by RPL that improve prediction of the chance of a subsequent ongoing pregnancy.
- In **chapters 6 and 7**, we zoom in on the role of seminal plasma in relation to RPL. In **chapter 6**, the association between seminal plasma cytokine expression profiles and clinical and lifestyle characteristics is discussed. To evaluate the immunomodulating effect of seminal plasma on female immune cells, we performed an in vitro study, which is described in **chapter 7**.
- In **chapter 8**, we focus on supportive care for couples with RPL. The results of a questionnaire study are presented, which quantified both female and male preferences for supportive care after RPL.
- Finally, in **chapter 9**, a summary of all studies presented in this thesis is provided and the findings are discussed and placed in a broader perspective, including opportunities for future research.

1

REFERENCES

1. Hartsoeker N. Essai de Dioptrique. Paris, 1694.
2. Lawrence CR. Hartsoeker's Homunculus Sketch from Essai de Dioptrique 2008 [Available from: http://embryo.asu.edu/handle/10776/1924].
3. Pinto-Correia C. The Ovary of Eve: Egg and Sperm and Preformation. Chicago: University of Chicago Press; 1997.
4. Alijotas-Reig J, Garrido-Gimenez C. Current concepts and new trends in the diagnosis and management of recurrent miscarriage. Obstet Gynecol Surv. 2013;68(6):445-66.
5. Bender Atik R, Christiansen OB, Elson J, Kolte AM, Lewis S, Middeldorp S, et al. ESHRE guideline: recurrent pregnancy loss. Hum Reprod. 2018;2018(2).
6. Quenby S, Gallos ID, Dhillon-Smith RK, Podesek M, Stephenson MD, Fisher J, et al. Miscarriage matters: the epidemiological, physical, psychological, and economic costs of early pregnancy loss. The Lancet. 2021;397(10285):1658-67.
7. Kolte AM, Bernardi LA, Christiansen OB, Quenby S, Farquharson RG, Goddijn M, et al. Terminology for pregnancy loss prior to viability: a consensus statement from the ESHRE early pregnancy special interest group. Hum Reprod Online. 2015;30(3):495-8.
8. Youssef A, Lashley L, Dieben S, Verburg H, van der Hoorn ML. Defining recurrent pregnancy loss: associated factors and prognosis in couples with two versus three or more pregnancy losses. Reprod Biomed Online. 2020;41(4):679-85.
9. Ziakas PD, Pavlou M, Voulgarelis M. Heparin treatment in antiphospholipid syndrome with recurrent pregnancy loss: a systematic review and meta-analysis. Obstet Gynecol. 2010;115(6):1256-62.
10. Rikken JFW, Kowalik CR, Emanuel MH, Bongers MY, Spinder T, Jansen FW, et al. Septum resection versus expectant management in women with a septate uterus: an international multicentre open-label randomized controlled trial. Hum Reprod. 2021;36(5):1260-7.
11. Alexander EK, Pearce EN, Brent GA, Brown RS, Chen H, Dosiou C, et al. 2017 Guidelines of the American Thyroid Association for the Diagnosis and Management of Thyroid Disease During Pregnancy and the Postpartum. Thyroid. 2017;27(3):315-89.
12. Coomarasamy A, Dhillon-Smith RK, Papadopoulou A, Al-Memar M, Brewin J, Abrahams VM, et al. Recurrent miscarriage: evidence to accelerate action. The Lancet. 2021;397(10285):1675-82.
13. Lashen H, Fear K, Sturdee DW. Obesity is associated with increased risk of first trimester and recurrent miscarriage: matched case-control study. Hum Reprod. 2004;19(7):1644-6.
14. Lo W, Rai R, Hameed A, Brailsford SR, Al-Ghamdi AA, Regan L. The effect of body mass index on the outcome of pregnancy in women with recurrent miscarriage. J Family Community Med. 2012;19(3):167-71.
15. Maconochie N, Doyle P, Prior S, Simmons R. Risk factors for first trimester miscarriage--results from a UK-population-based case-control study. BJOG. 2007;114(2):170-86.
16. Andersen AM, Andersen PK, Olsen J, Grønbæk M, Strandberg-Larsen K. Moderate alcohol intake during pregnancy and risk of fetal death. Int J Epidemiol. 2012;41(2):405-13.
17. Avalos LA, Roberts SCM, Kaskutas LA, Block G, Li D-K. Volume and type of alcohol during early pregnancy

and the risk of miscarriage. Subst Use Misuse. 2014;49(11):1437-45.

18. Greenwood DC, Alwan N, Boylan S, Cade JE, Charvill J, Chipps KC, et al. Caffeine intake during pregnancy, late miscarriage and stillbirth. Eur J Epidemiol. 2010;25(4):275-80.
19. Stefanidou EM, Caramellino L, Patriarca A, Menato G. Maternal caffeine consumption and sine causa recurrent miscarriage. Eur J Obstet Gynecol Reprod Biol. 2011;158(2):220-4.
20. Leviton A. Biases Inherent in Studies of Coffee Consumption in Early Pregnancy and the Risks of Subsequent Events. Nutrients. 2018;10(9):1152.
21. Lynch CD, Sundaram R, Buck Louis GM. Biomarkers of preconception stress and the incidence of pregnancy loss. Hum Reprod. 2018;33(4):728-35.
22. Zhang L, Liu W, Hou K, Lin J, Zhou C, Tong X, et al. Air pollution-induced missed abortion risk for pregnancies. Nature Sustainability. 2019;2(11):1011-7.
23. Naidoo S, London L, Burdorf A, Naidoo R, Kromhout H. Spontaneous miscarriages and infant deaths among female farmers in rural South Africa. Scand J Work Environ Health. 2011;37(3):227-36.
24. Pathak R, Mustafa M, Ahmed RS, Tripathi AK, Guleria K, Banerjee BD. Association between recurrent miscarriages and organochlorine pesticide levels. Clin Biochem. 2010;43(1-2):131-5.
25. Fatemi HM, Lawrenz B. The search must go on-for patients with recurrent pregnancy loss. Fertil Steril. 2020;113(4):769-70.
26. Oldereid NB, Wennerholm UB, Pinborg A, Loft A, Laivuori H, Petzold M, et al. The effect of paternal factors on perinatal and paediatric outcomes: a systematic review and meta-analysis. Hum Reprod Update. 2018;24(3):320-89.
27. Nybo Andersen AM, Hansen KD, Andersen PK, Davey Smith G. Advanced paternal age and risk of fetal death: a cohort study. Am J Epidemol. 2004;160(12):1214-22.
28. Kleinhaus K, Perrin M, Friedlander Y, Paltiel O, Malaspina D, Harlap S. Paternal age and spontaneous abortion. Obstet Gynecol. 2006;108(2):369-77.
29. Nguyen BT, Chang EJ, Bendikson KA. Advanced paternal age and the risk of spontaneous abortion: an analysis of the combined 2011-2013 and 2013-2015 National Survey of Family Growth. Am J Obstet Gynecol. 2019;221(5):476.e1-.e7.
30. Windham GC, Von Behren J, Waller K, Fenster L. Exposure to environmental and mainstream tobacco smoke and risk of spontaneous abortion. Am J Epidemiol. 1999;149(3):243-7.
31. Venners SA, Wang X, Chen C, Wang L, Chen D, Guang W, et al. Paternal smoking and pregnancy loss: a prospective study using a biomarker of pregnancy. Am J Epidemiol. 2004;159(10):993-1001.
32. 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.
33. Henriksen TB, Hjollund NH, Jensen TK, Bonde JP, Andersson AM, Kolstad H, et al. Alcohol consumption at the time of conception and spontaneous abortion. Am J Epidemiol. 2004;160(7):661-7.
34. Ruixue W, Hongli Z, Zhihong Z, Rulin D, Dongfeng G, Ruizhi L. The impact of semen quality, occupational exposure to environmental factors and lifestyle on recurrent pregnancy loss. J Assist Reprod Genet. 2013;30(11):1513-8.
35. Ioannou D, Miller D, Griffin DK, Tempest HG. Impact of sperm DNA chromatin in the clinic. J Assist Reprod

Genet 2016;33(2):157-66.

36. Champroux A, Torres-Carreira J, Gharagozloo P, Drevet JR, Kocer A. Mammalian sperm nuclear organization: resiliencies and vulnerabilities. Basic Clin Androl. 2016;26(1):17.
37. Leduc F, Nkoma GB, Boissonneault G. Spermiogenesis and DNA Repair: A Possible Etiology of Human Infertility and Genetic Disorders. Syst Biol Reprod Med. 2008;54(1):3-10.
38. Tanphaichitr N, Sobhon P, Taluppeth N, Chalermisarachai P. Basic nuclear proteins in testicular cells and ejaculated spermatozoa in man. Exp Cell Res. 1978;117(2):347-56.
39. Hammoud SS, Nix DA, Zhang H, Purwar J, Carrell DT, Cairns BR. Distinctive chromatin in human sperm packages genes for embryo development. Nature. 2009;460(7254):473-8.
40. Kocer A, Henry-Berger J, Noblanc A, Champroux A, Pogorelcnik R, Guiton R, et al. Oxidative DNA damage in mouse sperm chromosomes: Size matters. Free Radic Biol Med. 2015;89:993-1002.
41. Jones RE, Lopez KH. Chapter 9 - Gamete Transport and Fertilization. In: Jones RE, Lopez KH, editors. Human Reproductive Biology (Fourth Edition). San Diego: Academic Press; 2014. p. 159-73.
42. Fraczek M, Kurpisz M. Cytokines in the male reproductive tract and their role in infertility disorders. J Reprod Immunol. 2015;108:98-104.
43. 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.
44. Nederlof I, Meuleman T, van der Hoorn MLP, Claas FHJ, Eikmans M. The seed to success: The role of seminal plasma in pregnancy. J Reprod Immunol. 2017;123:24-8.
45. Schjenken JE, Robertson SA. The Female Response to Seminal Fluid. Physiol Rev. 2020;100(3):1077-117.
46. WHO. WHO laboratory manual for the examination and processing of human semen 2010, 5th ed. [Available from: https://www.who.int/docs/default-source/reproductive-health/srhr-documents/infertility/examination-and-processing-of-human-semen-5ed-eng.pdf.]
47. Bhattacharya SM. Association of various sperm parameters with unexplained repeated early pregnancy loss--which is most important? Int Urol Nephrol. 2008;40(2):391-5.
48. Brahem S, Mehdi M, Landolsi H, Mougou S, Elghezal H, Saad A. Semen parameters and sperm DNA fragmentation as causes of recurrent pregnancy loss. Urology. 2011;78(4):792-6.
49. Imam SN, Shamsi MB, Kumar K, Deka D, Dada R. Idiopathic recurrent pregnancy loss: role of paternal factors; a pilot study. J Reprod Infertil. 2011;12(4):267-76.
50. Talebi AR, Vahidi S, Aflatoonian A, Ghasemi N, Ghasemzadeh J, Firoozabadi RD, et al. Cytochemical evaluation of sperm chromatin and DNA integrity in couples with unexplained recurrent spontaneous abortions. Andrologia. 2012;44 Suppl 1:462-70.
51. Zhang L, Wang L, Zhang X, Xu G, Zhang W, Wang K, et al. Sperm chromatin integrity may predict future fertility for unexplained recurrent spontaneous abortion patients. Int J Androl. 2012;35(5):752-7.
52. Khadem N, Poorhoseyni A, Jalali M, Akbary A, Heydari ST. Sperm DNA fragmentation in couples with unexplained recurrent spontaneous abortions. Andrologia. 2014;46(2):126-30.
53. Kaare M, Painter JN, Ulander VM, Kaaja R, Aittomäki K. Sex chromosome characteristics and recurrent miscarriage. Fertil Steril. 2008;90(6):2328-33.
54. Wettasinghe TK, Jayasekara RW, Dissanayake VH. Y chromosome microdeletions are not associated with spontaneous recurrent pregnancy loss in a Sinhalese population in Sri Lanka. Hum Reprod.

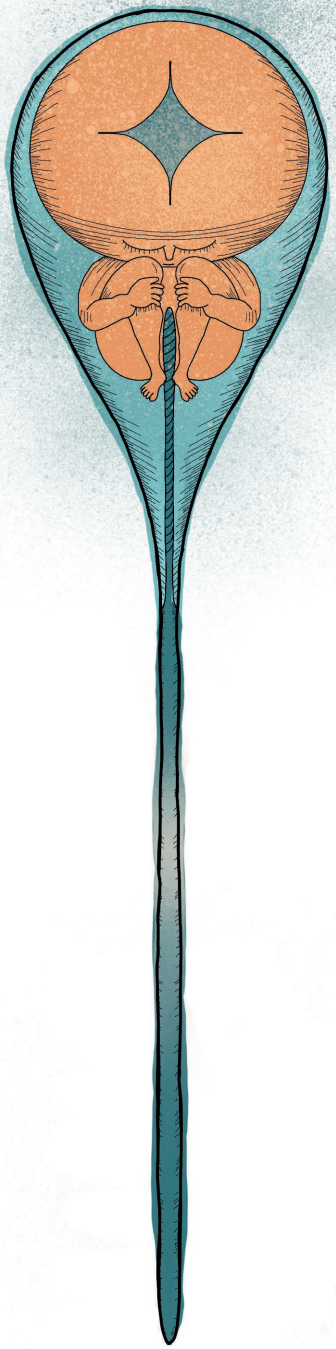
2010;25(12):3152-6.

55. Robinson L, Gallos ID, Conner SJ, Rajkhowa M, Miller D, Lewis S, et al. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. Human Reprod. 2012;27(10):2908-17.
56. Zhao J, Zhang Q, Wang Y, Li Y. Whether sperm deoxyribonucleic acid fragmentation has an effect on pregnancy and miscarriage after in vitro fertilization/intracytoplasmic sperm injection: a systematic review and meta-analysis. Fertil Steril. 2014;102(4):998-1005.e8.
57. Havrylyuk A, Chopyak V, Boyko Y, Kril I, Kurpisz M. Cytokines in the blood and semen of infertile patients. Cent Eur J Immunol. 2015;40(3):337-44.
58. Seshadri S, Bates M, Vince G, Jones DI. Cytokine expression in the seminal plasma and its effects on fertilisation rates in an IVF cycle. Andrologia. 2011;43(6):378-86.
59. Lopes S, Sun JG, Jurisicova A, Meriano J, Casper RF. Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. Fertil Steril. 1998;69(3):528-32.
60. Gandini L, Lombardo F, Paoli D, Caruso F, Eleuteri P, Leter G, et al. Full-term pregnancies achieved with ICSI despite high levels of sperm chromatin damage. Hum Reprod. 2004;19(6):1409-17.
61. Aitken RJ, De Iuliis GN, McLachlan RI. Biological and clinical significance of DNA damage in the male germ line. Int J Androl. 2009;32(1):46-56.
62. Cozzubbo T, Neri QV, Rosenwaks Z, Palermo GD. To what extent can oocytes repair sperm DNA fragmentation? Fertil Steril. 2014;102(3):e61.
63. McQueen DB, Zhang J, Robins JC. Sperm DNA fragmentation and recurrent pregnancy loss: a systematic review and meta-analysis. Fertil Steril. 2019.
64. 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.
65. Sakkas D, Alvarez JG. Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. Fertil Steril. 2010;93(4):1027-36.
66. Bayr H. Reactive oxygen species. Crit Care Med. 2005;33(12).
67. Agarwal A, Saleh RA. Role of oxidants in male infertility: rationale, significance, and treatment. Urol Clin North Am. 2002;29(4):817-27.
68. Tremellen K. Oxidative stress and male infertility—a clinical perspective. Hum Reprod Update. 2008;14(3):243-58.
69. Xavier MJ, Nixon B, Roman SD, Scott RJ, Drevet JR, Aitken RJ. Paternal impacts on development: identification of genomic regions vulnerable to oxidative DNA damage in human spermatozoa. Hum Reprod. 2019;34(10):1876-90.
70. Aitken RJ, Baker MA, Sawyer D. Oxidative stress in the male germ line and its role in the aetiology of male infertility and genetic disease. Reprod Biomed Online. 2003;7(1):65-70.
71. Aitken RJ, Baker MA. The Role of Genetics and Oxidative Stress in the Etiology of Male Infertility-A Unifying Hypothesis? Front Endocrinol. 2020;11:581838.
72. De M, Choudhuri R, Wood GW. Determination of the number and distribution of macrophages, lymphocytes, and granulocytes in the mouse uterus from mating through implantation. J Leukoc Biol.

1991;50(3):252-62.

73. Robertson SA, Mau VJ, Tremellen KP, Seamark RF. Role of high molecular weight seminal vesicle proteins in eliciting the uterine inflammatory response to semen in mice. J Reprod Fertil. 1996;107(2):265-77.
74. Robertson SA, Sharkey DJ. Seminal fluid and fertility in women. Fertil Steril. 2016;106(3):511-9.
75. Moldenhauer LM, Diener KR, Thring DM, Brown MP, Hayball JD, Robertson SA. Cross-presentation of male seminal fluid antigens elicits T cell activation to initiate the female immune response to pregnancy. J Immunol. 2009;182(12):8080-93.
76. Robertson SA, Guerin LR, Bromfield JJ, Branson KM, Ahlstrom AC, Care AS. Seminal fluid drives expansion of the CD4+CD25+ T regulatory cell pool and induces tolerance to paternal alloantigens in mice. Biol Reprod. 2009;80(5):1036-45.
77. Guerin LR, Prins JR, Robertson SA. Regulatory T-cells and immune tolerance in pregnancy: a new target for infertility treatment? Hum Reprod Update. 2009;15(5):517-35.
78. Sharkey DJ, Tremellen KP, Jasper MJ, Gemzell-Danielsson K, Robertson SA. Seminal fluid induces leukocyte recruitment and cytokine and chemokine mRNA expression in the human cervix after coitus. J Immunol. 2012;188(5):2445-54.
79. Sharkey DJ, Macpherson AM, Tremellen KP, Robertson SA. Seminal plasma differentially regulates inflammatory cytokine gene expression in human cervical and vaginal epithelial cells. Mol Hum Reprod. 2007;13(7):491-501.
80. 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.
81. 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.
82. Choi SJ, Park JY, Lee YK, Choi HI, Lee YS, Koh CM, et al. Effects of cytokines on VEGF expression and secretion by human first trimester trophoblast cell line. Am J Reprod Immunol. 2002;48(2):70-6.
83. Sharkey DJ, Tremellen KP, Briggs NE, Dekker GA, Robertson SA. Seminal plasma pro-inflammatory cytokines interferon-γ (IFNG) and C-X-C motif chemokine ligand 8 (CXCL8) fluctuate over time within men. Hum Reprod. 2017;32(7):1373-81.
84. 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.
85. Meaney S, Corcoran P, Spillane N, O'Donoghue K. Experience of miscarriage: an interpretative phenomenological analysis. BMJ Open. 2017;7(3):e011382.
86. Bardos J, Hercz D, Friedenthal J, Missmer SA, Williams Z. A national survey on public perceptions of miscarriage. Obstet Gynecol. 2015;125(6):1313-20.
87. Brier N. Grief following miscarriage: a comprehensive review of the literature. J Womens Health (2002). 2008;17(3):451-64.
88. Kagami M, Maruyama T, Koizumi T, Miyazaki K, Nishikawa-Uchida S, Oda H, et al. Psychological adjustment and psychosocial stress among Japanese couples with a history of recurrent pregnancy loss. Hum Reprod. 2012;27(3):787-94.
89. Kolte AM, Olsen LR, Mikkelsen EM, Christiansen OB, Nielsen HS. Depression and emotional stress is highly prevalent among women with recurrent pregnancy loss. Hum Reprod.2015;30(4):777-82.

90. Hedegaard S, Landersoe SK, Olsen LR, Krog MC, Kolte AM, Nielsen HS. Stress and depression among women and men who have experienced recurrent pregnancy loss: focusing on both sexes. Reprod Biomed Online. 2021.
91. 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.
92. Koert E, Malling GMH, Sylvest R, Krog MC, Kolte AM, Schmidt L, et al. Recurrent pregnancy loss: couples' perspectives on their need for treatment, support and follow up. Hum Reprod. 2018;34(2):291-6.
93. 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.
94. 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.
95. van den Berg MMJ, Dancet EAF, Erlikh T, van der Veen F, Goddijn M, Hajenius PJ. Patient-centered early pregnancy care: a systematic review of quantitative and qualitative studies on the perspectives of women and their partners. Hum Reprod Update. 2018;24(1):106-18.



CHAPTER 2

Advanced paternal age is associated with an increased risk of spontaneous miscarriage: a systematic review and meta-analysis

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ABSTRACT

Background

Although spontaneous miscarriage is the most common complication of human pregnancy, potential contributing factors are not fully understood. Advanced maternal age has long been recognised as a major risk factor for miscarriage, being strongly related with fetal chromosomal abnormalities. The relation between paternal age and the risk of miscarriage is less evident, yet it is biologically plausible that an increasing number of genetic and epigenetic sperm abnormalities in older males may contribute to miscarriage. Previous meta-analyses showed associations between advanced paternal age and a broad spectrum of perinatal and paediatric outcomes. This is the first systematic review and meta-analysis on paternal age and spontaneous miscarriage.

Objective and rationale

The aim of this systematic review and meta-analysis is to evaluate the effect of paternal age on the risk of spontaneous miscarriage.

Search methods

PubMed, Embase and Cochrane databases were searched to identify relevant studies up to August 2019. The following free text and MeSH terms were used: paternal age, father's age, male age, husband's age, spontaneous abortion, spontaneous miscarriage, abortion, miscarriage, pregnancy loss, fetal loss and fetal death. PRISMA guidelines for systematic reviews and meta-analysis were followed. Original research articles in English language addressing the relation between paternal age and spontaneous miscarriage were included. Exclusion criteria were studies that solely focused on pregnancy outcomes following artificial reproductive technology (ART) and studies that did not adjust their effect estimates for at least maternal age. Risk of bias was qualitatively described for three domains: bias due to confounding, information bias and selection bias.

Outcomes

The search resulted in 975 original articles. Ten studies met the inclusion criteria and were included in the qualitative synthesis. Nine of these studies were included in the quantitative synthesis (meta-analysis). Advanced paternal age was found to be associated with an increased risk of miscarriage. Pooled risk estimates for miscarriage for age categories 30-34, 35-39, 40-44 and ≥45 years of age were 1.04 (95% CI 0.90, 1.21), 1.15 (0.92, 1.43), 1.23 (1.06, 1.43) and 1.43 (1.13, 1.81) respectively (reference category 25-29 years). A second meta-analysis was performed for the subgroup of studies investigating first trimester miscarriage. This showed similar pooled risk estimates for the first three age categories and a slightly higher pooled risk estimate for age category ≥45 years (1.74; 95% CI 1.26, 2.41).

Wider implications

Over the last decades, childbearing at later ages has become more common. It is known that frequencies of adverse reproductive outcomes, including spontaneous miscarriage, are higher in women with advanced age. We show that advanced paternal age is also associated with an increased risk of spontaneous miscarriage. Although the paternal age effect is less pronounced than that observed with advanced maternal age and residual confounding by maternal age cannot be excluded, it may have implications for preconception counselling of couples comprising an older aged male.

INTRODUCTION

Advanced maternal age is an extensively studied risk factor for adverse reproductive outcome.(1-10) The reproductive risks associated with advanced maternal age (usually defined as age ≥ 35 years) form an integral part of preconception counselling and are well known to the general public.(11). Moreover, clinical policy is based on this knowledge, for instance, maternal age-related access criteria for in vitro fertilisation (IVF) treatment.(12) In contrast, less attention has been paid to the potential effect of paternal age. There are, however, studies indicating that this is unjustified. In 2018, Oldereid et al. evaluated the influence of paternal factors on a broad spectrum of perinatal and paediatric outcomes.(13) They found associations between advanced paternal age and adverse outcomes in the offspring, particularly with psychiatric disorders like autism spectrum disorders and schizophrenia but also with stillbirth and several birth defects. The age of the father and the mutation rate in the offspring are found to be strongly related, possibly due to the larger number of germline divisions that have occurred in older males.(14, 15) Next to a higher frequency of point mutations, there is evidence suggesting that increasing paternal age is associated with sperm DNA strand breaks, genetic imprinting errors and chromosomal anomalies, all of which are factors related to miscarriage.(16-18) As such, from a biological point of view, it seems justified to consider paternal age as an independent risk factor for miscarriage.

Spontaneous miscarriage is the most common complication of human pregnancy; it is estimated that at least 30% of all pregnancies and 10–15% of clinically recognised pregnancies end in miscarriage.(4, 19) Miscarriage refers to a spontaneous demise of pregnancy before the fetus reaches viability (before 24 weeks of gestational age); however, in many studies it is defined as a pregnancy loss that occurs before 20 completed weeks of gestational age.(20, 21) The majority of studies on miscarriage and its associated factors are focused on female factors. Cytogenetic and chromosomal microarray analysis studies on miscarriage specimens have shown that genetic abnormalities play a role in 50–70% of cases.(22-24) The prevalence of genetic abnormalities is highest in miscarriage samples from the first trimester, particularly in miscarriage samples of embryonic stage.(23) Advanced maternal age is strongly related with fetal chromosomal abnormalities, mainly aneuploid conceptions.(4, 25, 26) Besides maternal age, other factors such as uterine anomalies, poorly controlled diabetes and thyroid autoimmunity are related to miscarriage.(25, 27-29) In addition, associations have been found with behavioural and environmental factors including maternal obesity, smoking, alcohol and caffeine consumption, the use of non-steroidal anti-inflammatory drugs and acute and chronic stress.(30-35)

Despite our current knowledge, the cause of miscarriage is not always well-understood, especially in couples with recurrent miscarriages.(36, 37) Since the male partner contributes half of the genetic material of the embryo, studying paternal factors will possibly contribute to unravelling the complex aetiology of pregnancy loss. This may help to provide answers to affected couples, of whom many experience a high psychological impact and emotional burden.(38)

This is the first systematic review and meta-analysis evaluating the effect of paternal age on spontaneous miscarriage. We provide an overview of epidemiological studies evaluating the association between paternal age and spontaneous miscarriage and we discuss possible underlying explanatory mechanisms.

METHODS

We have conducted a systematic review and meta-analysis following the PRISMA guidelines.(39) This systematic review was registered and accepted for inclusion in the international prospective register of systematic reviews PROSPERO (ID CRD42019132886).

Systematic search

A systematic search of PubMed, Embase and Cochrane electronic databases was performed to identify relevant studies from inception until 12 August 2019. We used the following free text and MeSH terms: paternal age, father's age, male age, husband's age, spontaneous abortion, spontaneous miscarriage, abortion, miscarriage, pregnancy loss, fetal loss, fetal death. The full electronic search strategy for PubMed is shown in Supplementary Table 1. Additional searches in Google Scholar were conducted, and reference lists of identified articles were manually searched for additional relevant references.

The literature search was performed by two researchers (N.F. and E.L.) and a librarian. The results of the search were exported to a citation manager (EndNote), and duplicates were removed. The screening was performed by two researchers (N.F. and E.L.). There were two stages of screening for study inclusion: in the first stage, titles and abstracts were screened and in the second stage, full manuscripts of the articles identified in the initial screening were retrieved and read in detail. Any discordance on selecting studies and assessing risk of bias (see further) was resolved by consensus. If no agreement was obtained, the opinion of a third observer (M.H.) was sought to gain consensus.

Eligibility criteria

Inclusion criteria were original research articles in English language addressing the relation between paternal age and spontaneous miscarriage. Exclusion criteria were studies that solely focused on pregnancy outcomes after artificial reproductive technology (ART) and studies that did not adjust their effect estimates for at least maternal age.

Data extraction

Two reviewers (N.F. and E.L.) extracted data from all selected articles on study design, country, publication year, study period, population characteristics, inclusion and exclusion criteria, exposure and outcome definitions, outcome ascertainment, sample size, type of effect measures, adjusted effect estimates with 95% confidence interval (CI) or P value, variables adjusted for in the analyses and statistical methods of adjustment for maternal age.

Risk of bias assessment

There is lack of a single obvious candidate tool for assessing quality of observational epidemiological studies.(40) Moreover, as stated by Dekkers et al. in the COSMOS-E (Conducting Systematic Reviews and Meta-Analyses of Observational Studies of Etiology) guideline(41), a 'one size fits all' approach for assessing quality of these studies is probably misguided, considering the large heterogeneity in observational research. Therefore, it has been recommended to develop a set of criteria for each observational systematic review and meta-analysis and to assess risk of bias in a qualitative manner.(41)

For the research question of this systematic review, we distinguished three relevant domains of risk of bias: bias due to confounding, information bias and selection bias (including bias due to loss of follow-up or missing data). Risk of bias was assessed by two reviewers (N.F. and E.L.). For each individual study, risk of bias within domains and across domains was assessed and described.

Statistical analysis

The selected studies reported outcomes in adjusted odds ratios (AORs), adjusted hazard ratios (AHRs) and adjusted rate ratios (ARRs) with 95% confidence intervals (CI) or P values. These effect measures were treated equally as risk measures. When standard errors were not reported, we calculated them from 95% CIs or P values. To assess the effect of paternal age on first trimester miscarriage separately, we performed a second meta-analysis for the subgroup of studies that focused on miscarriage <13 weeks.

Most studies used the age category of 25–29 years as the reference category. Two studies(42-44) used <25 years as reference; for these studies the reported AORs were rescaled by dividing the AOR by the reported AOR in age category 25–29 years.

Meta-analyses were stratified by the following paternal age categories: 30–34, 35–39, 40–44 and ≥45 years (similar to that in Oldereid et al.(13)). If a study reported more subcategories (i.e. 45–49 years and ≥50 years), the effect sizes of these categories were pooled using a within study fixed effect meta-analysis. One study(45) reported one odds ratio for the age category 29–39 years. We used the same estimate for both 30–34 and 35–39 years, and we adjusted the standard errors, assuming equal sample sizes in both categories.

Two studies analysed different combinations of paternal age and maternal age ('couple age'). To obtain overall AORs and ARRs for paternal age categories adjusted for maternal age, a weighted regression analysis (using fixed effect regression meta-analysis software) was performed with the estimated log AOR as dependent variable and paternal age and maternal age categories as independent variables.

Evidence of publication bias was assessed through qualitative inspection of a funnel plot. Statistical heterogeneity among studies was assessed by inspecting the heterogeneity (I2) statistics. Because of heterogeneity of study populations and study designs, random-effects meta-analysis with DerSimonian and Laird estimation was used for the main analysis (command metan in Stata 14: StataCorp LLC, TX, USA). For sensitivity analysis, fixed-effect estimates were calculated as well. A second sensitivity analysis was conducted to evaluate the influence of the study with the most extreme estimates, by repeating the meta-analysis with exclusion of this study.

RESULTS

Study selection

Details of the study selection process are shown in the PRISMA Flow Diagram (Fig. 1). The systematic search retrieved a total of 1343 articles: 1337 were identified by the search strategy and six additional articles were identified by hand searching other sources. After removing duplicates, 975 articles remained for first-stage screening. After first-stage screening by reviewing titles and abstracts, 954 articles were excluded and 21 articles were identified to assess the full text for eligibility. After this second stage of screening, 11 articles were excluded for reasons that are shown in Fig. 1. Finally, 10 articles met all the inclusion criteria. These were included in this review and were potentially appropriate to be included in meta-analysis. One study was excluded from meta-analysis, because of a different reference category and extremely high risk estimates, which is further explained in the narrative synthesis section.

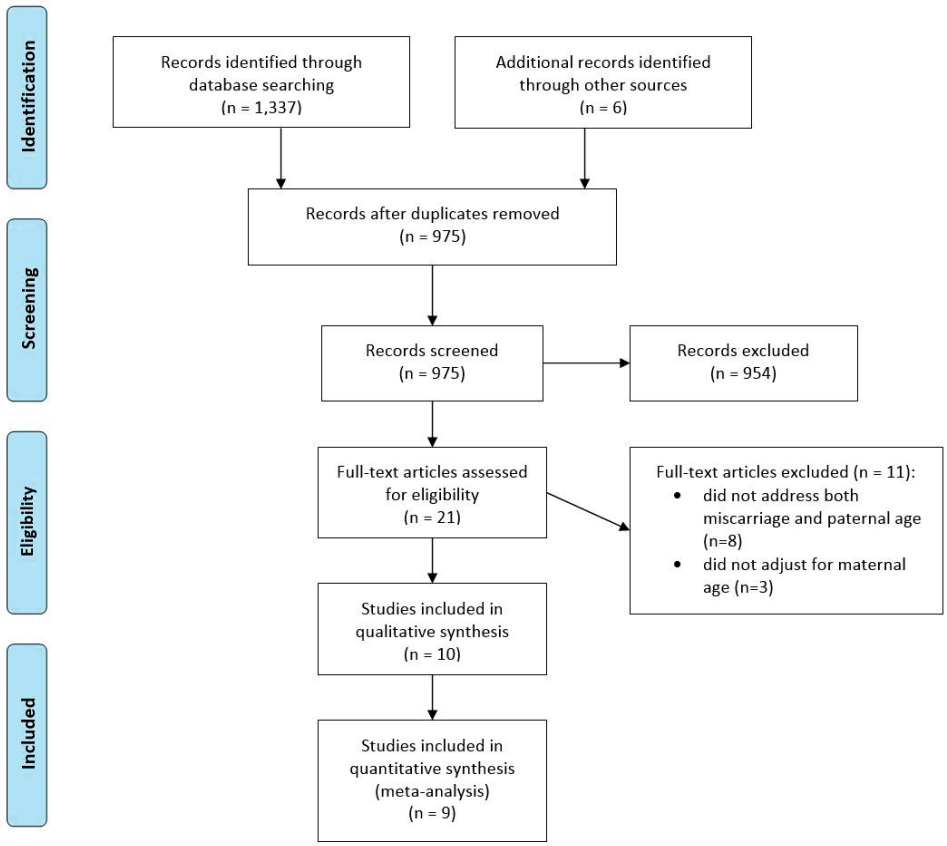


Figure 1. Flow diagram of study selection process

Ten articles met all inclusion criteria and were included in qualitative synthesis. Nine studies were included in the meta-analysis; one study was excluded for reasons explained in the narrative synthesis section.

Study characteristics

Detailed descriptions of key characteristics for all included studies are summarized in Table 1. With regard to the study designs of the ten included studies; four were cohort studies(42, 46-48) and six were case-control studies(43-45, 49-51). Two of the cohort studies(46, 47) were retrospective studies and two were prospective studies(42, 48). Two of the case-control studies were nested case-control studies(44, 49). As shown in Table 1, three studies took place in the USA(42, 44, 51) (one of these studies used data derived from a historic cohort; the Jerusalem Perinatal Study(44)), two in France(46, 47) (one of these studies was based on the European Study of Infertility and Subfecundity, including data from Denmark, Germany, Italy and Spain(46)), and one each in Denmark(48), the UK(49), Japan(45), China(43) and Pakistan(50). Seven studies were population-based(42, 44, 46-49, 51) and three were hospital-based(43, 45, 50). The sample sizes varied from 600 participants in a case-control study(50) to 23,821 in the Danish study by Nybo Andersen et al.(48). Two studies(44, 51) included only spontaneous pregnancies. In three studies(45, 48, 49) a specified proportion of pregnancies (the highest proportion being 13% in the study of Baba et al.(45)) were conceived after ART, while in one study(46) it was stated that part of the population had fertility problems but not further explained. In four other studies(42, 43, 47, 50) the mode of conception was not stated.

Definition of outcome

Miscarriage is defined as the spontaneous demise of intrauterine pregnancy before 24 weeks of gestational age.(52, 53) In the studies selected for this review, miscarriage was defined by different gestational age ranges. Two studies(42, 47) used a lower threshold for five or six weeks of gestational age, while a common upper threshold was 20 weeks(42, 44, 47, 48, 51). Four studies(43, 45, 49, 50) focused on first trimester miscarriages only (<12 weeks or <13 weeks). Two studies(46, 50) did not specifically define gestational age ranges for miscarriage.

Risk of bias

Risk of bias assessment was carried out for each included study, and the results of this assessment are shown in Supplementary Table II.

Bias due to confounding

When evaluating the effect of paternal age on the risk of miscarriage, maternal age is a major confounding factor, being strongly associated with both the exposure and the outcome. Hence, we decided to include only studies in this review that controlled for maternal age. For other factors, it is less evident whether they are confounding the relation between paternal age and miscarriage or whether they are in the causal pathway. For instance, prior miscarriage is a strong risk factor for a subsequent miscarriage. Six studies(43-46, 48, 49) considered this factor as a potential confounder. However, as

stated by Slama et al.(42, 54), a previous miscarriage might have been caused by an elevated paternal age during the previous pregnancy. From that perspective, it should be thought of as an intermediate variable (or a proxy for an intermediate variable) instead of a confounder. Other factors controlled for in some of the selected studies were maternal smoking(42-48) and alcohol consumption(42, 43, 45, 47, 48). Furthermore, some authors did adjust for potential confounding factors such as education level(44), occupational status(45, 48) and ethnicity(51)

Information bias and selection bias

The studies in this review can be subdivided into two types of designs: population-based studies and hospital-based studies. An advantage of large population-based studies(46-48) is a low risk of selection bias, although as a drawback they often have to rely on self-reports of the women regarding their pregnancy outcomes. This means that miscarriages have not been confirmed. In addition, self-reporting could be subject to recall bias or social desirability bias.(55) In hospital-based case-control studies(43, 45, 50), miscarriages are ascertained by hospital diagnosis. However, conducting a study in a hospital setting may introduce a selection bias, since only a subset of women that miscarried is recruited and this subset may not be representative for all women experiencing a miscarriage. Risk of selection bias due to loss to follow-up or missing data was low for all studies.

Narrative synthesis

We included ten studies in this review and seven studies(42, 44, 46, 47, 49-51) found a significant effect of paternal age on the risk of miscarriage. De la Rochebrochard et al.(46) analysed data of 3,174 couples from four European countries about last planned pregnancies that ended in live birth or miscarriage. They stratified paternal and maternal age in 5-year age classes, with 25–29 years designated as the reference group. Maternal and paternal age were analysed together, defined by the variable 'couple age', consisting of a combination of the age classes of both partners. A significant increased AOR for miscarriage was found if the woman was 30–34 years and the man ≥40 years of age, compared to same-aged women and younger men. When we recalculated the reported AORs to obtain AORs for paternal age effects adjusted for maternal age, we found an increased risk for age category 40–64 years, although this was not significant (AOR 1.31; 95% CI 0.75, 2.28).

In a retrospective study by Slama et al.(47) 1,151 randomly selected French women were interviewed about their pregnancy outcomes between 1985 and 2000. The authors developed a survival model to predict the probability of spontaneous miscarriage as a function of the woman's and man's age. This model showed an increased ARR of 1.95 (95% CI 0.97, 3.92) for spontaneous miscarriage in women aged 25 years with a partner of 35 years or older, compared to women aged 25 years whose partner was younger than 35 years.

Table I. Characteristics of included studies

Author, year, country	Study period	Study design	Study setting	Number of pregnancies or cases and controls	Proportion of ART pregnancies	Definition of miscarriage
De la Rochebrochard et al. (2002), France	1991 - 1993	Retro-spective cohort	Population-based (European Study of Infertility and Subfecundity: Denmark, Germany, Italy, Spain)	3,174 pregnancies	Part of study population had infertility problems, otherwise not stated	Not defined
Slama et al. (2003), France	1985 - 2000	Retro-spective cohort	Population-based	2,414 pregnancies	Not stated	Unplanned termination of pregnancy between 5 and 20 weeks

Miscarriage ascertainment			Adjusted risk estimates	Risk factors adjusted for	Methods of adjustment for maternal age
Self-reports	Paternal age	Maternal age	AOR (95% CI)	Country, number of the pregnancy, time to pregnancy, maternal and paternal smoking, history of miscarriage, history of ectopic pregnancy, history of induced abortion	Logistic regression Definition of new variable 'couple age', consisting of classes of maternal and paternal age combinations
	20-29	20-29	1.0 (reference)		
	30-34	20-29	1.06 (0.61-1.86)		
	35-39	20-29	1.31 (0.56-3.07)		
	40-64	20-29	1.80 (0.52-6.24)		
	20-29	30-34	1.72 (0.62-4.74)		
	30-34	30-34	1.62 (0.93-2.82)		
	35-39	30-34	1.06 (0.52-2.17)		
	40-64	30-34	2.90 (1.26-6.67)		
	20-29	35-44	9.18 (1.80-46.66)		
	30-34	34-44	3.87 (1.24-12.02)		
	35-39	35-44	3.38 (1.76–6.47)		
	40-64	35-44	6.73 (3.50-12.95)		
	20-29		*1.0 (reference)*		
	30-34		*0.93 (0.60-1.4)*[a]		
	35-39		*0.68 (0.42-1.12)*[a]		
	40-64		*1.31 (0.75-2.28)*[a]		
Self-reports	Paternal age	Maternal age	ARR (p-value or 95% CI)	Area of recruitment	Discrete time survival model Adjusted for maternal age as continuous variable; used age, age squared and age cubed as covariates in the model
	<25	<20	0.8 (0.64)		
	25-29	<20	0.7 (0.71)		
	30-34	<20	2.6 (0.39)		
	<25	20-24	1.2 (0.52)		
	25-29	20-24	1.0 (reference)		
	30-34	20-24	0.5 (0.23)		
	35-39	20-24	5.3 (0.01)		
	<25	25-29	1.3 (0.61)		
	25-29	25-29	1.1 (0.81)		
	30-34	25-29	0.90 (0.70)		
	35-39	25-29	1.1 (0.91)		
	>40	25-29	0.70 (0.77)		
	25-29	30-34	1.5 (0.27)		
	30-34	30-34	1.2 (0.51)		
	35-39	30-34	1.5 (0.28)		
	>40	30-34	1.5 (0.62)		
	25-29	35-39	7.0 (0.03)		
	30-34	35-39	3.3. (0.01)		
	35-39	35-39	2.2 (0.03)		
	>40	35-39	1.1 (0.91)		
	35-39	>40	1.6 (0.68)		
	>40	>40	11.2 (0.00)		
	20	<35	1.36 (0.98-1.90)	Area of recruitment, maternal smoking, maternal alcohol consumption in first trimester, previous history of urogenital disorder	
	25	<35	1.0 (reference)		
	25	≥35	1.95 (0.97-3.92)		
	30	<35	1.12 (0.93-1.35)		
	30	≥35	1.32 (0.84-2.07)		
	35	<35	2.31 (1.42-3.75)		
	35	≥35	1.40 (0.89-2.20)		
	40	≥35	2.76 (1.51-5.04)		
	42	≥35	4.46 (1.90-10.49)		
	25-29		*1.0 (reference)*	Area of recruitment, maternal age	
	30-34		*0.92 (0.57-1.52)*[a]		
	35-39		*1.21 (0.66-2.22)*[a]		
	>40		*1.01 (0.35-2.92)*[a]		

Table I. Continued

Author, year, country	Study period	Study design	Study setting	Number of pregnancies or cases and controls	Proportion of ART pregnancies	Definition of miscarriage
Nybo Andersen et al. (2004), Denmark	1997 - 1999	Prospective cohort	Population-based (Danish National Birth Cohort Recruitment)	23,821 pregnancies	6% of total study population	Early fetal death <20 weeks
Slama et al. (2005), France	1990 - 1991	Prospective cohort	Population-based (Pregnancy Outcome Study: California)	5,121 pregnancies	Not stated	Spontaneous abortion between 6 and 20 weeks
Kleinhaus et al. (2006), USA	1964 - 1976	Nested case-control	Population-based (Jerusalem Perinatal Study)	Cases: n=1,506 Controls: n=12,359 (live births)	Only fertile women, otherwise not stated	Spontaneous abortion <20 weeks
Maconochie et al. (2007), UK	2001	Nested case-control	Population-based (National Women's Health Study)	Cases: n=603 Controls: n=6,116 (ongoing pregnancy >12 weeks)	Cases: 7% Controls: 3%	Early miscarriage <13 weeks
Baba et al. (2011), Japan	2001 - 2005	Matched case-control	Hospital-based	Cases: n=430 Controls: n=830 (term delivery)	Cases: 13% Controls: 12%	Early miscarriage <12 weeks

Miscarriage ascertainment		Adjusted risk estimates	Risk factors adjusted for	Methods of adjustment for maternal age
Hospital diagnosis	Paternal age	AHR (95% CI)	Maternal age, parity, number of previous abortions, maternal alcohol and coffee consumption during pregnancy, maternal and paternal smoking, maternal and paternal occupational status	Cox regression model Maternal age entered in model in three different ways; using age continuously with restricted cubic splines instead of 5-year or 1-year groups yielded similar estimates for paternal age effects
	≤24	1.17 (0.84-1.63)		
	25-29	1 (reference)		
	30-34	0.86 (0.72-1.03)		
	35-39	0.99 (0.79-1.25)		
	40-44	0.77 (0.55-1.09)		
	45-49	0.97 (0.56-1.69)		
	≥50	1.38 (0.66-2.88)		
Hospital diagnosis	Paternal age	AHR (95% CI)	Maternal age, maternal smoking, maternal alcohol consumption, maternal caffeine consumption, paternal smoking in first trimester	Cox regression model Adjusted for maternal age as continuous variable, using a fractional polynomial approach
	<25	1 (reference)		
	25-29	1.47 (1.04-2.08)		
	30-34	1.25 (0.84-1.88)		
	35-39	1.74 (1.12-1.72)		
	40-44	1.45 (0.85-2.46)		
	≥45	1.87 (1.01-3.44)		
	25-29	*1 (reference)*		
	30-34	*0.85 (0.57-1.28)*[b]		
	35-39	*1.18 (0.76-1.85)*[b]		
	40-44	*0.99 (0.58-1.67)*[b]		
	≥45	*1.27 (0.69-2.34)*[b]		
Self-reports	Paternal age	AOR (95% CI)	Maternal age, maternal diabetes, maternal smoking, history of spontaneous abortions, parity, interval from interview to previous pregnancy, maternal and paternal education, history of induced abortions	Unconditional logistic regression Adjusted for maternal age as a continuous variable; used orthogonal coding of parental ages
	<25	0.59 (0.45-0.76)		
	25-29	1 (reference)		
	30-34	1.4 (1.2-1.6)		
	35-39	1.9 (1.6-2.3)		
	≥40	1.6 (1.2-2.0)		
Self-reports	Paternal age	AOR (95% CI)	Maternal age, year of conception, pregnancy order, history of miscarriage, history of live births	Logistic regression Coding of maternal age not stated
	<25	1.18 (0.80-1.73)		
	25	1 (reference)		
	30	1.05 (0.83-1.33)		
	35	1.22 (0.94-1.59)		
	40	1.04 (0.71-1.53)		
	≥45	1.63 (1.08-2.47)		
Hospital diagnosis	Paternal age	AOR (95% CI)	Maternal age[c], year of the event, history of spontaneous abortion, history of induced abortion, treatment of infertility, maternal BMI, maternal smoking, maternal alcohol consumption, maternal employment, paternal smoking	Conditional logistic regression Matched for maternal age ± 3 years
	<29	1 (reference)		
	29-39	1.14 (0.75-1.74)		
	≥40	1.65 (0.94-2.88)		

Table I. Continued

Author, year, country	Study period	Study design	Study setting	Number of pregnancies or cases and controls	Proportion of ART pregnancies	Definition of miscarriage
Jaleel et al. (2013), Pakistan	2007 - 2010	Case-control	Hospital-based	Cases: n=200 Controls: n=400 (ongoing pregnancy >24 weeks)	Not stated	Early miscarriage (otherwise not defined)
Xu et al. (2014), China	2009 - 2012	Matched case-control	Hospital-based	Cases: n=620 Controls: n=1,240 (ongoing pregnancy >12 weeks)	Not stated	Early miscarriage <13 weeks
Nguyen et al. (2019), USA	2011 - 2015	Case-control	Population-based (National Survey of Family Growth)	Cases: 2,300 pregnancies Controls: 10,410 pregnancies (live birth ≥37 weeks)	Only spontaneous pregnancies	Loss of clinically recognized pregnancy ≤12 weeks and <20 weeks

[a] *Recalculated from the risk estimates reported for the combinations of paternal and maternal age, as described in Statistical analysis;* [b] *Rescaled to reference category 25-29, as described in Statistical analysis;*
[c] *Matched for maternal age (±3 years)*
ART, artificial reproductive technology; AOR, adjusted odds ratio; AHR, adjusted hazard ratio; ARR, adjusted rate ratio; CI, confidence interval

Miscarriage ascertainment		Adjusted risk estimates	Risk factors adjusted for	Methods of adjustment for maternal age
Hospital diagnosis	Paternal age	AOR (95% CI)	Maternal age, paternal genital tract infection	Logistic regression Coding of maternal age not stated
	≤35	1 (reference)		
	36-40	16.44 (6.612-40.896)		
	41-45	13.738 (4.376-43.127)		
	>45	7.042 (1.269-39.090)		
Hospital diagnosis	Paternal age	AOR (95% CI)	Maternal age[c], history of early miscarriage, history of induced abortion, vitamin supplementation, maternal smoking and alcohol consumption, maternal night shift work, frequent staying up late, physical exercise	Conditional logistic regression Matched for maternal age ± 3 years
	<25	1 (reference)		
	25-29	0.94 (0.81-1.28)		
	30-34	1.04 (0.85-1.32)		
	35-39	0.97 (0.79-1.37)		
	≥40	1.16 (0.86-1.42)		
	25-29	*1 (reference)*		
	30-34	*1.11 (0.90-1.40)[b]*		
	35-39	*1.03 (0.84-1.46)[b]*		
	≥40	*1.23 (0.91-1.51)[b]*		
Self-reports	Paternal age	AOR (95% CI)	Maternal age, ethnicity, income, marital status, pregnancy intention	Generalized estimating equations logistic regression Maternal age entered in model in four age categories
	<20 weeks			
	<25	1.03 (0.85-1.25)		
	25-29	1 (reference)		
	30-34	1.04 (0.83-1.29)		
	35-39	1.11 (0.81-1.52)		
	40-44	1.10 (0.70-1.74)		
	45-49	1.49 (0.71-3.13)		
	≥50	2.05 (1.06-3.93)		
	≤12 weeks			
	<25	1.07 (0.86-1.32)		
	25-29	1 (reference)		
	30-34	1.10 (0.86-1.39)		
	35-39	1.08 (0.76-1.52)		
	40-44	1.10 (0.67-1.82)		
	45-49	1.49 (0.65-3.40)		
	≥50	2.30 (1.17-4.52)		

Nybo Andersen et al.(48) used data of 23,281 pregnancies from a Danish prospective cohort study to assess the association between paternal age and fetal death. They stratified for early (<20 weeks of gestation) and late (≥20 weeks of gestation) fetal death. Paternal age was categorised in 5-year age groups with the last group covering ≥50 years. The authors found an increased hazard ratio for early fetal death for fathers ≥50 years (AHR 1.38; 95% CI 0.66, 2.88), using 25–29 years as the reference group. They entered maternal age in three different ways in the model. Treating maternal age continuously with restricted cubic splines instead of 5- or 1-year age groups yielded similar estimates for paternal age effects, implying that there was no strong residual confounding by maternal age. To ensure that the effect of paternal age was not due to confounding by subfertility or infertility, they performed a second analysis restricted to couples who conceived without fertility treatment and they found comparable AHRs.

A second study of Slama et al.(42) with a prospective design assessed the risk of spontaneous miscarriage between 6 and 20 weeks of pregnancy in a Cox model. The risk of spontaneous miscarriage was 1.27 times increased for fathers with a paternal age of 35 years and more, compared to fathers younger than 35 years old (AHR 1.27; 95% CI 1.00, 1.60). When they coded paternal age in smaller age groups (and maternal age continuously, using a fractional polynomial approach), they found the highest risk of spontaneous miscarriage for men aged >45 years (AHR 1.87; 95% CI 1.01, 3.44, reference group men aged 18–24 years). We rescaled the AHRs using 25–29 years as the reference category, and this yielded lower AHRs of 0.99 (95% CI 0.58, 1.67) in category 40–44 and 1.27 (95% CI 0.69, 2.34) in the ≥45-year age group.

In a nested case-control study derived from the Jerusalem Perinatal Study, Kleinhaus et al.(44) compared 1506 couples with previous pregnancy ending in spontaneous miscarriage with a control group comprising 12359 couples with prior live birth. They used paternal age categories of 5 years, with 25–29 years being the reference group. The AORs for miscarriage <20 weeks of gestation for the age groups 30–34 (AOR 1.4; 95% CI 1.2, 1.6), 35–39 (AOR 1.9; 95% 1.6–2.3) and ≥40 years (AOR 1.6; 95% CI 1.2–2.0) were all significantly increased.

Maconochie et al.(49) studied various socio-demographic and behavioural factors in relation to last pregnancy outcomes. Cases consisted of 603 women whose most recent pregnancy was a first trimester (<13 weeks) miscarriage. Controls were 6116 women whose most recent pregnancy had progressed beyond 12 weeks. In fathers ≥45 years of age the AOR for first trimester miscarriage was significantly increased (AOR 1.63; 95% CI 1.08, 2.47; reference group 25–29 years).

Baba et al.(45) and Xu et al.(43) conducted similarly designed studies to identify risk

factors for first trimester miscarriage. These hospital-based case-control studies were matched for maternal age, with total sample sizes of 1290 and 1860, respectively. For fathers aged ≥40, Baba et al. found an AOR for miscarriage of 1.65 (95% CI 0.94, 2.88) and Xu et al. an AOR of 1.16 (95% CI 0.86, 1.42). In both studies, only women who miscarried and were hospitalised for a medical procedure were selected as cases; women with spontaneous miscarriages without additional treatment were not included. Baba et al. used women who underwent term deliveries in the same hospital as controls. The control group of Xu et al. consisted of women who attended the outpatient clinic for prenatal care and were past 13 weeks of gestation.

In a case-control study conducted in a hospital in Karachi, Pakistan, pregnant women aged 20-35 years were included.(50) Cases were women with first trimester miscarriage and controls were those admitted for delivery beyond 24 weeks of gestation. Studied factors were maternal age, paternal age, parental tobacco use and male genital tract infection. The final logistic regression model yielded extremely large effects of paternal age on the risk of first trimester miscarriage compared to all other studies, with AORs of 16.44 (95% CI 6.61, 40.90) in age category 36–40 years, 13.74 (95% CI 4.38, 43.13) in age category 41–45 years and 7.04 (95% CI 1.27, 39.09) in age category >45 years. In contrast to the other studies, paternal age≤35 years and maternal age≤31 years were used as reference categories. The reported data was insufficient to rescale the AORs to reference category 25–29 years, as we did for other studies. Part of the explanation for the deviating risk estimates could be that in this study population, there was less correlation between maternal and paternal ages, meaning there were relatively many couples consisting of older fathers and young mothers. We did not include this study in our meta-analyses, as this study might involve a selected population, reflected by the extreme and potentially unrealistic effects of paternal age that could not be compared to other studies because of the different reference category that was used.

The most recent study of Nguyen et al.(51) used data of 12 710 pregnancies from the US National Survey of Family Growth and assessed the risk of miscarriage <20 and ≤12 weeks separately. They used pregnancies ending in a live birth ≥37 weeks as controls. Pregnancies resulting in spontaneous miscarriage had 2.05 (95% CI 1.06–3.93) times the odds of being from a father aged ≥50 years. For first trimester miscarriage, the AOR for this age category was 2.30 (95% CI 1.17–4.52).

Quantitative synthesis of paternal age effects

The overall meta-analysis (Fig. 2), including nine studies, showed an increasing risk of miscarriage with advancing paternal age. Significant effects in age categories 40–44 years (pooled estimate 1.23; 95% CI 1.06, 1.43) and ≥45 years (1.43; 95% CI 1.13, 1.81) were found. The reference group was 25–29 years for all studies, except for Baba et

al.(45) (<29 years) and de la Rochebrochard et al.(46) (20–29 years).

A second meta-analysis (Fig. 3) was performed including the four studies that were restricted to first trimester miscarriage. A similar pattern of the paternal age effect was found, with a pooled estimate of 1.74 (95% CI 1.26, 2.41) in the highest age category.

In both meta-analyses, there was substantial heterogeneity in the two lower age categories, while in the more advanced age categories the effects across studies were more similar, as indicated by I^2. In Supplementary Fig. S1, funnel plots are displayed for each age category separately, including all nine studies. No clear evidence of small study effects or publication bias was found.

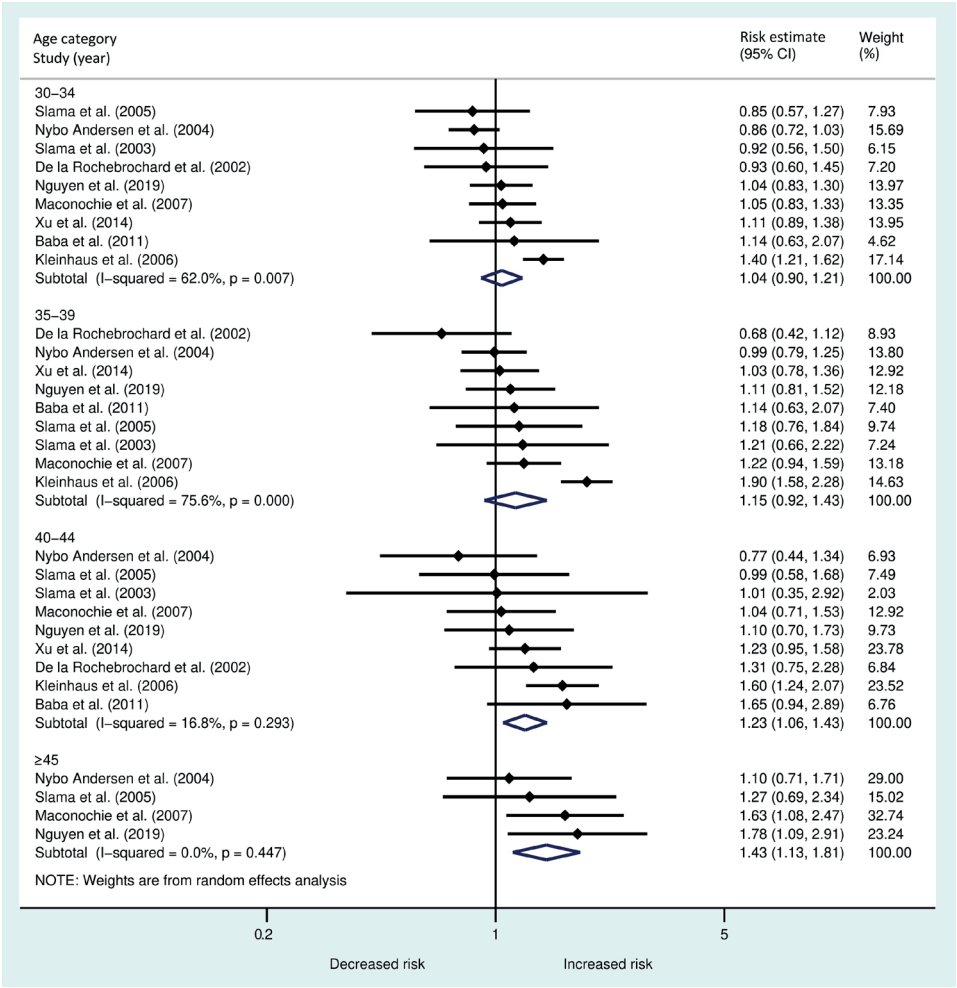


Figure 2. Forest plot describing the association between paternal age in different age categories and the risk of miscarriage <20 weeks

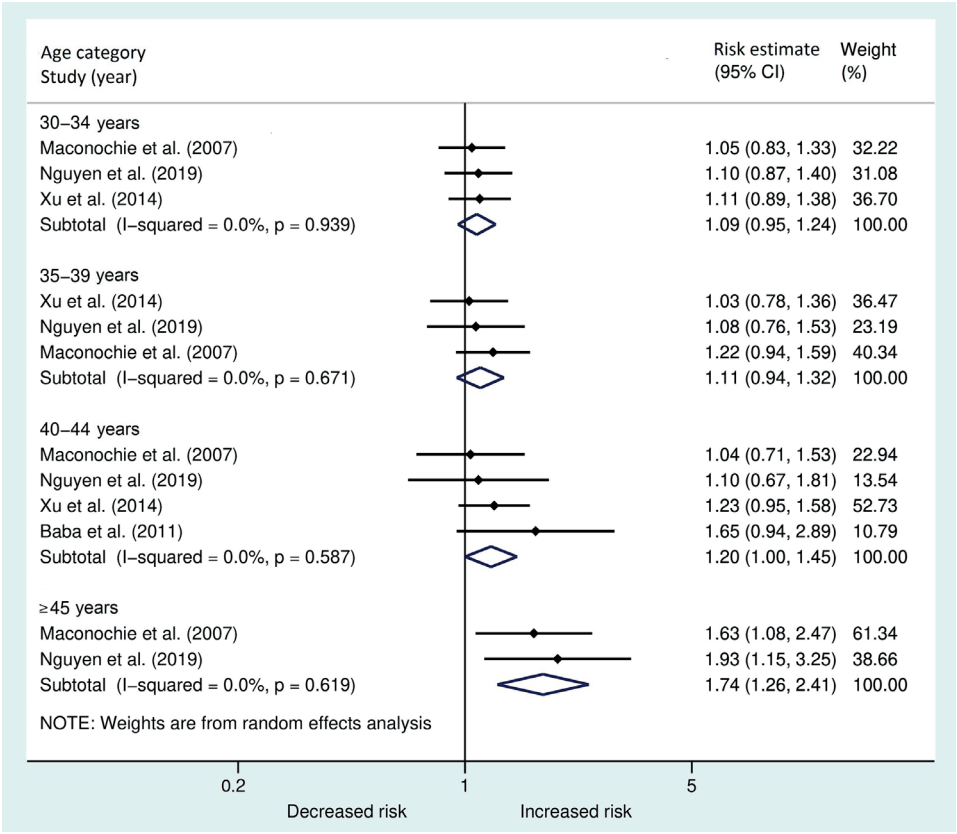


Figure 3. Forest plot describing the association between paternal age in different age categories and the risk of early miscarriage <13 weeks

Maternal age effects

Besides analysis of the paternal age effect, four of the included studies(42, 44, 49, 51) evaluated the effect of maternal age on the risk of miscarriage. They reported risk estimates for the maternal age effect, analysed on the same data as used for the paternal age effect. One study(42) provided risk estimates for maternal age, adjusted for paternal age. The other studies did not adjust the maternal age effects for paternal age. For two studies(46, 47) that analysed combinations of paternal and maternal age (couple-age), it was possible to obtain risk estimates for maternal age categories, adjusted for paternal age (in the same way as performed for the paternal age effect, described in the statistical analysis). Maternal risk estimates with a reference category other than 25–29 years were rescaled to reference category 25–29 years when possible. An overview of maternal age effects on the risk of spontaneous miscarriage is shown in Table II.

Table II. Maternal age effects

Author, year, country		Adjusted risk estimates	Risk factors adjusted for
De la Rochebrochard et al. (2002), France	Maternal age	AOR (95% CI)	Paternal age, country, number of the pregnancy, time to pregnancy, maternal and paternal smoking, history of miscarriage, history of ectopic pregnancy, history of induced abortion
	20-29	*1.0 (reference)*	
	30-34	*1.76 (1.10-2.82)*[a]	
	35-44	*6.49 (4.43-9.51)*[a]	
Slama et al. (2003), France	Maternal age	ARR (95% CI)	Paternal age, area of recruitment
	25-29	*1 (reference)*	
	30-34	*1.34 (0.81-2.20)*[a,b]	
	35-39	*2.39 (1.21-4.69)*[a,b]	
	≥40	*6.23 (1.48-26.17)*[a,b]	
Slama et al. (2005),	Maternal age	AHR (95% CI)	Paternal age, maternal smoking, maternal alcohol consumption, maternal caffeine consumption, paternal smoking in first trimester
	<22.5	1.27 (1.04-1.55)	
	22.5-27.4	1	
	27.5-32.4	0.98 (0.84-1.13)	
	32.5-37.4	1.30 (1.03-1.66)	
	37.5-42.4	2.63 (1.86-3.71)	
	≥42.5	8.80 (4.73-16.73)	
Kleinhaus et al. (2006), USA	Maternal age	AOR (95% CI)	Parity, time interval from index pregnancy to interview, history of miscarriage
	25-29	*1 (reference)*	
	30-34	*2 (1.68-2.36)*[b]	
	≥35	*3.77 (3.05-4.68)*[b]	
Maconochie et al. (2007), UK	Maternal age	AOR (95% CI)	Year of conception, history of miscarriage, history of live birth
	<25	1.09 (0.81-1.45)	
	25-29	1 (reference)	
	30-34	1.06 (0.85-1.31)	
	35-39	1.75 (1.37-2.22)	
	≥40	5.16 (3.54-7.52)	
Nguyen et al. (2019), USA	Maternal age	AOR (95% CI)	Ethnicity, income, marital status, pregnancy intention
	<20 weeks		
	<25	0.89 (0.72-1.10)	
	25-29	1 (reference)	
	30-34	0.98 (0.72-1.33)	
	≥35	1.52 (1.04-2.20)	
	≤12 weeks		
	<25	0.86 (0.69-1.09)	
	25-29	1 (reference)	
	30-34	0.92 (0.68-1.24)	
	≥35	1.66 (1.12-2.44)	

[a] Recalculated from the risk estimates reported for the combinations of paternal and maternal age, as described in Statistical analysis; [b] Rescaled to reference category 25-29, as described in Statistical analysis

Significant effects of maternal age ≥35 years were found in all of the above studies, varying from AOR 1.52 (95% CI 1.04-2.20, age category ≥35 years)(51) to AHR 8.80 (95% CI 4.73-16.73, age category ≥42.5 years)(42).

Because of the small number of studies and substantial differences in adjustments of the estimates and used age categories, a meta-analysis of the risk estimates of the maternal age effect was not performed.

Additional analyses

There were no major differences between the pooled estimates of the paternal age effect provided by models with random and fixed effects (Supplementary Fig. S2).

In the sensitivity analysis excluding the study(44) that consequently yielded relatively extreme estimates, the pooled estimates for the paternal age effect in age categories 35-39 and 40-44 years were slightly decreased (-8%). The pattern of the association between paternal age and risk of miscarriage was similar as observed in the main analysis (Supplementary material 3, Fig. S3).

DISCUSSION

In this systematic review and meta-analysis of 10 population-based cohort and case-control studies, advanced paternal age beyond 40 years was found to be significantly associated with an increased risk of spontaneous miscarriage, adjusted for maternal age. This paternal age effect was also observed in a subgroup of studies focusing on first trimester miscarriage.

A major strength of this systematic review and meta-analysis is that we could increase statistical power by combining data of the extreme paternal age categories of different studies. In the individual studies, the analyses were limited by small patient numbers in the more advanced age groups. Often increased risk estimates were found within these categories, although they were not statistically significant. By pooling the effect measures of different studies, we were able to find significant paternal age effects for both the 40–44 and ≥45 age classes.

It is important to mention that investigating a paternal age effect on the risk of miscarriage is challenging, due to the high level of collinearity between paternal and maternal age. To prevent confounding by maternal age, we only selected studies that did control for this variable. However, residual confounding by maternal age may still be present, especially when maternal age is treated as a discrete variable in broad age classes.(46, 56) We evaluated the methods used for adjustment of maternal age in the included studies. The majority of studies carefully adjusted for maternal age, either by matching cases and controls according to maternal age(43, 45), or treating maternal age as a continuous variable, using orthogonal coding of parental ages(44), a fractional polynomial approach(42, 47) or restricted cubic splines(48). Two studies(46, 51) entered maternal age in their model as a categorical variable and two other studies(49, 50) did not state how they treated maternal age in their models.

Other factors taken into account by several authors in the statistical adjustments were maternal smoking and alcohol consumption. The association of these maternal behaviours with spontaneous miscarriage is well-established(31, 32, 57-59). It is debatable to what extent maternal smoking and alcohol consumption are correlated with paternal age, which is another criterion for considering these factors as confounding factors. When such correlations do indeed exist in a study population, as suggested in some of the articles included in this review(42, 44, 48), these factors could potentially bias the estimated association between paternal age and miscarriage. However, it is conceivable that some of the included studies controlled for too many variables. If a study adjusts for a variable that is, instead of being a confounder, in the causal pathway between paternal age and miscarriage, the total causal effect cannot be consistently

estimated (i.e. the effect will be underestimated).(54, 60-62)

In contrast to the risk of overadjustment bias for maternal factors, there might exist residual confounding by paternal factors. Six of the included studies have taken into account at least one paternal factor other than age(42, 44-46, 48, 50). It is, however, possible that the encountered relation between paternal age and miscarriage is biased by other, unmeasured, paternal characteristics.(63-65).

Apart from the risk of confounding, conducting studies that aim to identify the risk of paternal age on spontaneous miscarriage comes with more challenges. Each study design has its own opportunities and obstacles. Population-based studies typically provide more generalisable results. At the same time, they are prone to information bias since they depend on the women's declaration of miscarriage; especially early miscarriage is hard to establish. Furthermore, as previously suggested by other authors, some of the reported miscarriages may actually have been induced abortions.(44, 46, 47) Hospital-based studies have less of a problem with case ascertainment. Nevertheless, these studies are more susceptible to selection bias since they exclusively recruit women who have received medical service for their miscarriage. From the studies included in this review, the cohort studies appear to have more conservative estimates compared to the case-control studies. This finding does not seem to be clearly related to differences in study setting or patient selection. Some of the case-control studies are population-based and others are hospital-based, while the cohort studies are all population-based. Also, the number of variables adjusted for does not substantially differ between the two clusters of studies. Because of the limited number of studies, especially when stratified per age group, sensitivity analysis on study design or meta-regression was not performed.

Supporting the observed epidemiological associations, it is plausible from a biological perspective that advanced paternal age increases the risk of adverse reproductive outcome. In women, the age-related decline in reproductive capacity is explained by a gradual decrease in ovarian reserve and oocyte integrity(66). More frequent chromosome segregation errors result in oocyte aneuploidy, and this is thought to be primarily responsible for maternal age-related miscarriage. In contrast to the process of oogenesis, where germ cell replication is completed at birth, male germ cells divide continuously throughout a man's reproductive lifespan. From entering puberty on, spermatogenic stem cells divide approximately 23 times per year and by the age of 50 years, more than 800 replications have occurred.(14) Therefore, advancing paternal age most likely increases the probability of replication errors in the germ line, resulting in an accumulation of de novo mutations.(15) This process is exacerbated when DNA repair mechanisms are also deteriorating with age.(67) Kong et al. performed whole genome

sequencing on 78 trios of parents and their children and demonstrated a clear association between advanced paternal age and increased number of de novo genetic mutations in the offspring, probably contributing to autosomal dominant disorders and complex disorders such as autism spectrum disorders.(13, 15) Advanced paternal age may also be linked to increased sperm aneuploidy; however, inconsistent findings have been reported in the literature.(68-70) It is suggested that due to continual spermatogenesis, the male gamete is less vulnerable to age-related non-disjunction aneuploidies than its female counterpart.(71)

The influence of paternal age on miscarriage is perhaps acting mostly at the level of sperm DNA integrity. Multiple studies have shown elevated levels of sperm DNA fragmentation in older men, with a more than doubling DNA fragmentation index (DFI) between 20 and 60 years old.(72-74) This is probably due to a combination of age-related mechanisms and inherent characteristics of spermatozoa, such as accumulation of reactive oxygen species, absence of antioxidant capacity and paucity of DNA repair mechanisms.(75) Although conventional sperm parameters such as volume, motility and morphology are declining with rising paternal age(76), those are relatively poor predictors of male fertility potential and miscarriage.(77, 78) In contrast, sperm DNA fragmentation seems directly associated with reproductive outcome. There is solid evidence that an increased level of sperm DNA fragmentation is associated with (recurrent) pregnancy loss.(79-82) In the case of fertilisation, sperm DNA fragmentation can to some extent be repaired by the oocyte. However, with advancing age the oocyte quality is deteriorating, together with its repair capacity.(83) This supports the hypothesis that the impact of paternal age on miscarriage, mediated by increased DFI, is more present in interaction with higher maternal age. This is in line with epidemiological studies that demonstrated such an interaction between advanced paternal and maternal age for the risk of miscarriage.(46) Furthermore, a recent study in IVF/ICSI couples observed a higher miscarriage rate in women beyond 35 years and partners with high sperm DFI, compared to couples with similarly high sperm DFI and younger women.(84) It is noteworthy that quality of sperm, measured either by conventional parameters or DNA integrity, has not been taken into account by any of the studies included in this review. An ongoing prospective study is currently investigating the predictive role of sperm DNA damage in RPL, as well as the relation with paternal age and lifestyle factors.(85)

In this review, we excluded studies that were restricted to couples who conceived after ART, since we were interested in the association between paternal age and miscarriage in the general population. The relationship between advanced parental age, infertility and miscarriage is complex. In some studies, miscarriage rates appear to be higher among ART pregnancies compared to natural pregnancies(86), however, this is not easily interpreted. Assisted pregnancies are usually closely monitored and, as a consequence,

pregnancy losses, especially from early stages, will probably be detected more often than in the general population. In addition, ART-treated couples are generally of more advanced age, which predisposes them to an increased risk of miscarriage. For these reasons, it is difficult to distinguish whether an increased risk of miscarriage in couples receiving fertility treatment is a consequence of the treatment itself, or due to underlying patient characteristics. Studies investigating the effect of paternal age on miscarriage after different forms of ART reported inconclusive results.(87-93) These contradictory data may be explained by the heterogeneity of these studies, the small proportions of older men they included and by the exclusion of women with advanced age or using young oocyte donors in some studies.(71) Furthermore, studies that did not observe an effect of paternal aging on the risk of pregnancy loss were mainly in IVF/ICSI pregnancies from a very heterogeneous population of men with extensive variations in sperm parameters and cause and severity of infertility, which may have diluted an age effect.(94)

While advanced maternal age is generally agreed upon as age≥35, there is currently no consensus for the definition of advanced paternal age. However, ageing is a complex process and it is hard to determine a clear cutoff point, the more because age effects are likely to occur gradually and thresholds are not necessarily the same for all different outcomes that are affected by paternal age. Most studies suggest that infertility and reproductive risks start to increase after the paternal age of 40.(95) This is in accordance with the results of our meta-analyses. Based on our findings, it should be considered to counsel couples with older males about the increased risk of miscarriage at preconception visits. Furthermore, our results are of value for patients with recurrent miscarriages. This condition remains unexplained in the majority of cases(36, 37), and for a proportion of the idiopathic cases, advanced paternal age could be responsible. Currently, there are no studies that did specifically focus on the relation between paternal age and recurrent miscarriages and this should certainly be addressed in future research. Although it is challenging to distinguish paternal age effects from maternal age effects, most studies included in this review made relevant efforts and collectively they suggest the existence of an, albeit small, independent effect of paternal age on the risk of spontaneous miscarriage. Since there are strong biological hypotheses for this paternal effect, it is likely that future studies will establish it even more. Both large population-based registry studies and hospital-based case-control studies may help to validate the paternal age effect on pregnancy loss, provided that they carefully control for maternal age in their statistical analyses. There is a trend toward delayed childbearing in western societies and it has become more common to father children at older age.(96). Hence, we consider it important to not merely focus on the effects of maternal aging on reproductive outcome, but to be aware of risks associated with advanced paternal age as well.

REFERENCES

1. Hassold T, Chiu D. Maternal age-specific rates of numerical chromosome abnormalities with special reference to trisomy. Hum Genet. 1985;70(1):11-7.
2. Aldous MB, Edmonson MB. Maternal age at first childbirth and risk of low birth weight and preterm delivery in Washington State. JAMA. 1993;270(21):2574-7.
3. van Katwijk C, Peeters LL. Clinical aspects of pregnancy after the age of 35 years: a review of the literature. Hum Reprod Update. 1998;4(2):185-94.
4. Nybo Andersen AM, Wohlfahrt J, Christens P, Olsen J, Melbye M. Maternal age and fetal loss: population based register linkage study. BMJ. 2000;320(7251):1708-12.
5. Bacak SJ, Callaghan WM, Dietz PM, Crouse C. Pregnancy-associated hospitalizations in the United States, 1999-2000. Am J Obstet Gynecol. 2005;192(2):592-7.
6. Cleary-Goldman J, Malone FD, Vidaver J, Ball RH, Nyberg DA, Comstock CH, et al. Impact of maternal age on obstetric outcome. Obstet Gynecol. 2005;105(5 Pt 1):983-90.
7. Delpisheh A, Brabin L, Attia E, Brabin BJ. Pregnancy late in life: a hospital-based study of birth outcomes. J Womens Health. 2008;17(6):965-70.
8. Nelson SM, Telfer EE, Anderson RA. The ageing ovary and uterus: new biological insights. Hum Reprod Update. 2013;19(1):67-83.
9. Waldenstrom U, Cnattingius S, Vixner L, Norman M. Advanced maternal age increases the risk of very preterm birth, irrespective of parity: a population-based register study. BJOG. 2017;124(8):1235-44.
10. Lisonkova S, Potts J, Muraca GM, Razaz N, Sabr Y, Chan WS, et al. Maternal age and severe maternal morbidity: A population-based retrospective cohort study. PLoS Med. 2017;14(5):e1002307.
11. Heffner LJ. Advanced Maternal Age — How Old Is Too Old? 2004;351(19):1927-9.
12. National Collaborating Centre for Women's and Children's Health. National Institute for Health and Clinical Excellence: Guidance. Fertility: Assessment and Treatment for People with Fertility Problems. London: Royal College of Obstetricians & Gynaecologists

National Collaborating Centre for Women's and Children's Health, 2013.

13. Oldereid NB, Wennerholm UB, Pinborg A, Loft A, Laivuori H, Petzold M, et al. The effect of paternal factors on perinatal and paediatric outcomes: a systematic review and meta-analysis. Hum Reprod Update. 2018;24(3):320-89.
14. Crow JF. The origins, patterns and implications of human spontaneous mutation. Nat Rev Genet. 2000;1(1):40-7.
15. Kong A, Frigge ML, Masson G, Besenbacher S, Sulem P, Magnusson G, et al. Rate of de novo mutations and the importance of father's age to disease risk. Nature. 2012;488(7412):471-5.
16. Sartorius GA, Nieschlag E. Paternal age and reproduction. Hum Reprod Update. 2010;16(1):65-79.
17. Robinson L, Gallos ID, Conner SJ, Rajkhowa M, Miller D, Lewis S, et al. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. Hum Reprod. 2012;27(10):2908-17.
18. Kobayashi N, Miyauchi N, Tatsuta N, Kitamura A, Okae H, Hiura H, et al. Factors associated with aberrant imprint methylation and oligozoospermia. Sci Reo. 2017;7:42336-.
19. Wilcox AJ, Weinberg CR, O'Connor JF, Baird DD, Schlatterer JP, Canfield RE, et al. Incidence of early loss of

pregnancy. N Engl J Med. 1988;319(4):189-94.
20. ESHRE Guideline Group on RPL. Recurrent Pregnancy Loss. Guideline of the European Society of Human Reproduction and Embryology, 2017.
21. Zegers-Hochschild F, Adamson GD, de Mouzon J, Ishihara O, Mansour R, Nygren K, et al. The International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) Revised Glossary on ART Terminology, 2009. Hum Reprod. 2009;24(11):2683-7.
22. Levy B, Sigurjonsson S, Pettersen B, Maisenbacher MK, Hall MP, Demko Z, et al. Genomic imbalance in products of conception: single-nucleotide polymorphism chromosomal microarray analysis. Obstet Gynecol. 2014;124(2 Pt 1):202-9.
23. Romero ST, Geiersbach KB, Paxton CN, Rose NC, Schisterman EF, Branch DW, et al. Differentiation of genetic abnormalities in early pregnancy loss. Ultrasound Obstet Gynecol. 2015;45(1):89-94.
24. Soler A, Morales C, Mademont-Soler I, Margarit E, Borrell A, Borobio V, et al. Overview of Chromosome Abnormalities in First Trimester Miscarriages: A Series of 1,011 Consecutive Chorionic Villi Sample Karyotypes. Cytogenet Genome Res. 2017;152(2):81-9.
25. Magnus MC, Wilcox AJ, Morken NH, Weinberg CR, Haberg SE. Role of maternal age and pregnancy history in risk of miscarriage: prospective register based study. Bmj. 2019;364:l869.
26. Group TECW. Genetic aspects of female reproduction. Hum Reprod Update. 2008;14(4):293-307.
27. Maraka S, Ospina NM, O'Keeffe DT, Espinosa De Ycaza AE, Gionfriddo MR, Erwin PJ, et al. Subclinical Hypothyroidism in Pregnancy: A Systematic Review and Meta-Analysis. Thyroid. 2016;26(4):580-90.
28. Dorman JS, Burke JP, McCarthy BJ, Norris JM, Steenkiste AR, Aarons JH, et al. Temporal trends in spontaneous abortion associated with Type 1 diabetes. Diabetes Res Clin Pract. 1999;43(1):41-7.
29. Saravelos SH, Cocksedge KA, Li TC. Prevalence and diagnosis of congenital uterine anomalies in women with reproductive failure: a critical appraisal. Hum Reprod Update. 2008;14(5):415-29.
30. Metwally M, Ong KJ, Ledger WL, Li TC. Does high body mass index increase the risk of miscarriage after spontaneous and assisted conception? A meta-analysis of the evidence. Fertil Steril. 2008;90(3):714-26.
31. Pineles BL, Park E, Samet JM. Systematic review and meta-analysis of miscarriage and maternal exposure to tobacco smoke during pregnancy. Am J Epidemiol. 2014;179(7):807-23.
32. Sundermann AC, Zhao S, Young CL, Lam L, Jones SH, Velez Edwards DR, et al. Alcohol Use in Pregnancy and Miscarriage: A Systematic Review and Meta-Analysis. Alcohol Clin Exp Res. 2019;43(8):1606-16.
33. Hahn KA, Wise LA, Rothman KJ, Mikkelsen EM, Brogly SB, Sorensen HT, et al. Caffeine and caffeinated beverage consumption and risk of spontaneous abortion. Hum Reprod. 2015;30(5):1246-55.
34. 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.
35. Qu F, Wu Y, Zhu YH, Barry J, Ding T, Baio G, et al. The association between psychological stress and miscarriage: A systematic review and meta-analysis. Sci Rep. 2017;7(1):1731.
36. Stephenson MD. Frequency of factors associated with habitual abortion in 197 couples. Fertil Steril. 1996;66(1):24-9.
37. Jaslow CR, Carney JL, Kutteh WH. Diagnostic factors identified in 1020 women with two versus three or more recurrent pregnancy losses. Fertil Steril. 2010;93(4):1234-43.
38. Farren J, Mitchell-Jones N, Verbakel JY, Timmerman D, Jalmbrant M, Bourne T. The psychological impact

of early pregnancy loss. Hum Reprod Update. 2018;24(6):731-49.

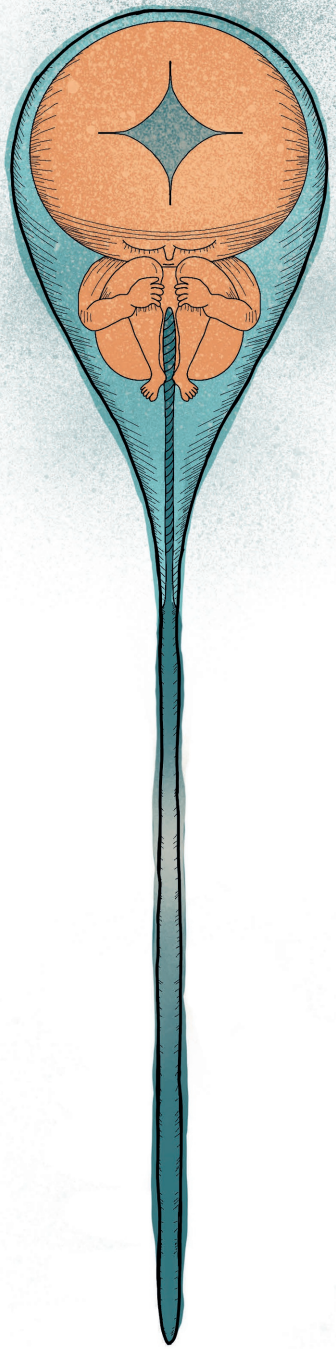
39. Moher D, Liberati A, Tetzlaff J, Altman DG. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. 2009;339:b2535.
40. Sanderson S, Tatt ID, Higgins JP. Tools for assessing quality and susceptibility to bias in observational studies in epidemiology: a systematic review and annotated bibliography. Int J Epidemiol. 2007;36(3):666-76.
41. Dekkers OM, Vandenbroucke JP, Cevallos M, Renehan AG, Altman DG, Egger M. COSMOS-E: Guidance on conducting systematic reviews and meta-analyses of observational studies of etiology. PLoS Medicine. 2019;16(2):e1002742.
42. Slama R, Bouyer J, Windham G, Fenster L, Werwatz A, Swan SH. Influence of paternal age on the risk of spontaneous abortion. Am J Epidemiol. 2005;161(9):816-23.
43. Xu G, Wu Y, Yang L, Yuan L, Guo H, Zhang F, et al. Risk factors for early miscarriage among Chinese: a hospital-based case-control study. Fertil Steril. 2014;101(6):1663-70.
44. Kleinhaus K, Perrin M, Friedlander Y, Paltiel O, Malaspina D, Harlap S. Paternal age and spontaneous abortion. Obstet Gynecol. 2006;108(2):369-77.
45. Baba S, Noda H, Nakayama M, Waguri M, Mitsuda N, Iso H. Risk factors of early spontaneous abortions among Japanese: a matched case-control study. Hum Reprod. 2011;26(2):466-72.
46. de la Rochebrochard E, Thonneau P. Paternal age and maternal age are risk factors for miscarriage; results of a multicentre European study. Hum Reprod. 2002;17(6):1649-56.
47. Slama R, Werwatz A, Boutou O, Ducot B, Spira A, Hardle W. Does male age affect the risk of spontaneous abortion? An approach using semiparametric regression. Am J Epidemiol. 2003;157(9):815-24.
48. Nybo Andersen AM, Hansen KD, Andersen PK, Davey Smith G. Advanced paternal age and risk of fetal death: a cohort study. Am J Epidemiol. 2004;160(12):1214-22.
49. Maconochie N, Doyle P, Prior S, Simmons R. Risk factors for first trimester miscarriage--results from a UK-population-based case-control study. BJOG. 2007;114(2):170-86.
50. Jaleel R, Khan A. Paternal factors in spontaneous first trimester miscarriage. Pak J Med Sci. 2013;29(3):748-52.
51. Nguyen BT, Chang EJ, Bendikson KA. Advanced paternal age and the risk of spontaneous abortion: an analysis of the combined 2011-2013 and 2013-2015 National Survey of Family Growth. Am J Obstet Gynecol. 2019.
52. Bender Atik R, Christiansen OB, Elson J, Kolte AM, Lewis S, Middeldorp S, et al. ESHRE guideline: recurrent pregnancy loss. Human Reproduction Open. 2018;2018(2).
53. Kolte AM, on behalf of the ESHRE Special Interest Group EP, Bernardi LA, on behalf of the ESHRE Special Interest Group EP, Christiansen OB, on behalf of the ESHRE Special Interest Group EP, et al. Terminology for pregnancy loss prior to viability: a consensus statement from the ESHRE early pregnancy special interest group. Hum Reprod. 2014;30(3):495-8.
54. Slama R, Ballester F, Casas M, Cordier S, Eggesbo M, Iniguez C, et al. Epidemiologic tools to study the influence of environmental factors on fecundity and pregnancy-related outcomes. Epidemiol Rev. 2014;36:148-64.
55. Althubaiti A. Information bias in health research: definition, pitfalls, and adjustment methods. J

Multidiscip Healthc. 2016;9:211-7.
56. Reijneveld SA. Age in epidemiological analysis. J Epidemiol Community Health. 2003;57(6):397.
57. DiFranza JR, Lew RA. Effect of maternal cigarette smoking on pregnancy complications and sudden infant death syndrome. J Fam Pract. 1995;40(4):385-94.
58. Nielsen A, Hannibal CG, Lindekilde BE, Tolstrup J, Frederiksen K, Munk C, et al. Maternal smoking predicts the risk of spontaneous abortion. Acta Obstet Gynecol Scand. 2006;85(9):1057-65.
59. Andersen AM, Andersen PK, Olsen J, Gronbaek M, Strandberg-Larsen K. Moderate alcohol intake during pregnancy and risk of fetal death. Int J Epidemiol. 2012;41(2):405-13.
60. Schisterman EF, Cole SR, Platt RW. Overadjustment bias and unnecessary adjustment in epidemiologic studies. Epidemiology. 2009;20(4):488-95.
61. Ananth CV, Schisterman EF. Confounding, causality, and confusion: the role of intermediate variables in interpreting observational studies in obstetrics. Am J Obstet Gynecol. 2017;217(2):167-75.
62. Howards PP, Schisterman EF, Poole C, Kaufman JS, Weinberg CR. "Toward a clearer definition of confounding" revisited with directed acyclic graphs. Am J Epidemiol. 2012;176(6):506-11.
63. Venners SA, Wang X, Chen C, Wang L, Chen D, Guang W, et al. Paternal smoking and pregnancy loss: a prospective study using a biomarker of pregnancy. Am J Epidemiol. 2004;159(10):993-1001.
64. Henriksen TB, Hjollund NH, Jensen TK, Bonde JP, Andersson AM, Kolstad H, et al. Alcohol consumption at the time of conception and spontaneous abortion. Am J Epidemiol. 2004;160(7):661-7.
65. Raad G, Hazzouri M, Bottini S, Trabucchi M, Azoury J, Grandjean V. Paternal obesity: how bad is it for sperm quality and progeny health? Basic and clinical andrology. 2017;27:20-.
66. te Velde ER, Pearson PL. The variability of female reproductive ageing. Hum Reprod Update. 2002;8(2):141-54.
67. Wiener-Megnazi Z, Auslender R, Dirnfeld M. Advanced paternal age and reproductive outcome. Asian J Androl. 2012;14(1):69-76.
68. Garcia-Ferreyra J, Luna D, Villegas L, Romero R, Zavala P, Hilario R, et al. High Aneuploidy Rates Observed in Embryos Derived from Donated Oocytes are Related to Male Aging and High Percentages of Sperm DNA Fragmentation. Clin Med Insights Reprod Health. 2015;9:21-7.
69. Luetjens CM, Rolf C, Gassner P, Werny JE, Nieschlag E. Sperm aneuploidy rates in younger and older men. Hum Reprod. 2002;17(7):1826-32.
70. Coates A, Hesla JS, Hurliman A, Coate B, Holmes E, Matthews R, et al. Use of suboptimal sperm increases the risk of aneuploidy of the sex chromosomes in preimplantation blastocyst embryos. Fertil Steril. 2015;104(4):866-72.
71. Brandt JS, Cruz Ithier MA, Rosen T, Ashkinadze E. Advanced paternal age, infertility, and reproductive risks: A review of the literature. Prenat Diagn. 2019;39(2):81-7.
72. Schmid TE, Grant PG, Marchetti F, Weldon RH, Eskenazi B, Wyrobek AJ. Elemental composition of human semen is associated with motility and genomic sperm defects among older men. Hum Reprod. 2013;28(1):274-82.
73. Plastira K, Msaouel P, Angelopoulou R, Zanioti K, Plastiras A, Pothos A, et al. The effects of age on DNA fragmentation, chromatin packaging and conventional semen parameters in spermatozoa of oligoasthenoteratozoospermic patients. J Assist Reprod Genet. 2007;24(10):437-43.

74. Wyrobek AJ, Eskenazi B, Young S, Arnheim N, Tiemann-Boege I, Jabs EW, et al. Advancing age has differential effects on DNA damage, chromatin integrity, gene mutations, and aneuploidies in sperm. Proc Natl Acad Sci U S A. 2006;103(25):9601-6.
75. Martin JH, Aitken RJ, Bromfield EG, Nixon B. DNA damage and repair in the female germline: contributions to ART. Human Reproduction Update. 2018;25(2):180-201.
76. Johnson SL, Dunleavy J, Gemmell NJ, Nakagawa S. Consistent age-dependent declines in human semen quality: a systematic review and meta-analysis. Ageing Res Rev. 2015;19:22-33.
77. Guzick DS, Overstreet JW, Factor-Litvak P, Brazil CK, Nakajima ST, Coutifaris C, et al. Sperm morphology, motility, and concentration in fertile and infertile men. N Engl J Med. 2001;345(19):1388-93.
78. Keel BA. Within- and between-subject variation in semen parameters in infertile men and normal semen donors. Fertility and Sterility. 2006;85(1):128-34.
79. 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.
80. McQueen DB, Zhang J, Robins JC. Sperm DNA fragmentation and recurrent pregnancy loss: a systematic review and meta-analysis. Fertil Steril. 2019;112(1):54-60.e3.
81. Zhao J, Zhang Q, Wang Y, Li Y. Whether sperm deoxyribonucleic acid fragmentation has an effect on pregnancy and miscarriage after in vitro fertilization/intracytoplasmic sperm injection: a systematic review and meta-analysis. Fertil Steril. 2014;102(4):998-1005.e8.
82. Robinson L, Gallos ID, Conner SJ, Rajkhowa M, Miller D, Lewis S, et al. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. Hum Reprod. 2012;27(10):2908-17.
83. Cozzubbo T, Neri QV, Rosenwaks Z, Palermo GD. To what extent can oocytes repair sperm DNA fragmentation? Fertil Steril. 2014;102(3):e61.
84. Liang X, Mao Y, Wang Y, Liu S, Yan J. Female age affects the utility of sperm DNA fragmentation in predicting the outcomes of in vitro fertilization and intracytoplasmic sperm injection. Reprod Biomed Online. 2019.
85. du Fossé N, van der Hoorn ML, Eikmans M, Heidt S, le Cessie S, Mulders A, et al. Evaluating the role of paternal factors in etiology and prognosis of recurrent pregnancy loss: study protocol for a multicenter case-control study and cohort study (the REMI III project) BMJ Open. 2019;9(11):e033095.
86. Sunderam S, Kissin DM, Crawford SB, Folger SG, Jamieson DJ, Warner L, et al. Assisted Reproductive Technology Surveillance- United States, 2013. MMWR Surveill Summ. 2015;64(11):1-25.
87. Spandorfer SD, Avrech OM, Colombero LT, Palermo GD, Rosenwaks Z. Effect of parental age on fertilization and pregnancy characteristics in couples treated by intracytoplasmic sperm injection. Hum Reprod. 1998;13(2):334-8.
88. Klonoff-Cohen HS, Natarajan L. The effect of advancing paternal age on pregnancy and live birth rates in couples undergoing in vitro fertilization or gamete intrafallopian transfer. Am J Obstet Gynecol. 2004;191(2):507-14.
89. Whitcomb BW, Turzanski-Fortner R, Richter KS, Kipersztok S, Stillman RJ, Levy MJ, et al. Contribution of male age to outcomes in assisted reproductive technologies. Fertil Steril. 2011;95(1):147-51.
90. Gallardo E, Simon C, Levy M, Guanes PP, Remohi J, Pellicer A. Effect of age on sperm fertility potential:

oocyte donation as a model. Fertil Steril. 1996;66(2):260-4.

91. Paulson RJ, Milligan RC, Sokol RZ. The lack of influence of age on male fertility. Am J Obstet Gynecol. 2001;184(5):818-22; discussion 22-4.
92. Bellver J, Garrido N, Remohi J, Pellicer A, Meseguer M. Influence of paternal age on assisted reproduction outcome. Reprod Biomed Online. 2008;17(5):595-604.
93. Belloc S, Cohen-Bacrie P, Benkhalifa M, Cohen-Bacrie M, De Mouzon J, Hazout A, et al. Effect of maternal and paternal age on pregnancy and miscarriage rates after intrauterine insemination. Reprod Biomed Online. 2008;17(3):392-7.
94. Belloc S, Hazout A, Zini A, Merviel P, Cabry R, Chahine H, et al. How to overcome male infertility after 40: Influence of paternal age on fertility. Maturitas. 2014;78(1):22-9.
95. Ramasamy R, Chiba K, Butler P, Lamb DJ. Male biological clock: a critical analysis of advanced paternal age. Fertil Steril. 2015;103(6):1402-6.
96. Billari FC, Kohler H-P, Andersson G, Lundström H. Approaching the Limit: Long-Term Trends in Late and Very Late Fertility. Popul Dev Rev. 2007;33(1):149-70.



CHAPTER 3

Paternal smoking is associated with an increased risk of pregnancy loss in a dose-dependent manner: a systematic review and meta-analysis

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ABSTRACT

Objective

To study the association between paternal lifestyle factors in the preconception period and the risk of pregnancy loss.

Evidence Review

The Preferred Reporting Items for Systematic Reviews and Meta-analysis guidelines for systematic reviews and meta-analysis were followed. PubMed and Embase databases were searched up to August 2020. Original articles in English language addressing the relation between paternal exposure status in the preconception period and pregnancy loss were included. Paternal lifestyle factors examined were: smoking, alcohol consumption and body mass index (BMI). Studies that only examined exposure status during pregnancy (and not in the preconception period) and those that solely focused on pregnancy outcome after artificial reproductive technology (ART) were excluded. The qualitative risk of bias assessments were performed. Meta-analysis using a random-effects model was performed if sufficient data were available, with the risk of pregnancy loss as the primary outcome.

Results

The systematic search included 3386 articles of which 11 articles met the inclusion criteria. In a meta-analysis of 8 studies, paternal smoking of >10 cigarettes per day in the preconception period was found to be associated with an increased risk of pregnancy loss, after adjustment for maternal smoking status (1-10 cigarettes per day: 1.01; 95% confidence interval [CI] 0.97-1.06; 11-19 cigarettes per day: 1.12; 95% CI 1.08-1.16; ≥20 cigarettes per day: 1.23; 95% CI 1.17-1.29). No clear association was found between paternal alcohol consumption and pregnancy loss, based on 5 available studies. No studies were identified evaluating the association between paternal BMI and spontaneous pregnancy loss.

Conclusion

Awareness of the association between paternal smoking in the preconception period and the risk of pregnancy loss should be raised. More well-designed studies are needed to further investigate the effects of other paternal lifestyle factors on the risk of pregnancy loss.

INTRODUCTION

Although cigarette smoking, alcohol consumption and obesity are generally known health hazards with a significant impact on general health and well-being, they remain highly prevalent. There is substantial evidence that these modifiable lifestyle risk factors also affect reproductive health, including the risk of pregnancy loss. Pregnancy loss comprises spontaneous demise of the pregnancy before the fetus reaches viability and is a common complication of pregnancy occurring in 15% of clinically recognized pregnancies and 30% of all pregnancies.(1, 2) Active maternal smoking, maternal obesity and alcohol consumption have been consistently associated with an increased risk of pregnancy loss.(3-5)

While maternal risk factors for pregnancy loss are well-established, studies on potentially contributing paternal factors remain sparse. Recently, a significant association was found between advanced paternal age and pregnancy loss, persisting after adjustment for maternal age.(6) In another systematic review and meta-analysis paternal smoking was related to birth defects including congenital heart defects and orofacial clefts.(7) As knowledge on the impact of paternal lifestyle risk factors on the risk of pregnancy loss is still limited, it is essential to gain more insights into this. Biological evidence indicates that male lifestyle behaviors in the preconception period exert their effects on spermatozoa and may, thereby, influence pregnancy outcome. Cigarette smoking, excessive alcohol consumption and obesity have all been linked with systemic oxidative stress, which may result in sperm oxidative DNA damage and eventually lead to both short-term pregnancy complications and long-term outcomes in the offspring.(8, 9)

This systematic review aimed to provide a detailed analysis of the existing literature on the association between paternal lifestyle factors during the preconception period and the risk of pregnancy loss. The paternal factors that were evaluated included cigarette smoking, alcohol consumption and body mass index (BMI).

MATERIALS AND METHODS

This systematic review and meta-analysis was conducted following the Preferred Reporting Item for Systematic Reviews and Meta-analysis Statement and registered in the international prospective register of systematic reviews PROSPERO (ID CRD42020206057).(10)

Search and Selection Strategy

A systematic search of PubMed and Embase electronic databases was performed on August 23, 2020. The following free text and MeSH terms were used: pregnancy loss, abortion, spontaneous miscarriage, male, paternal, father, body mass index, BMI, obesity, smoking, alcohol, drinking behavior, lifestyle. The full search strategy for PubMed is shown in the Supplemental Material (available online). Additional searches in Google Scholar were conducted and reference lists of identified articles were manually searched for additional references.

The literature search was performed by two researchers (N.A.dF. and N.H.B.) and a librarian. The screening was performed by two researchers (N.A.dF. and N.H.B.). In the first stage, titles and abstracts were screened, and in the second stage, full manuscripts of the identified articles were read in detail. Any discordance on selection of studies and assessing risk of bias (described in the following) was resolved by consensus. If no agreement was obtained, the opinion of a third observer (E.E.L.O.L) was sought to gain consensus.

Eligibility Criteria

The inclusion criteria were original articles in English language addressing the relation between pregnancy loss and one or more of the following paternal exposure factors during the preconception period: smoking behavior, alcohol consumption and BMI. Pregnancy loss is generally defined as the spontaneous loss of conception before 20 or 24 weeks of gestation, including both biochemical and ultrasonically or histologically confirmed losses.(11-13) However, several studies used diverse definitions. We did not use a specific definition for pregnancy loss as a strict inclusion criterion, but we described the exact definitions used in all of the included studies. The preconception period in men has previously been described as around 10 weeks prior to conception, in line with the spermatogenic cycle.(14) We did not use a specific definition for the preconception period, but we described the exact definitions used in all of the included studies. Studies that only examined exposure status during pregnancy (and not in the preconception period) were excluded. To be included, a risk estimate for the relation between exposure and outcome had to be provided in the article. As we were interested in the relation between paternal lifestyle factors and pregnancy loss in the general population, studies

that solely focused on pregnancy outcomes after artificial reproductive technology were excluded.

Data Extraction

Two researchers (NF and NB) extracted data from all selected articles on: publication year, country, study period, study design, population characteristics, inclusion and exclusion criteria, exposure and outcome definitions, exposure and outcome ascertainment, sample size, type of effect measures, adjusted effect estimates with 95% confidence intervals (CI) and variables adjusted for in the analyses.

Risk of Bias Assessment

As stated by Dekkers et al.(15) in the Conducting Systematic Reviews and Meta-analyses of Observational Studies of Etiology guideline, it is not recommended to use a standard tool for assessing quality of observational epidemiologic studies. Because of the large heterogeneity in observational research, it is considered more appropriate to develop a tailored set of criteria for each observational systematic review to assess risk of bias in a qualitative matter.

For the current research question, we distinguished 3 relevant domains for risk of bias: bias due to confounding, information bias, and selection bias (including bias due to missing data or loss-to-follow-up). Risk of bias was assessed by 2 reviewers (N.A.dF. and N.H.B.). For each individual study, the risk of bias assessment is shown in the Supplemental Material.

Statistical analysis

The outcomes of the included studies were reported as adjusted odds ratios (AORs) or adjusted hazard ratios (AHRs) with 95% CIs. For meta-analysis, these effect measures were treated equally as risk measures. Standard errors were calculated from 95% CIs. Meta-analysis was only performed for the association between paternal smoking and pregnancy loss because insufficient data were available for paternal alcohol consumption and paternal BMI (as further explained in the Results section).

The meta-analysis for paternal smoking was stratified in four categories: 1-10 cigarettes per day, 11-19 cigarettes per day, ≥20 cigarettes per day and “any smoking” (regardless of the quantity of smoking). To prevent bias due to confounding by maternal smoking behavior, only studies that provided risk estimates adjusted for maternal smoking or studies that were conducted in nonsmoking women were included in the meta-analysis. One study reported AORs for different combinations of maternal and paternal smoking status.(16) The AOR for nonsmoking women with smoking male partners were used for meta-analysis.

If a study reported additional subcategories (e.g., 1-5 cigarettes per day and 5-10 cigarettes per day), the risk estimates of these categories were pooled using a within-study fixed-effect meta-analysis and included as such in the final meta-analysis. If a study used a broader category (e.g., 1-20 cigarettes per day), we used the same estimates for the subcategories (e.g., 1-10 cigarettes per day and 10-20 cigarettes per day) and standard errors were adjusted, assuming equal sample sizes in both subcategories. Some studies reported a risk estimate for smoking in general, that is, without specifying the quantity of smoking. These risk estimates were included in the meta-analysis in the category "any smoking". For studies that did not report a risk estimate for smoking in general, the risk estimates of the different subcategories for smoking used in that particular study were pooled using a within-study fixed-effect meta-analysis and this pooled risk estimate was used for "any smoking" in the final meta-analysis. One study included the average amount of cigarettes per day as a continuous variable in a multivariable model. (17) The AHR with 95% CI that was presented in the article was used to calculate risk estimates with 95% CIs for the subcategories 1-10, 11-19 and ≥20 cigarettes per day.

Evidence of publication bias was assessed through qualitative inspection of a funnel plot. Considering heterogeneity of study populations and study designs, random-effects meta-analyses with DerSimonian and Laird estimation were used (command metan in Stata 14: StataCorp LLC, TX).

RESULTS

Study selection

An overview of the study selection process is shown in the Preferred Reporting Items for Systematic Reviews and Meta-analysis Flow Diagram (Fig. 1). The systematic search retrieved a total of 3,386 original articles. After first-stage screening by reviewing titles and abstracts, 3,365 studies were excluded and 21 articles were identified to assess the full text for eligibility. After the assessment of full manuscripts, 10 articles were excluded for several reasons shown in Figure 1. Finally, 11 studies met all the inclusion criteria. Six studies evaluated the association between preconceptional paternal smoking behaviour and pregnancy loss, 2 studies studies focussed on paternal alcohol consumption and pregnancy loss and 3 studies adressed both exposures. No studies were retrieved that investigated the relation between paternal BMI and pregnancy loss.

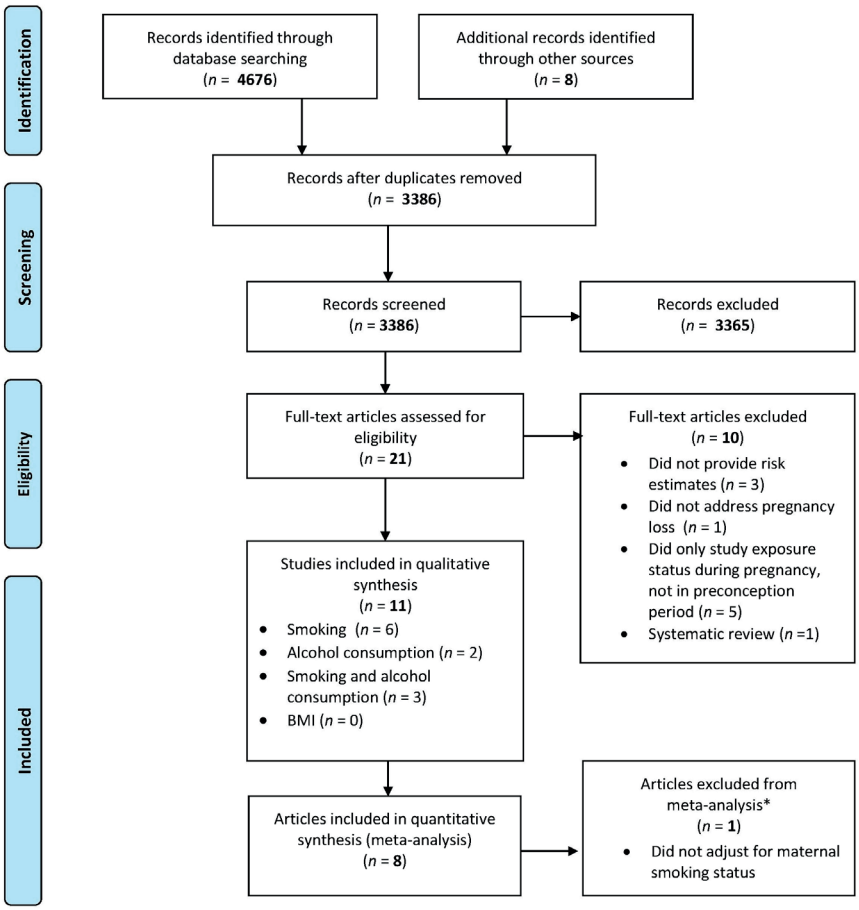


Figure 1. Flow diagram of study selection process

*Meta-analysis is only performed for the association between paternal smoking behavior and pregnancy loss, as explained in the Results section.

Characteristics of included studies

Six studies(16, 18-22) were case-control studies, 4 studies were prospective cohort studies, and 1 study was a retrospective cohort study.(16-26) Sample sizes varied from 107 participants in a case-control study to nearly 6 million pregnancies in the largest cohort study.(16, 26) Five studies were conducted in the USA, 2 in China, and 1 each in Italy, Denmark, Mexico and the United Kingdom.(16-26) The key characteristics of all included studies are summarized in Table 1.

Definition of outcome

In the studies included in this systematic review, pregnancy loss was mostly defined as a loss of conception before 20 weeks of gestation.(16, 21-23, 25) Three studies used <28 or <22 weeks of gestation and 3 studies focused on first trimester pregnancy loss, with gestational age <13 or <12 weeks.(17-20, 24, 26)

Risk of bias

Risk of bias was assessed for all of the included studies. The results of this assessment are shown in the Supplemental Material.

Bias due to confounding

When evaluating paternal lifestyle factors on pregnancy outcome, maternal lifestyle behaviors are important confounding factors. Of 11 included studies, 7 were adjusted for maternal smoking behavior and alcohol consumption.(17-19, 21, 22, 24) Three studies were restricted either to nonsmoking or non-alcohol-consuming women (depending on the studied paternal exposure).(23, 25, 26) One study provided a risk estimate for a subgroup of couples all consisting of smoking men and nonsmoking women.(16) One study that reported ORs for both paternal smoking and alcohol consumption did not adjust for the equivalent maternal factors and was, therefore, not included in the meta-analysis.(20) All studies adjusted for maternal age, being a well-established major risk factor for pregnancy loss. However, it is equivocal to what extent age is related to lifestyle factors and, thus, whether it should be considered as a confounding factor. Five studies controlled for 1 or more potentially confounding paternal factors, including lifestyle factors and exposure to toxins.(16, 17, 21, 23, 26)

Information bias

In 6 of the included studies, data on preconception paternal exposure status were collected during the preconception period or during early pregnancy.(16, 17, 23-26) In 5 studies, these data were collected in retrospect; that is, after outcome of the pregnancy. In these same 5 retrospective studies plus 1 prospective study, information on paternal exposure status was acquired from the female partners.(18-22, 25) In all other (prospective) studies, paternal exposure status was directly reported by the male

partners.(16, 17, 23, 24, 26) Regarding ascertainment of pregnancy outcome, 5 studies only included cases with hospital-confirmed pregnancy loss.(18, 19, 21, 22, 25) In 2 studies early pregnancy loss was detected by daily urine hCG assays and losses beyond 6 weeks were clinically confirmed.(17, 23) One study used daily hCG assays during early pregnancy, whereas later pregnancy outcomes were gained from questionnaires.(24) Two studies completely relied on self-reports of pregnancy outcomes, and 1 study did not state the ascertainment of pregnancy outcomes.(16, 20, 26)

Selection bias

Four studies were hospital-based, and 7 studies were population-based. All of the hospital-based studies were restricted to women that underwent a medical procedure for their miscarriage.

Loss to follow-up was low for all studies, except for the study of Blanco-Muñoz et al.(16), who reported an attrition rate of 28% after confirmation of pregnancies. Missing data were low for all studies that reported missing data. Two studies did not report missing data.(19, 24)

Narrative synthesis

Paternal smoking

Windham et al.(21) conducted a case-control study in the United States to assess the relation between cigarette smoking and the risk of pregnancy loss. The AORs for all categories of paternal smoking (1-10, 11-20, and >20 cigarettes per day) approximated unity. Information on paternal smoking during the 3 months before pregnancy was based on maternal reporting. In a small subsample, male partners were also interviewed to validate maternal reporting. Maternal reporting of paternal smoking status showed good agreement, whereas the quantity of smoking tended to correspond less well. Seven years later, the same authors performed a second study within a prospective cohort only including nonsmoking women.(25) Similar to their previous study, no association between paternal smoking and pregnancy loss was found.

The Italian hospital-based case-control study by Chatenoud et al.(18) examined the association between paternal smoking status and loss <12 weeks of gestation. They did not find any significant relationship between paternal smoking habits before conception and the risk of pregnancy loss (AOR for >10 cigarettes per day 0.9; 95% CI 0.7-1.1). Data on paternal smoking habits were acquired from the female partner.

Table 1. Characteristics of included studies

Author, year, country	Studied factor(s)	Study period	Study design	Study setting	No. of pregnancies or no. of cases and controls	Definition and ascertainment of pregnancy loss
Windham et al. (1992), United States	Paternal smoking	1986-1987	Case-control	Hospital-based	Cases: *n* = 626 Controls: *n* = 1,300 (live birth)	<20-wk gestation Pathology specimen submitted to the hospital laboratory
Chatenoud et al. (1998), Italy	Paternal smoking	1993-1998	Case-control	Hospital-based	Cases: *n* = 782 Controls: *n* = 1,543 (live birth >37 wk)	<12-wk gestation Uterine curettage and pathological examination
Windham et al. (1999), USA	Paternal smoking	1990-1991	Prospective cohort	Population-based (recruited from a large prepaid health plan)	4,196 pregnancies	<20-wk gestation Medical records
Venners et al. (2004), USA	Paternal smoking	1996-1998	Prospective cohort	Reproductive health study in China	526 women	<20-wk gestation Early pregnancy loss (<6 wk) detected by daily urinary hCG assay; later pregnancy losses clinically confirmed
Blanco-Muñoz et al. (2009), Mexico	Paternal smoking	2001-2004	Nested case-control	Recruited during the state's obligatory prenuptial marriage counselling in four municipalities in Mexico	Cases: *n* = 23 Controls: *n* = 84 (ongoing pregnancy >20 wk)	<20-wk gestation Ascertainment of pregnancy loss not stated
Wang et al. (2018), China	Paternal smoking	2010-2016	Retrospective cohort	Population-based (National Free Pre-Pregnancy Checkups Project)	5,770,691 pregnancies	<28-wk gestation Self-reports (recontacted within 1 year after confirmation of pregnancy)

Definition and ascertainment of exposure		Adjusted risk estimates	Risk factors adjusted for
Average amount smoked in 3 mo before pregnancy	Cigarettes/day in three months before pregnancy	AOR (95% CI)	Maternal age, race, caffeine, alcohol, bottled water, tobacco consumption, prior fetal loss, marital status, insurance coverage
Indirectly by the female partner; a small subsample of men (n = 94) was interviewed for validation	None	1 (reference)	
	1-10	0.9 (0.6-1.3)	Paternal age, race, education, alcohol consumption
	11-20	1.1 (0.7-1.5)	
	>20	1.0 (0.6-1.5)	
	Any smoking	1.1 (0.9-1.4)	
Average amount smoked before conception	Smoking status	AOR (95% CI)	Centre, age, education, marital status, maternal family history of spontaneous abortion, history of miscarriages, nausea, maternal alcohol and coffee intake and smoking in the first trimester
	Never	1 (reference)	
Indirectly reported by the female partner	Former	0.8 (0.6-1.1)	
	Current	0.8 (0.7-1.0)	
	Cigarettes/day before conception		
	≤10	0.8 (0.6-1.0)	
	>10	0.9 (0.7-1.1)	
Average amount smoked in 3 mo before pregnancy	Cigarettes/day during three months before pregnancy	AOR (95% CI)	Maternal age, prior fetal loss, alcohol and caffeine consumption, gestational age at interview
Indirectly reported by the female partner	None	1 (reference)	
	1-20	0.98 (0.73-1.3)	*Only non-smoking women were included*
	>20	0.97 (0.41-2.3)	
Average amount smoked before the date of stopping use of contraceptive methods	Smoking status	AOR (95% CI)	Maternal age, education, perceived life stress, exposures to dust and noise, BMI, tea drinking
	Non-smoker	1 (reference)	
	<20 cigarettes/day	1.01 (0.68-1.50)	
Directly reported by the male partner	≥20 cigarettes/day	1.45 (0.82-2.56)	Paternal age, alcohol consumption, previous smoking, exposure to toxins
			Only non-smoking women and non-alcohol consuming women were included
Average amount smoked at the prenuptial marriage counselling	Smoking status	AOR (95% CI)	Maternal age, occupation, intake of coffee
	Man non-smoker	1 (reference)	
Directly reported by the male partner	Man smoker	2.89 (0.99-8.45)	Paternal occupation
	M-F+	1 (reference)	
	M-F+	1.96 (0.40, 10.1)	
	M+F-	3.60 (0.80, 16.3)	
	M+F+	4.61 (1.04, 20.5)	
Average amount smoked at preconception health examination	Cigarettes/day before conception	AOR (95% CI)	Maternal age, last menstrual period, maternal higher education, Han ethnicity, preconception BMI, alcohol drinking, passive smoking, region of provinces
	No	1	
	Yes	1.11 (1.08-1.14)	
Directly reported by the male partner	1-4	1.03 (0.96-1.11)	Paternal age, paternal passive smoking
	5-9	1.02 (0.97-1.08)	
	10-14	1.11 (1.06-1.16)	*Only non-smoking women were included*
	15-19	1.21 (1.09-1.33)	
	≥20	1.23 (1.17-1.30)	

Table 1. Continued.

Author, year, country	Studied factor(s)	Study period	Study design	Study setting	No. of pregnancies or no. of cases and controls	Definition and ascertainment of pregnancy loss
Maconochie et al. (2007), UK	Paternal smoking and alcohol consumption	1980-2000	Case-control	Population-based (National Women's Health Study)	Cases: *n* = 603 Controls: *n* = 6,116 (ongoing pregnancy >12 weeks)	<13-wk gestation Self-reports (questionnaire)
Xu et al. (2014), China	Paternal smoking and alcohol consumption	2009-2012	Matched case-control	Hospital-based	Cases: *n* = 620 Controls: *n* = 1,240 (ongoing pregnancy >12 weeks)	<13-wk gestation Clinically confirmed
Buck Louis et al. (2016), USA	Paternal smoking, alcohol consumption and BMI	2005-2009	Prospective cohort	Population-based (16 counties in Michigan and Texas)	344 pregnancies	<22-wk gestation Conversion to negative hCG test or clinical confirmation
Windham et al. (1992), USA	Paternal alcohol consumption	1986-1987	Case-control	Hospital-based	Cases: *n* = 626 Controls: *n* = 1,300 (live birth)	<20-wk gestation Pathology specimen submitted to the hospital laboratory
Henriksen et al. (2004), Denmark	Paternal alcohol consumption	1992-1994	Prospective cohort	Population-based (members of four trade unions in Denmark)	186 pregnancies	<28-wk gestation Early pregnancy loss detected by daily urinary hCG assay; outcomes of clinically recognized pregnancies collected by questionnaires (self-reports)

AHR = adjusted hazard ratio; AOR = adjusted odds ratio; BMI = body mass index; CI = confidence interval; F = female; hCG = human chorionic gonadotropin; M = male

	Definition and ascertainment of exposure		Adjusted risk estimates	Risk factors adjusted for
	Average amount of cigarettes per day and alcohol per week) in 3 mo before pregnancy Indirectly reported by the female partner	Cigarettes/day No Yes <5 5-10 11-20 >20 Alcohol/week (standard UK units) No drinking <1 1-10 10-21 21-35 >35	AOR (95% CI) 1 (reference) 1.04 (0.87-1.25) 0.68 (0.43-1.07) 1.03 (0.71-1.50) 1.13 (0.88-1.44) 1.19 (0.86-1.66) 1 (reference) 0.77 (0.48-1.26) 0.73 (0.49-1.07) 0.87 (0.58-1.29) 0.95 (0.61-1.50) 0.84 (0.51-1.40)	Year of conception, maternal age, previous miscarriage, previous live birth
	Average amount of cigarettes per day and alcohol per week in 3 mo before pregnancy Indirectly reported by the female partner	Cigarettes/day No smoking 1-10 11-20 >20 Amount of alcohol per week (mL) No drinking or <200 200-500 >500	AOR (95% CI) 1 (reference) 1.05 (0.81-1.27) 1.01 (0.79-1.33) 1.23 (0.87-1.47) 1 (reference) 0.90 (0.68-1.15) 1.01 (0.80-1.23)	History of miscarriage, previous induced abortion, maternal vitamin supplementation, frequency of night shift, frequent staying up late, regular physical exercise, smoking, alcohol consumption Controls matched by maternal age ±3 years
	Average amount of cigarettes per day and alcoholic consumptions per week in three months before pregnancy Directly reported by the male partner	Average cigarette smoking Average alcohol consumption	AHR (95% CI)* 1.01 (0.95-1.07) 0.97 (0.72-2.81)	Maternal age, BMI, difference in partner's ages, prior pregnancy loss, smoking, alcohol consumption, caffeine consumption, vitamin adherence, average intercourse frequency Paternal BMI, smoking, alcohol consumption, caffeine consumption, vitamin adherence
	Average amount of alcohol consumptions per week in three months before pregnancy Indirectly reported by the female partner; a small subsample of men (n = 94) was interviewed for validation	Alcoholic consumptions/week <1/2 1-6 7-13 ≥14	AOR (95% CI) 1 (reference) 1.2 (0.95-1.6) 1.0 (0.74-1.4) 1.2 (0.84-1.7)	Maternal age, maternal smoking, passive smoking, nausea, maternal alcohol consumption
	Amount of alcohol consumptions in the cycle before conception Directly reported by the male partner	Alcoholic consumptions/week 0 1-4 5-9 ≥10	AHR (95% CI) 1 (reference) 2.7 (0.6-2.4) 1.6 (0.3-7.7) 4.3 (0.9-19.3)	Maternal caffeine intake, alcohol consumption, smoking, age, menstrual cycle length

Venners et al.(23) conducted a prospective study in a cohort of Chinese textile workers. Paternal smoking behavior was reported through a questionnaire, completed by the male partners. Both early pregnancy losses, detected by daily urine hCG assays, and clinically detected spontaneous miscarriages were taken into account. Compared to nonsmoking men, AORs for total pregnancy loss were 1.12 (95% CI 0.77-1.65) for smoking 1-20 cigarettes per day and 1.64 (95% CI 0.92-2.93) for smoking ≥20 cigarettes per day.

Blanco-Muñoz et al.(16) reported a nested-case control study in couples who were included during the obligatory prenuptial marriage counselling in Mexico. They found an increased risk of pregnancy loss in couples consisting of smoking men and nonsmoking women compared to couples consisting of 2 nonsmoking partners, although this was not statistically significant (AOR 3.60; 95% CI 0.80-16.3). The amount of smoking was not specified.

The most recent (2018) and largest study on paternal smoking and the risk of pregnancy loss was a Chinese population-based retrospective cohort study of nearly 6 million pregnancies by Wang et al.(26) The data used for this study derived from couples who participated in the National Free Pre-Pregnancy Checkup Project. During preconception health examinations, both partners were interviewed about their smoking behavior. Only nonsmoking women and their partners were included. Reported AORs for pregnancy loss increased from 1.03 (95% CI 0.96-1.11) for paternal smoking of 1-4 cigarettes per day to 1.23 (1.17-1.30) for ≥20 cigarettes per day, with nonsmoking men being the reference group.

Paternal smoking and paternal alcohol consumption

Maconochie et al. studied a wide range of socio-demographic and lifestyle behaviors in relation to first trimester pregnancy loss in the UK, including paternal smoking and alcohol consumption.(20) All data was collected from the participating women. They did not find any significant associations between these two factors and the risk of pregnancy loss. The odds ratios were adjusted for maternal age, year of conception, and previous pregnancy outcomes, but not for maternal lifestyle factors.

In a maternal age-matched case-control study in China, Xu et al.(19) evaluated a variety of potential risk factors for early pregnancy loss. They reported AORs for clinically confirmed first trimester pregnancy loss ranging from 1.05 (95% CI 0.81-1.27) for preconceptional paternal of smoking 1-10 cigarettes per day to 1.23 (95% 0.87-1.47) for >20 cigarettes per day, compared with nonsmoking men. All information on lifestyle factors was obtained through a questionnaire completed by the participating women.

Buck Louis et al.(17) investigated associations between couples' lifestyle behaviors in the preconception period and pregnancy loss in a prospective cohort study in the USA. Both members of the participating couples recorded their daily use of cigarettes. Pregnancy loss was detected by conversion to a negative pregnancy test or by clinical confirmation upon gestation. The investigators presented a multivariable model with AHRs for female and male lifestyle factors. The average daily number of cigarettes and alcoholic consumptions were included in the model as continuous variables, with AHRs of 1.01 (95% CI 0.95-1.07) and 0.97 (0.73-1.28), respectively.

Paternal alcohol consumption

Two studies entirely focused on alcohol consumption and the effect on pregnancy loss. In an American hospital-based case-control study, Windham et al.(22) found an AOR of 1.2 (95% CI 0.84-1.7) for men consuming 14 or more alcoholic consumptions per week during the preconception period (drinking behavior was reported by their partners). Henriksen et al.(24) conducted a prospective cohort study in Denmark and interviewed both members of the couples. They reported an AHR for pregnancy loss of 4.3 (95% CI 0.9-19.3) when men consumed 10 or more alcoholic consumptions per week, compared to non-drinking men.

Quantitative synthesis

Paternal smoking

Eight studies that evaluated the association between paternal smoking behavior in the preconception period and the risk of pregnancy loss were included in the meta-analysis.(16-19, 21, 23, 25, 26) One study was not included in the meta-analysis because it reported risk estimates without adjustment for maternal smoking status.(20) The meta-analysis (Fig. 2) showed significant increased risks of pregnancy loss if fathers smoked more than 10 cigarettes per day (pooled estimates 1.12; 95% CI 1.08-1.16 for 11-19 cigarettes per day and 1.23; 95% CI 1.17-1.29 for ≥20 cigarettes per day). No effects were found for smoking 1-10 cigarettes per day or for "any smoking" (i.e., taking into account all smoking fathers, regardless of the quantity of smoking).

A sensitivity analysis (Supplemental Fig. 1, available online) was performed by repeating the meta-analysis with exclusion of the study of Wang et al.(26), as the sample size and, thus, the weight of this study in the meta-analysis were relatively large compared to all of the other studies. A similar pattern of the paternal smoking association was observed, with a pooled estimate of 1.19 (95% CI 0.97-1.46) for smoking ≥20 cigarettes per day.

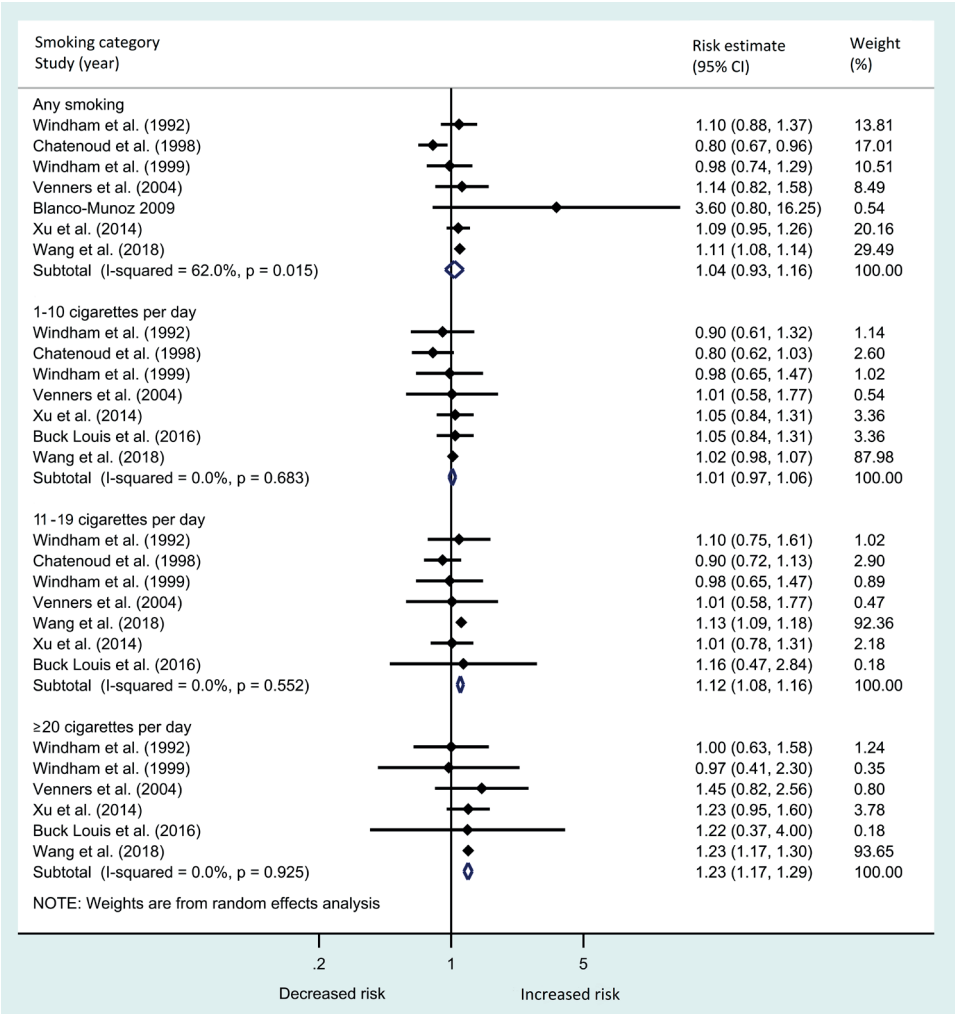


Figure 2. Forest plot describing the association between paternal smoking behavior in different categories of paternal smoking and the risk of spontaneous pregnancy loss

As indicated by I^2 (a statistic that indicates the percentage of variance in a meta-analysis that is attributable to study heterogeneity), heterogeneity was small for smoking categories 1-10, 11-19 and ≥20 cigarettes per day, whereas heterogeneity was substantial in the category "any smoking" because of the relatively extreme risk estimated reported by Blanco-Munoz et al.(16) A funnel plot showed some underrepresentation of small studies with negative effects (Supplemental Fig. 2, available online). There were no major differences in the pooled estimates provided by models with random and fixed effects (data not shown).

Paternal alcohol consumption

Five of the included studies evaluated the association between paternal alcohol consumption in the preconception period and the risk of pregnancy loss. Three studies(17, 19, 21) did not find any increased risks of pregnancy loss associated with paternal alcohol consumption, regardless of the quantity of alcohol consumption. Two studies(22, 24) reported increased AORs for large numbers of alcoholic consumptions per week, although these effects were not statistically significant (Windham et al.(22): AOR 1.2; 95% CI 0.84-1.7 for ≥14 alcoholic consumptions per week and Henriksen et al.(24): AOR 1.6; 95% CI 0.3-7.7 for 5-9 alcoholic consumptions per week and AOR 4.3; 95% CI 0.9-19.3 for ≥10 alcoholic consumptions per week). Because of the limited number of studies and substantial differences between studies in used subcategories for quantity and unity of alcohol consumption, no meta-analysis was performed.

DISCUSSION

In this systematic review and meta-analysis, paternal smoking in the preconception period of >10 cigarettes per day was found to be associated with a significantly increased risk of pregnancy loss, independent of maternal smoking habits. The study of Wang et al.(26) had relatively much weight in the meta-analysis due to its large sample size; however, we assessed this study as well conducted and with a low risk of bias. A sensitivity analysis excluding this study showed also a similar pattern of the paternal smoking effect. Based on few available studies, no clear evidence was found for a link between paternal alcohol consumption and pregnancy loss. No studies were identified that evaluated the association between paternal BMI and the risk of pregnancy loss.

Investigating the relation between paternal lifestyle factors and the risk of pregnancy loss from an etiological perspective is challenging for several reasons. First, the risk of bias due to confounding should be taken into account. For example, because smokers are more likely to have partners who smoke and maternal smoking is a known risk factor for pregnancy loss, it is crucial to control for the smoking status of the female partner when evaluating the paternal smoking effect.(27) For this reason, we restricted our meta-analysis to studies that appropriately adjusted their risk estimates for maternal smoking or that were conducted in non-smoking women. On the other hand, a risk may appear in controlling for too many variables. Risk of bias due to confounding occurs when there is a failure to adjust for common causes of both the exposure and outcome. Prior pregnancy loss, for instance, is a strong predictor for a next pregnancy loss but should not be treated as a confounder as explained by Weinberg(28) and Howards et al.(29). If one assumes that the exposure of interest (e.g., paternal smoking) is a cause of both prior and current pregnancy losses, controlling for prior pregnancy loss will result in overadjustment bias: the estimate of the total causal effect will be biased toward the null.(28-30) Likewise, some of the studies adjusted for socioeconomic status, which is associated with the risk of pregnancy loss.(31) However, indicators of socioeconomic status (e.g., education and income) are most likely non-causally related to pregnancy loss and mediated by lifestyle and behavioral factors.(30) From that perspective, not adjusting for socioeconomic status is appropriate to prevent overadjustment. Overadjustment bias may have been induced in some of the studies included in this review.

A second issue is that different study designs may introduce different types of bias. Although hospital-based case-control studies have the advantage of more certainty about the diagnosis of pregnancy loss, only a selection of all women who underwent a medical procedure for their pregnancy loss are included in these studies; women with early pregnancy loss are usually less well represented. Furthermore, in studies where the exposure status is obtained in retrospect (i.e., after the occurrence of pregnancy

loss or live birth), differential recall bias may arise. Women who miscarried (and their partners) could be more likely to report higher levels of possibly damaging exposures. (32) In addition, in retrospective studies, the selection of exposed or unexposed subjects may be somehow related to the outcome of interest, since women who suspect a relation between their exposure status and their pregnancy loss may be more inclined to participate. Besides this, self-reported socially undesirable lifestyle exposures are prone to underreporting, which may result in some nondifferential misclassification bias.(33) As shown by Windham et al.(21), maternal reporting of paternal smoking behavior may as well lead to non-differential misclassification, making a potential association more difficult to detect.

A third challenge is to differentiate between the impact of exposure in the preconception stage and exposure during pregnancy. For example, maternal passive smoking (second-hand smoke derived from their partner) may be a confounder for the direct effect of preconceptional paternal smoking on pregnancy loss. To assess the true effect of preconceptional paternal smoking, Wang et al.(26) did a separate analysis with exclusion of women whose partners still smoked during the early pregnancy follow-up. The effect estimates derived from this analysis were slightly lower compared to the non-restricted analysis (AOR for ≥20 cigarettes 1.23; 95% CI 1.1.17-1.30 compared to 1.33; 95% 1.30-1.35), suggesting that some confounding by maternal passive smoking was present indeed.

Despite these caveats, there are solid biological arguments supporting a causal relation between preconceptional paternal lifestyle factors and pregnancy loss. It has been shown that tobacco smoke constituents react directly with spermatozoa and can cause DNA damage.(34) Male cigarette smokers exhibit higher levels of reactive oxygen species in their seminal plasma, which may overwhelm seminal plasma antioxidant capacity and cause oxidative stress-mediated sperm DNA fragmentation.(35, 36) Similarly, both obesity and excessive alcohol intake have been linked to sperm DNA damage.(37, 38) Because of the minimal repair capacity of ejaculated sperm, changes in genomic integrity of spermatozoa may persist upon conception. A recent study showed that paternal lifestyle characteristics, potentially mediated by sperm DNA fragmentation, have significant effects on embryo developmental kinetics in couples that underwent intracytoplasmic sperm injection treatment.(39) In addition, impaired sperm DNA integrity has been associated with pregnancy loss in both spontaneous and assisted pregnancies.(40, 41) As the paternal genome is activated after 4-8 cell embryo stages, the effect of high sperm DNA damage is presumed to manifest after fertilization, in the later stages of embryonic development.(42) Defects in sperm DNA may impact blastocyst development and may as well lead to (post)implantation failures.(42-44)

The male contribution to adverse pregnancy outcome has been under evaluated for a long time. Here we show that paternal smoking in the preconception period is associated with an increased risk of pregnancy loss in a dose-dependent manner, irrespective of maternal smoking habits. This significant finding has implications for preconception counselling and is also of interest for couples with unexplained recurrent pregnancy loss. More research into other paternal lifestyle exposures, including alcohol consumption and obesity (or dietary intake), is needed since these factors have hardly been studied in the context of pregnancy loss. Future studies should preferably have a prospective design, appropriate ascertainment of exposures and outcomes and adequate adjustment for confounders. In addition to epidemiologic research, basic studies are desired to further explore underlying mechanisms.

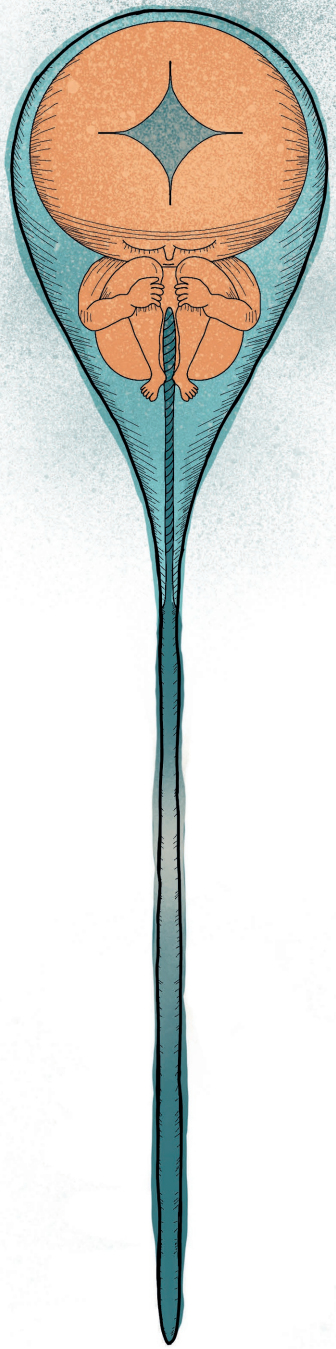
REFERENCES

1. Wilcox AJ, Weinberg CR, O'Connor JF, Baird DD, Schlatterer JP, Canfield RE et al. Incidence of early loss of pregnancy. N Engl J Med 1988;319:189-94.
2. Nybo Andersen AM, Wohlfahrt J, Christens P, Olsen J, Melbye M. Maternal age and fetal loss: population based register linkage study. BMJ 2000;320:1708-12.
3. Pineles BL, Park E, Samet JM. Systematic review and meta-analysis of miscarriage and maternal exposure to tobacco smoke during pregnancy. Am J Epidemiol 2014;179:807-23.
4. Metwally M, Ong KJ, Ledger WL, Li TC. Does high body mass index increase the risk of miscarriage after spontaneous and assisted conception? A meta-analysis of the evidence. Fertil Steril 2008;90:714-26.
5. Sundermann AC, Velez Edwards DR, Slaughter JC, Wu P, Jones SH, Torstenson ES et al. Week-by-week alcohol consumption in early pregnancy and spontaneous abortion risk: a prospective cohort study. Am J Obstet Gynecol 2020.
6. du Fossé NA, van der Hoorn M-LP, van Lith JMM, le Cessie S, Lashley EELO. Advanced paternal age is associated with an increased risk of spontaneous miscarriage: a systematic review and meta-analysis. Hum Reprod Update 2020;26:650-69.
7. Oldereid NB, Wennerholm UB, Pinborg A, Loft A, Laivuori H, Petzold M et al. The effect of paternal factors on perinatal and paediatric outcomes: a systematic review and meta-analysis. Hum Reprod Update 2018;24:320-89.
8. Tremellen K. Oxidative stress and male infertility—a clinical perspective. Hum Reprod Update 2008;14:243-58.
9. Martin JH, Aitken RJ, Bromfield EG, Nixon B. DNA damage and repair in the female germline: contributions to ART. Hum Reprod Update 2018;25:180-201.
10. Moher D, Liberati A, Tetzlaff J, Altman DG. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. 2009;339:b2535.
11. Kolte AM, on behalf of the ESHRE Special Interest Group EP, Bernardi LA, on behalf of the ESHRE Special Interest Group EP, Christiansen OB, on behalf of the ESHRE Special Interest Group EP et al. Terminology for pregnancy loss prior to viability: a consensus statement from the ESHRE early pregnancy special interest group. Hum Reprod 2014;30:495-8.
12. 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.
13. .ACOG Practice Bulletin No. 200: Early Pregnancy Loss. Obstet Gynecol 2018;132.
14. Steegers-Theunissen RP, Twigt J, Pestinger V, Sinclair KD. The periconceptional period, reproduction and long-term health of offspring: the importance of one-carbon metabolism. Hum Reprod Update 2013;19:640-55.
15. Dekkers OM, Vandenbroucke JP, Cevallos M, Renehan AG, Altman DG, Egger M. COSMOS-E: Guidance on conducting systematic reviews and meta-analyses of observational studies of etiology. PLoS Med 2019;16:e1002742.
16. Blanco-Muñoz J, Torres-Sánchez L, López-Carrillo L. Exposure to maternal and paternal tobacco consumption and risk of spontaneous abortion. Public Health Rep 2009;124:317-22.

17. Buck Louis GM, Sapra KJ, Schisterman EF, Lynch CD, Maisog JM, Grantz KL et al. Lifestyle and pregnancy loss in a contemporary cohort of women recruited before conception: The LIFE Study. Fertil Steril 2016;106:180-8.
18. Chatenoud L, Parazzini F, di Cintio E, Zanconato G, Benzi G, Bortolus R et al. Paternal and maternal smoking habits before conception and during the first trimester: relation to spontaneous abortion. Ann Epidemiol 1998;8:520-6.
19. Xu G, Wu Y, Yang L, Yuan L, Guo H, Zhang F et al. Risk factors for early miscarriage among Chinese: a hospital-based case-control study. Fertil Steril 2014;101:1663-70.
20. Maconochie N, Doyle P, Prior S, Simmons R. Risk factors for first trimester miscarriage—results from a UK-population-based case–control study. BJOG 2007;114:170-86.
21. Windham GC, Swan SH, Fenster L. Parental cigarette smoking and the risk of spontaneous abortion. Am J Epidemiol 1992;135:1394-403.
22. Windham GC, Fenster L, Swan SH. Moderate maternal and paternal alcohol consumption and the risk of spontaneous abortion. Epidemiology 1992;3:364-70.
23. Venners SA, Wang X, Chen C, Wang L, Chen D, Guang W et al. Paternal smoking and pregnancy loss: a prospective study using a biomarker of pregnancy. Am J Epidemiol 2004;159:993-1001.
24. Henriksen TB, Hjollund NH, Jensen TK, Bonde JP, Andersson AM, Kolstad H et al. Alcohol consumption at the time of conception and spontaneous abortion. Am J Epidemiol 2004;160:661-7.
25. Windham GC, Von Behren J, Waller K, Fenster L. Exposure to environmental and mainstream tobacco smoke and risk of spontaneous abortion. Am J Epidemiol 1999;149:243-7.
26. 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;72:783-9.
27. Okechukwu CA, Nguyen K, Hickman NJ. Partner smoking characteristics: Associations with smoking and quitting among blue-collar apprentices. Am J Ind Med. 2010;53:1102-8.
28. Weinberg CR. Toward a Clearer Definition of Confounding. American Journal of Epidemiology 1993;137:1-8.
29. Howards PP, Schisterman EF, Poole C, Kaufman JS, Weinberg CR. "Toward a clearer definition of confounding" revisited with directed acyclic graphs. Am J Epidemiol 2012;176:506-11.
30. Schisterman EF, Cole SR, Platt RW. Overadjustment bias and unnecessary adjustment in epidemiologic studies. Epidemiology. 2009;20:488-95.
31. Norsker FN, Espenhain L, á Rogvi S, Morgen CS, Andersen PK, Nybo Andersen A-M. Socioeconomic position and the risk of spontaneous abortion: a study within the Danish National Birth Cohort. BMJ Open 2012;2:e001077.
32. Leviton A. Biases Inherent in Studies of Coffee Consumption in Early Pregnancy and the Risks of Subsequent Events. Nutrients 2018;10:1152.
33. Gorber SC, Schofield-Hurwitz S, Hardt J, Levasseur G, Tremblay M. The accuracy of self-reported smoking: A systematic review of the relationship between self-reported and cotinine-assessed smoking status. Nicotine Tob Res. 2009;11:12-24.
34. Zenzes MT. Smoking and reproduction: gene damage to human gametes and embryos. Hum Rep Update

2000;6:122-31.

35. Esakky P, Moley KH. Paternal smoking and germ cell death: A mechanistic link to the effects of cigarette smoke on spermatogenesis and possible long-term sequelae in offspring. Mol Cell Endocrinol. 2016;435:85-93.
36. Beal MA, Yauk CL, Marchetti F. From sperm to offspring: Assessing the heritable genetic consequences of paternal smoking and potential public health impacts. Mutat Res Rev Mutat Res. 2017;773:26-50.
37. Wright C, Milne S, Leeson H. Sperm DNA damage caused by oxidative stress: modifiable clinical, lifestyle and nutritional factors in male infertility. Reprod Biomed Online 2014;28:684-703.
38. Aboulmaouahib S, Madkour A, Kaarouch I, Sefrioui O, Saadani B, Copin H et al. Impact of alcohol and cigarette smoking consumption in male fertility potential: Looks at lipid peroxidation, enzymatic antioxidant activities and sperm DNA damage. Andrologia 2018;50:e12926.
39. Hoek J, Schoenmakers S, Baart EB, Koster MPH, Willemsen SP, van Marion ES et al. Preconceptional Maternal Vegetable Intake and Paternal Smoking Are Associated with Pre-implantation Embryo Quality. Reprod Sci. 2020;27:2018-28.
40. McQueen DB, Zhang J, Robins JC. Sperm DNA fragmentation and recurrent pregnancy loss: a systematic review and meta-analysis. Fertil Steril. 2019;112:54-60.e3.
41. Robinson L, Gallos ID, Conner SJ, Rajkhowa M, Miller D, Lewis S et al. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. Hum Reprod 2012;27:2908-17.
42. Borges E, Jr., Zanetti BF, Setti AS, Braga DPdAF, Provenza RR, Iaconelli A, Jr. Sperm DNA fragmentation is correlated with poor embryo development, lower implantation rate, and higher miscarriage rate in reproductive cycles of non–male factor infertility. Fertil Steril. 2019;112:483-90.
43. Seli E, Gardner DK, Schoolcraft WB, Moffatt O, Sakkas D. Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after in vitro fertilization. Fertil Steril. 2004;82:378-83.
44. Borini A, Tarozzi N, Bizzaro D, Bonu MA, Fava L, Flamigni C et al. Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART. Hum Reprod. 2006;21:2876-81.



CHAPTER 4

Evaluating the role of paternal factors in aetiology and prognosis of recurrent pregnancy loss: study protocol for a hospital-based multicentre case–control study and cohort study (REMI III project)

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ABSTRACT

Introduction

Recurrent pregnancy loss (RPL) is defined as the spontaneous demise of two or more pregnancies before the fetus reaches viability. Despite investigation of multiple known maternal risk factors, in more than 50% of couples this condition remains unexplained. Studies focusing on paternal factors in RPL are scarce and, therefore, paternal evaluation in RPL is currently very limited. However, regarding single miscarriage, there are multiple publications suggesting a contributive role of paternal factors. In this project we aim to identify paternal factors associated with RPL and to improve couple-specific prediction of future pregnancy outcomes by developing a prediction model containing both maternal and paternal factors.

Methods and analysis

In a case-control design the relation between unexplained RPL and paternal age, lifestyle factors, sperm DNA damage and immunomodulatory factors in peripheral blood and semen will be studied. Prospectively, 135 couples with naturally conceived unexplained RPL (cases) and 135 fertile couples without a history of pregnancy loss (controls) will be included, with collection of paternal blood and semen samples and documentation of clinical and lifestyle characteristics. In addition, 600 couples from both groups will be included retrospectively. To adjust for confounders, multivariate logistic regression will be used. The predictive value of paternal and maternal factors will be studied in the total RPL cohort consisting of approximately 735 couples. The primary outcome of the cohort study is live birth within five years after initial visit of the clinic. Secondary outcomes are ongoing pregnancy, time interval until next pregnancy and pregnancy complications.

Ethics and dissemination

This project is approved by the Medical Research Ethics Committee of the Leiden University Medical Center. No risks or burden are expected from the study. The findings of this study will be disseminated via peer-reviewed publications and presentations at international conferences.

INTRODUCTION

Spontaneous pregnancy loss is the most common complication in human pregnancy, defined as the loss of conception before the fetus reaches viability (<24 weeks of gestation) and occurs in 10-15% of clinically recognized pregnancies.(1, 2) Pregnancy loss is also often referred to as miscarriage, however this term is recommended to be used for confirmed intrauterine pregnancy losses only.(3) Recurrent pregnancy loss (RPL) is defined as two or more losses in one couple.(1) This condition affects approximately 1-3% of all couples of reproductive age.(4, 5)

RPL is a highly heterogeneous condition. Among the multifaceted risk factors are maternal acquired thrombophilia (antiphospholipid syndrome), structural uterine abnormalities, thyroid autoimmunity and parental balanced chromosomal translocations.(6-12) Maternal age is a strong risk factor for pregnancy loss, mainly based on the increased prevalence of the fetal aneuploid abnormalities with advancing age.(13) Maternal lifestyle factors such as smoking, alcohol and caffeine consumption and adiposity are also associated with RPL.(14-19)

Despite extensive investigations, a potential underlying condition cannot be identified in 50-70% of couples that present with RPL.(20, 21) Limited understanding of underlying pathophysiological mechanisms means that options for effective interventions are lacking. Currently, no evidence-based therapeutic options are available for couples with unexplained RPL. Clinical management is either empirical or primarily focused on providing supportive care, which has been shown to have a beneficial effect.(22) Part of this supportive care is counseling on the prognosis and success rate of subsequent pregnancies in couples with RPL. Lund et al. evaluated the prognosis of 987 women with RPL and found that 67% achieved a live birth within 5 years after first consultation.(23) They showed that the chance of at least one subsequent live birth decreased significantly with increasing maternal age and cumulative number of preceding miscarriages. Other studies reported live birth rates ranging from 57-95%.(24-26) This large variation might be explained by the use of different definitions for RPL (2 vs. 3 losses, consecutive vs. non-consecutive, primary vs. secondary), by the degree of monitoring of the women and by in- or exclusion of biochemical pregnancies in the definition of RPL.(23) Nevertheless, these results demonstrate that although unidentified factors increasing the risk for pregnancy loss may exist, they do not necessarily prevent the development of a successful pregnancy. An essential part of the management of couples with RPL is to give trustworthy advice on the prognosis for a next pregnancy. However, the main limitation in current prognostic studies on unexplained RPL is the lack of adjustment for relevant risk factors, disabling the possibility of individual risk estimation.(23, 27)

The investigation of paternal contribution to RPL is currently limited to exploring the male karyotype. When considering counseling at an individual level, paternal factors may be included to establish a couple specified prognosis. Since the oocyte and the spermatozoon contribute equally to the genome of the embryo, it is biologically plausible to think that part of the idiopathic RPL cases could be explained by paternal factors. Some studies have evaluated the effect of paternal risk factors such as age, smoking and somatic health factors on the development of miscarriages, though these studies are mostly restricted to single miscarriage or to couples undergoing assisted reproductive techniques.(28-30) Following the absence of a consistent association between conventional semen parameters and RPL(31-38), the majority of recent studies addressing paternal factors and pregnancy losses focused on genetic defects, with sperm DNA fragmentation showing the most promising results. Both Robinson(39) and Zhao(40) showed in a meta-analysis that a high level of sperm DNA damage is associated with an increased miscarriage rate after in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) treatment. Two other recent meta-analyses found an increased mean difference in sperm DNA fragmentation of 12% in male partners of women with RPL compared to men whose partners had successful pregnancies.(41, 42) However, prospective studies in RPL couples evaluating the predictive value of sperm DNA fragmentation on future pregnancy outcomes are lacking.

In addition, imbalances in seminal immunomodulatory factors may contribute to the development of RPL. During pregnancy the maternal immune system has to tolerate the presence of semiallogeneic cells in maternal tissue. Seminal fluid contains various signalling molecules that are thought to induce lymphocyte proliferation, affect natural killer cell activity and modify cytokine release from antigen presenting cells, resulting in tolerance towards paternal allo-antigens.(43-45) An optimal balance of pro-inflammatory and immunomodulatory factors seems to be necessary for the induction of immunologic tolerance and the process of implantation and placentation.(46) Increased plasma levels of interleuking-18 (IL-18) and IL-8 and decreased levels of IL-11 were found to be negatively correlated to fertilization and implantation.(47, 48) In subfertile couples with normospermia, including a small subgroup with a history of RPL, decreased concentrations of IL-1β and increased interferon-γ (IFN-γ) were present in the seminal plasma.(49) The same study also suggests a correlation between levels of pro-inflammatory and anti-inflammatory cytokines in paternal peripheral blood and reproductive outcome. In case of such correlations, cytokine micropatterns in blood serum could serve as a proxy for those in the seminal plasma and could potentially be suitable as easily available prognostic markers in clinical practice. However, larger prospective studies are required to assess this.

In this study, we hypothesise that unexplained RPL is an issue stemming from both the female and the male. Our overall aims are to identify paternal factors that are associated with the development of this condition and to assess the predictive value of these factors for future reproductive outcomes in couples with RPL, in addition to maternal factors.

STUDY OBJECTIVES

Primary objectives

To identify paternal factors that are associated with unexplained RPL.
Paternal factors that will be assessed are: age, smoking, alcohol intake, recreational drugs intake, caffeine intake, body mass index (BMI), level of sperm DNA fragmentation and immunomodulatory factors in seminal plasma and paternal peripheral blood.

To assess the correlation between level of sperm DNA fragmentation and immunomodulatory factors in seminal plasma and paternal peripheral blood.

Secondary objectives

To assess the prognostic effect of paternal factors on reproductive outcomes in couples with unexplained RPL.

To develop a prediction model containing both maternal and paternal factors to predict the chance of a successful pregnancy for couples with unexplained RPL.

METHODS AND ANALYSIS

Study design

The primary objectives are focused on etiology and will be addressed in a case-control study. In this case-control study paternal factors are compared between couples with RPL and control couples. The expected duration of the case-control study is one year.

The secondary objectives will be addressed in a retrospective and prospective cohort study of couples with RPL. For all couples participating in the cohort study we aim to complete a follow-up on pregnancy outcomes of five years after first consultation.

A schematic overview of the study design is shown in Figure 1.

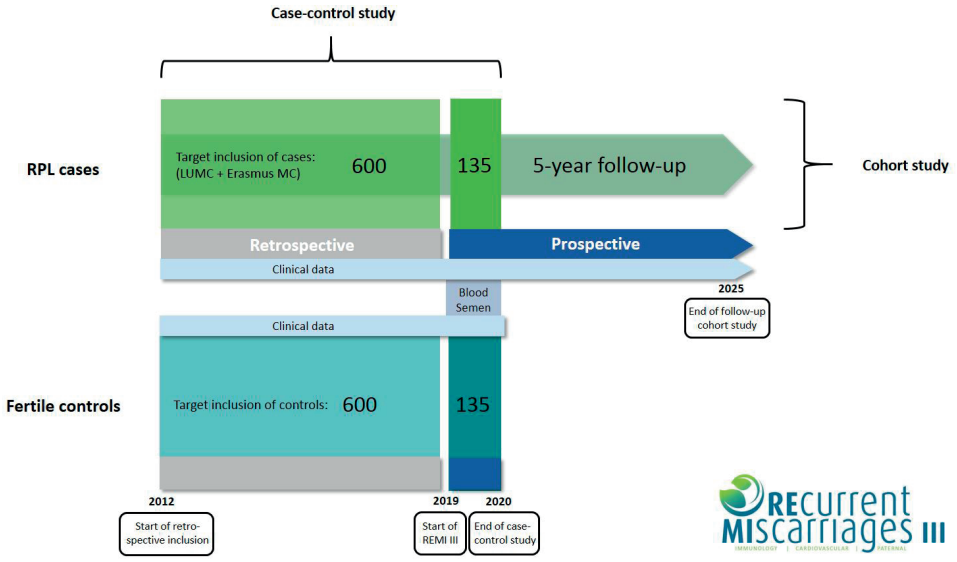


Figure 1. Schematic diagram of study design

Schematic diagram of study design. For the case–control study the target for inclusion is 735 couples in each arm. Of these 735 couples, 600 will be included retrospectively (2012–2018) and 135 will be included prospectively (2019–2020). Semen and blood will be collected from prospectively included men only. Couples with RPL (cases) are also part of a cohort study. We aim to complete a 5-year follow-up of these couples, starting from their individual point of inclusion. Control couples will not be in follow-up. LUMC, Leiden University Medical Center; MC, Medical Center; REMI, REcurrent MIscarriages; RPL, recurrent pregnancy loss.

Eligibility criteria

Inclusion criteria for RPL couples are:

- Unexplained RPL
  According to the ESHRE Recurrent Pregnancy Loss Guideline(1) defined as the loss of ≥2 pregnancies in the current relationship, without any of the following known risk factors: parental chromosomal abnormalities, uterine abnormalities, acquired thrombophilia, and thyroid autoimmunity. The definition includes all pregnancy losses before 24th week of gestation verified by ultrasonography or uterine curettage and histology and also non-visualized pregnancies (including biochemical pregnancy loss and/or resolved and treated pregnancies of unknown location) verified by positive urine or serum hCG. If identified as such, ectopic and molar pregnancies are not included. Pregnancy losses do not need to be consecutive.

Exclusion criteria for RPL couples are:

- Known risk factors for RPL as defined above;
- Mental or legal incapability of either the male or female;
- Pregnancy after ART;
- Pregnancy after oocyte, embryo or spermatozoa donation;
- Loss of < 2 pregnancies in the current relationship.

Inclusion criteria for control couples are:

- Proven fertility (i.e., pregnant at the time of inclusion or previously experienced pregnancy in the same relationship)

Exclusion criteria for control couples are:

- Previous spontaneous pregnancy loss;
- One of the following conditions: parental chromosomal abnormalities, uterine abnormalities, acquired thrombophilia and thyroid autoimmunity (this will not be investigated, however, couples are excluded when it is known);
- Mental or legal incapability of either the male or female;
- Pregnancy after ART;
- Pregnancy after oocyte, embryo or spermatozoa donation.

Study population and recruitment

Couples with RPL that visit the RPL outpatient clinic of Leiden University Medical Center (LUMC) or early pregnancy unit of Erasmus MC University Medical Center (Erasmus MC) will be assessed for eligibility. LUMC is the coordinating centre. Couples with RPL will be invited to participate at their intake visit (after they have been referred by their general practitioner or a referring hospital). After diagnostic investigations on known risk factors

of RPL are completed, couples with unexplained RPL will be selected for inclusion. In addition, couples that visited the participating clinics in the period 2012-2019 will be included in retrospect. Couples with RPL will participate in both the case-control study and the cohort study.

Eligible couples visiting the antenatal outpatient clinic of LUMC during their pregnancy will be invited to participate in the control group. Control couples will also be included in retrospect.

Study recruitment in the coordinating centre started in June 2019. Recruitment at Erasmus MC is expected to start in September 2019. All couples will receive written information about the study together with the informed consent form, which includes a request to obtain permission for gathering data from medical records and storage of biomaterial for additional analyses related to this study. Participants are informed that study participation is voluntary and that they are free to withdraw at any time without any consequences for subsequent care. In case of participation, the informed consent form should be signed prior to inclusion in the study.

Study procedures

Collection of clinical characteristics

Data about obstetric and general medical history and lifestyle factors of all participating couples will be documented (Table 1).

Table 1. Collection of data

	Parameters
Maternal characteristics	Date of birth, zip code, ethnicity, level of education, profession, body weight, height, general medical history, use of medication, family history, detailed obstetric history (parity, number of spontaneous pregnancy losses, ectopic pregnancies or induced abortions, modes of conception of previous births, modes of delivery of previous births, gestational age at previous births, complications during previous pregnancies and deliveries, birth weight, gender and Apgar score of children of previous births), lifestyle characteristics (smoking, alcohol, drugs and caffeine intake, physical exercise pattern).
Paternal characteristics	Date of birth, zip code, ethnicity, level of education, profession, body weight, height, general medical history, use of medication, family history, lifestyle characteristics (smoking, alcohol, drugs and caffeine intake, physical exercise pattern).
Results of (previous) investigations into known risk factors of RPL	Presence of antiphospholipid syndrome (anticardiolipin IgG and IgM, B2 glycoprotein I antibodies IgG and IgM, and lupus anticoagulans), parental chromosomal abnormalities, presence of thyroid antibodies, presence of uterine anomalies.

These data will be collected during consultations (in a semistandardised way using a template) and from medical records. Additional required data will be acquired via digital surveys that will be sent to participating couples. Data entry and generation of digital surveys will be performed using Castor EDC.(50)

Couples with RPL participating in the cohort study will be in follow-up for a total time of five years after initial consultation. These couples will receive a digital survey once a year. This survey contains questions about outcomes of new pregnancies if applicable and changes in medical history and lifestyle in the past year. When couples with RPL are still in regular clinical follow-up, data will be collected during regular consultations and it will not be necessary to send a digital survey. Retrospectively included couples from whom (part of) the follow-up period is missing in their medical records, will receive a survey to ask for pregnancy outcomes in the missing time period.

Clinical characteristics of couples participating in the control group will be collected at one time point (during consultation at the antenatal clinic), directly followed by a digital survey containing questions about lifestyle related to the period prior to the index pregnancy. There is no follow-up of control couples.

Collection and analysis of samples

Male partners of participating couples will be asked for a peripheral blood sample and sperm sample acquired through masturbation. Samples will be collected from all prospectively included men. This applies to both cases and controls. From all retrospectively included couples only clinical data will be documented.

All samples will be processed and analyzed in the laboratory of Reproductive Immunology at LUMC. Samples will be collected once. Samples from other participating centres will be sent to LUMC for storage and analysis.

Semen samples will be stored in -20^{o}C until time of analysis. Sperm DNA fragmentation will be detected by terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) assay (APO-DIRECT^{TM} Kit, BD Biosciences) following the manufacturer's instructions. The level (%) of sperm DNA fragmentation will be determined by flow cytometric analysis.

The level of immunomodulatory factors in seminal plasma and peripheral blood will be assessed by Bio-Plex Luminex™ system assay (Bio-Rad Laboratories) following the manufacturer's instructions. Samples will be analysed using a Bio- Plex™ Array Reader with Bio-Plex software. Through this assay quantification of cytokine levels including TNF-α, IFN-γ, TGF-β1, IL-1β, IL-8, IL-10, IL-11, IL-18, sHLA-G and PGE2 will be performed. These cytokines were selected because previous small studies suggested correlations between concentrations in seminal plasma and/or paternal peripheral blood and reproductive outcome. (47-49, 51)

Control of bias

Since the design of this study is observational, there is need to control and adjust for

confounding factors. For example, maternal age is an important confounder for the effect of paternal age on RPL. To control for confounders, stratification and regression models will be used. Selection bias is minimized by a clear definition of the study population. In addition, the control couples are selected independently of their exposure and they represent the source population that generates the cases. Finally, information bias is limited as much as possible by collecting information similarly from the cases and controls.

Sample size calculation

Case-control study

Since sperm DNA fragmentation could be seen as a proxy for advanced age and also for the presence of smoking, obesity and excessive exercise, this factor was used for sample size calculations. Zhao et al.(40) evaluated the association between sperm DNA fragmentation and miscarriages after IVF/ICSI treatment in 2756 couples and they found a combined odds ratio of 2.28 (95% CI 1.55-3.35) for miscarriage in patients with high sperm DNA fragmentation The rate of high sperm DNA fragmentation was significantly higher in the group with miscarriage (34%) compared to the group with live births (19%). To detect this difference, using $\alpha = 0.05$ and power = 80%, the sample size would be 135 in the RPL group and 135 in the control group. Also the recent meta-analyses of Tan et al.(41) and McQueen et al.(42) have been taken into consideration for sample size calculation. They evaluated the mean difference in % sperm DNA fragmentation between RPL patients and fertile controls. However, based on these mean differences (both of approximately 11%), the sample size would be very small (<10 per arm) and therefore not appropriate for this project, since we are not solely interested in sperm DNA fragmentation but also in other lifestyle and demographic factors.

Cohort-study

No straightforward accepted methods exist to estimate the required number of subjects to develop a multivariable prediction model. Ideally, prognostic studies include several hundreds of patients who develop the outcome event.(52) Various studies have suggested that for each candidate predictor studied, at least 10 events are required. (53, 54) Currently, female age and number of previous pregnancy losses are the only known factors consistently shown to impact prognosis for future pregnancy outcomes. (1) In addition to these factors, we intend to examine paternal factors for their predictive capacity. Assuming that at least two paternal factors will be included in the model, like age and BMI (and also maternal BMI), with four age categories (<30, 30-35, 35-40, >40 years), four categories for preceding pregnancy losses (2, 3, 4, ≥5) and four BMI categories (<18, 18-25, 25-30, >30 kg/m^2), a minimum of 20 x 10 = 200 patients with RPL and live birth in subsequent pregnancy are necessary. We estimate that the total RPL cohort will eventually consist of approximately 735 couples (with retrospective

and prospective inclusions together, shown in Figure 1) and we expect 70% of them to have a live birth within five years after initial consultation. Based on these numbers, it is feasible to develop a multivariable model to predict the chances for ongoing pregnancy and live birth within five years. We will include patients who visited the clinics between 2012-2019 and also the couples (cases) of the case-control study.

Study outcomes

In the case-control study the following exposures will be studied:

- Smoking: documented as average number of cigarettes per day. Also data on former smoking behavior will be documented;
- Alcohol consumption: documented as average number of units per week;
- Recreational drug consumption: specified by type of drug, quantity and frequency;
- Caffeine intake: documented as average number of caffeinated drinks per day;
- Physical exercise pattern; documented as moderate to intensive physical exercise in days per week and minutes per day.

In the cohort study the following outcomes will be studied:

- Live birth within five years after initial consultation (for this outcome we intend to develop a prediction model);
- Ongoing pregnancy (>24 weeks);
- Time interval until next pregnancy;
- Pregnancy complications including fetal growth restriction, preterm birth, pregnancy induced hypertension, preeclampsia, hemolysis elevated liver enzymes and low platelets (HELLP) syndrome and gestational diabetes mellitus.

Statistical analysis plan

Case-control study

For the case-control study, proportions will be calculated for the dichotomous and categorial exposures with 95% confidence intervals. Comparison between the cases and controls is performed by a Chi square test. Mean differences with 95% confidence intervals are calculated to compare continuous variables between the groups. To correct for confounders (including maternal factors), stratified analyses and multivariate logistic regression including paternal and maternal variables that are highly correlated will be performed.

Cohort study

To indicate a relation between live birth and paternal (and maternal) factors as described above, first univariate logistic regression will be used. To select the most prognostic set of variables logistic regression with shrinkage methods such as lasso will be used. Time to pregnancy is estimated using the Kaplan Meier method. Only in the subgroup

of prospectively included RPL couples (with collection of samples), blood and sperm investigations will be included in the analyses.

To cope with analysis of missing values, multiple imputation will be performed. Statistical analysis will be performed using SPSS Statistics V.25 (IBM SPSS Software) and/or R version V.3.6.0. For all tests, a two-sided $p < 0.05$ or 95% CI not including the null value is considered significant.

Patient and public involvement

During the development of the study protocol the Dutch association for patients with fertility problems (Freya) was consulted. Results will be presented during their thematic meetings to inform patients about study progress. Social media will be used to highlight new publications and conference presentations.

Ethics and dissemination

This study will be conducted according to the principles of the Declaration of Helsinki.(55) Ethics approval for this study was obtained at the Medical Research Ethics Committee of the Leiden University Medical Center. No risks or burden are expected from the study. No additional hospital visits are required.

Eligible couples obtain written information about the study objectives and procedures and they will have sufficient time to decide on participating. All clinical data and data derived from surveys will be saved in the Castor EDC REMI III database. No data directly traceable to patients will be included in this database. Every couple will be assigned a unique code. This code will also be used to associate clinical data with corresponding blood and semen samples.

The findings of this study will be disseminated via peer-reviewed publications and presentations at international conferences.

DISCUSSION

RPL is often accompanied by psychological morbidities such as depression and anxiety, making it a very distressing and costly condition.(56) In current practice, RPL is mostly considered an issue derived exclusively from female causes. However, it is questionable whether this female-centred approach is correct, especially considering the substantial proportion of RPL cases that remains unexplained. In November 2017 the European Society of Human Reproduction and Embryology (ESHRE) developed a new guideline for the management of RPL, to supply healthcare providers with the best available evidence for investigation and treatment of RPL. Future research on the paternal contribution in RPL, such as the impact of paternal lifestyle factors and sperm DNA damage, was recommended by the guideline committee.(1)

In this project, we hypothesise that besides maternal factors, paternal factors are associated with the development of RPL. Understanding the role of these factors contributing to the pathological mechanisms of RPL may provide new diagnostic tools and treatment options. To the best of our knowledge, this project includes the first large prospective cohort study evaluating the contribution of multiple paternal lifestyle and biological factors to unexplained RPL.

Limitations of all research on lifestyle factors using self-reported data are the phenomena of recall and response bias. Individuals might report biased estimates of self-assessed behaviour for different reasons, including misunderstanding or social-desirability. Although these types of bias will always be present to some extent, we try to minimize this by using standardized and well-structured surveys, by avoiding long recall periods as much as possible and by choosing an appropriate and well-defined control group.

Ultimately, we aim to develop a couple-specific model including both maternal and paternal factors to predict future reproductive outcomes in couples with unexplained RPL. Although not an intervention as such, counseling couples confronted with RPL about their individual prognosis is an essential part of the management of these couples and allows them to decide for or against future pregnancy attempts. Moreover, this study might also provide new starting points for future treatment options with regard to lifestyle interventions.

REFERENCES

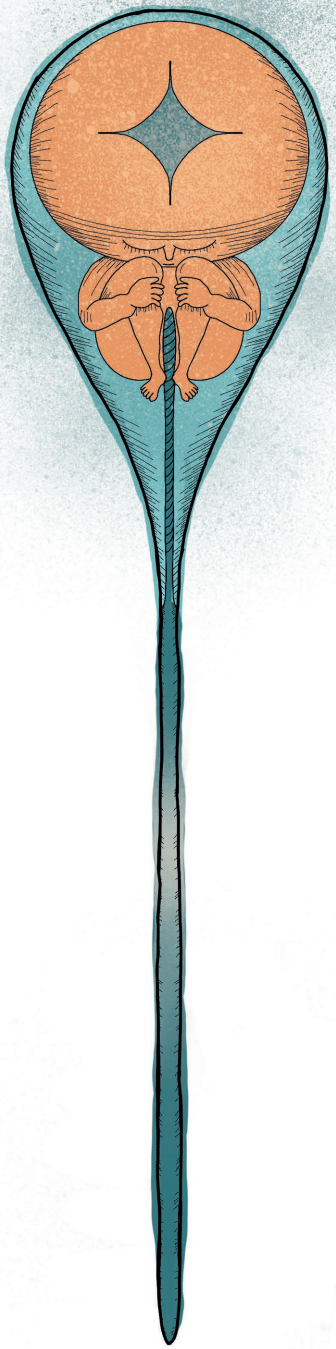
1. ESHRE Guideline Group on RPL. Recurrent Pregnancy Loss. Guideline of the European Society of Human Reproduction and Embryology 2017.
2. Nybo Andersen AM, Wohlfahrt J, Christens P, Olsen J, Melbye M. Maternal age and fetal loss: population based register linkage study. BMJ. 2000;320(7251):1708-12.
3. Kolte AM, on behalf of the ESHRE Special Interest Group EP, Bernardi LA, on behalf of the ESHRE Special Interest Group EP, Christiansen OB, on behalf of the ESHRE Special Interest Group EP, et al. Terminology for pregnancy loss prior to viability: a consensus statement from the ESHRE early pregnancy special interest group. Hum Reprod. 2014;30(3):495-8.
4. Rai R, Regan L. Recurrent miscarriage. Lancet. 2006;368(9535):601-11.
5. Jauniaux E, Farquharson RG, Christiansen OB, Exalto N. Evidence-based guidelines for the investigation and medical treatment of recurrent miscarriage. Hum Reprod. 2006;21(9):2216-22.
6. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost. 2006;4(2):295-306.
7. van den Boogaard E, Cohn DM, Korevaar JC, Dawood F, Vissenberg R, Middeldorp S, et al. Number and sequence of preceding miscarriages and maternal age for the prediction of antiphospholipid syndrome in women with recurrent miscarriage. Fertil Steril. 2013;99(1):188-92.
8. Woelfer B, Salim R, Banerjee S, Elson J, Regan L, Jurkovic D. Reproductive outcomes in women with congenital uterine anomalies detected by three-dimensional ultrasound screening. Obstet Gynecol. 2001;98(6):1099-103.
9. Salim R, Woelfer B, Backos M, Regan L, Jurkovic D. Reproducibility of three-dimensional ultrasound diagnosis of congenital uterine anomalies. Ultrasound Obstet Gynecol. 2003;21(6):578-82.
10. Lata K, Dutta P, Sridhar S, Rohilla M, Srinivasan A, Prashad GRV, et al. Thyroid autoimmunity and obstetric outcomes in women with recurrent miscarriage: a case–control study. 2013;2(2):118.
11. Hook EB, Healy NP, Willey AM. How much difference does chromosome banding make? Adjustments in prevalence and mutation rates of human structural cytogenetic abnormalities. Ann Hum Genet. 1989;53(3):237-42.
12. De Braekeleer M, Dao TN. Cytogenetic studies in couples experiencing repeated pregnancy losses. Hum Reprod. 1990;5(5):519-28.
13. Goddijn M, Leschot NJ. Genetic aspects of miscarriage. Baillieres Best Pract Res Clin Obstet Gynaecol. 2000;14(5):855-65.
14. Gardella JR, Hill JA, 3rd. Environmental toxins associated with recurrent pregnancy loss. Semin Reprod Med. 2000;18(4):407-24.
15. Christiansen OB, Nybo Andersen AM, Bosch E, Daya S, Delves PJ, Hviid TV, et al. Evidence-based investigations and treatments of recurrent pregnancy loss. Fertil Steril. 2005;83(4):821-39.
16. George L, Granath F, Johansson AL, Olander B, Cnattingius S. Risks of repeated miscarriage. Paediatr Perinat Epidemiol. 2006;20(2):119-26.
17. Lashen H, Fear K, Sturdee DW. Obesity is associated with increased risk of first trimester and recurrent

miscarriage: matched case-control study. Hum Reprod. 2004;19(7):1644-6.
18. Cnattingius S, Signorello LB, Anneren G, Clausson B, Ekbom A, Ljunger E, et al. Caffeine intake and the risk of first-trimester spontaneous abortion. N Engl J Med. 2000;343(25):1839-45.
19. George L, Granath F, Johansson AL, Anneren G, Cnattingius S. Environmental tobacco smoke and risk of spontaneous abortion. Epidemiology. 2006;17(5):500-5.
20. Stephenson MD. Frequency of factors associated with habitual abortion in 197 couples. Fertil Steril. 1996;66(1):24-9.
21. Jaslow CR, Carney JL, Kutteh WH. Diagnostic factors identified in 1020 women with two versus three or more recurrent pregnancy losses. Fertil Steril. 2010;93(4):1234-43.
22. Liddell HS, Pattison NS, Zanderigo A. Recurrent miscarriage--outcome after supportive care in early pregnancy. Aust N Z J Obstet Gynaecol. 1991;31(4):320-2.
23. 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.
24. Kaandorp SP, Goddijn M, van der Post JA, Hutten BA, Verhoeve HR, Hamulyak K, et al. Aspirin plus heparin or aspirin alone in women with recurrent miscarriage. N Engl J Med. 2010;362(17):1586-96.
25. Badawy AM, Khiary M, Sherif LS, Hassan M, Ragab A, Abdelall I. Low-molecular weight heparin in patients with recurrent early miscarriages of unknown aetiology. J Obstet Gynaecol. 2008;28(3):280-4.
26. Fawzy M, Shokeir T, El-Tatongy M, Warda O, El-Refaiey AA, Mosbah A. Treatment options and pregnancy outcome in women with idiopathic recurrent miscarriage: a randomized placebo-controlled study. Arch Gynecol Obstet. 2008;278(1):33-8.
27. Sugiura-Ogasawara M, Ozaki Y, Kitaori T, Suzumori N, Obayashi S, Suzuki S. Live birth rate according to maternal age and previous number of recurrent miscarriages. Am J Reprod Immunol. 2009;62(5):314-9.
28. de la Rochebrochard E, Thonneau P. Paternal age and maternal age are risk factors for miscarriage; results of a multicentre European study. Hum Reprod. 2002;17(6):1649-56.
29. Venners SA, Wang X, Chen C, Wang L, Chen D, Guang W, et al. Paternal smoking and pregnancy loss: a prospective study using a biomarker of pregnancy. Am J Epidemiol. 2004;159(10):993-1001.
30. Colaci DS, Afeiche M, Gaskins AJ, Wright DL, Toth TL, Tanrikut C, et al. Men's body mass index in relation to embryo quality and clinical outcomes in couples undergoing in vitro fertilization. Fertil Steril. 2012;98(5):1193-9.e1.
31. Sbracia S, Cozza G, Grasso JA, Mastrone M, Scarpellini F. Semen parameters and sperm morphology in men in unexplained recurrent spontaneous abortion, before and during a 3 year follow-up period. Hum Reprod. 1996;11(1):117-20.
32. Gopalkrishnan K, Padwal V, Meherji PK, Gokral JS, Shah R, Juneja HS. Poor quality of sperm as it affects repeated early pregnancy loss. Arch Androl. 2000;45(2):111-7.
33. Bhattacharya SM. Association of various sperm parameters with unexplained repeated early pregnancy loss--which is most important? Int Urol Nephrol. 2008;40(2):391-5.
34. Brahem S, Mehdi M, Landolsi H, Mougou S, Elghezal H, Saad A. Semen parameters and sperm DNA fragmentation as causes of recurrent pregnancy loss. Urology. 2011;78(4):792-6.
35. Imam SN, Shamsi MB, Kumar K, Deka D, Dada R. Idiopathic recurrent pregnancy loss: role of paternal

factors; a pilot study. J Reprod Infertil. 2011;12(4):267-76.

36. Talebi AR, Vahidi S, Aflatoonian A, Ghasemi N, Ghasemzadeh J, Firoozabadi RD, et al. Cytochemical evaluation of sperm chromatin and DNA integrity in couples with unexplained recurrent spontaneous abortions. Andrologia. 2012;44 Suppl 1:462-70.
37. Khadem N, Poorhoseyni A, Jalali M, Akbary A, Heydari ST. Sperm DNA fragmentation in couples with unexplained recurrent spontaneous abortions. Andrologia. 2014;46(2):126-30.
38. Zhang L, Wang L, Zhang X, Xu G, Zhang W, Wang K, et al. Sperm chromatin integrity may predict future fertility for unexplained recurrent spontaneous abortion patients. Int J Androl. 2012;35(5):752-7.
39. Robinson L, Gallos ID, Conner SJ, Rajkhowa M, Miller D, Lewis S, et al. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. Hum Reprod. 2012;27(10):2908-17.
40. Zhao J, Zhang Q, Wang Y, Li Y. Whether sperm deoxyribonucleic acid fragmentation has an effect on pregnancy and miscarriage after in vitro fertilization/intracytoplasmic sperm injection: a systematic review and meta-analysis. Fertil Steril. 2014;102(4):998-1005.e8.
41. 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. 2018.
42. McQueen DB, Zhang J, Robins JC. Sperm DNA fragmentation and recurrent pregnancy loss: a systematic review and meta-analysis. Fertil Steril. 2019.
43. Moldenhauer LM, Diener KR, Thring DM, Brown MP, Hayball JD, Robertson SA. Cross-presentation of male seminal fluid antigens elicits T cell activation to initiate the female immune response to pregnancy. J Immunol. 2009;182(12):8080-93.
44. Robertson SA. Seminal plasma and male factor signalling in the female reproductive tract. Cell Tissue Res. 2005;322(1):43-52.
45. 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.
46. Nederlof I, Meuleman T, van der Hoorn MLP, Claas FHJ, Eikmans M. The seed to success: The role of seminal plasma in pregnancy. J Reprod Immunol. 2017;123:24-8.
47. Nikolaeva MA, Babayan AA, Stepanova EO, Smolnikova VY, Kalinina EA, Fernandez N, et al. The relationship of seminal transforming growth factor-beta1 and interleukin-18 with reproductive success in women exposed to seminal plasma during IVF/ICSI treatment. J Reprod Immunol. 2016;117:45-51.
48. Seshadri S, Bates M, Vince G, Jones DI. Cytokine expression in the seminal plasma and its effects on fertilisation rates in an IVF cycle. Andrologia. 2011;43(6):378-86.
49. Havrylyuk A, Chopyak V, Boyko Y, Kril I, Kurpisz M. Cytokines in the blood and semen of infertile patients. Cent Eur J Immunol. 2015;40(3):337-44.
50. Ciwit BV. Castor Electronic Data Capture [Internet]. 2018.
51. Larsen MH, Bzorek M, Pass MB, Larsen LG, Nielsen MW, Svendsen SG, et al. Human leukocyte antigen-G in the male reproductive system and in seminal plasma. Mol Hum Reprod. 2011;17(12):727-38.
52. Moons KG, Royston P, Vergouwe Y, Grobbee DE, Altman DG. Prognosis and prognostic research: what, why, and how? BMJ. 2009;338:b375.
53. Laupacis A, Sekar N, Stiell IG. Clinical prediction rules. A review and suggested modifications of methodological standards. JAMA. 1997;277(6):488-94.

54. Harrell FE, Jr., Lee KL, Mark DB. Multivariable prognostic models: issues in developing models, evaluating assumptions and adequacy, and measuring and reducing errors. Stat Med. 1996;15(4):361-87.
55. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. JAMA. 2013;310(20):2191-4.
56. Serrano F, Lima ML. Recurrent miscarriage: psychological and relational consequences for couples. Psychol Psychother. 2006;79(Pt 4):585-94.



CHAPTER 5

Toward more accurate prediction of future pregnancy outcome in couples with unexplained recurrent pregnancy loss: taking both partners into account

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ABSTRACT

Objective

To identify, besides maternal age and the number of previous pregnancy losses, additional characteristics of couples with unexplained recurrent pregnancy loss (RPL) that improve the prediction of an ongoing pregnancy.

Design

Hospital-based cohort study in couples who visited specialised RPL units of two academic centres between 2012-2020.

Setting

Two academic centres in the Netherlands.

Patients

Clinical data from 526 couples with unexplained RPL were used in this study.

Intervention(s)

None.

Main Outcome Measure(s)

The final model to estimate the chance of a subsequent ongoing pregnancy was determined with a backward selection process and internally validated using bootstrapping. Model performance was assessed in terms of calibration and discrimination (area under the ROC curve; AUC).

Results

Subsequent ongoing pregnancy was achieved in 345/526 couples (66%). Number of previous pregnancy losses, maternal age, paternal age, maternal body mass index (BMI), paternal BMI, maternal smoking status and previous IVF/ICSI treatment were predictive for the outcome. The optimism corrected AUC was 0.63, compared to 0.57 when using only the number of previous pregnancy losses and maternal age.

Conclusion

The identification of additional predictors for a subsequent ongoing pregnancy after RPL, including male characteristics, is important for both clinicians and couples with RPL. At the same time we showed that the predictive ability of the current model is still limited and more research is warranted to develop a model that can be used in clinical practice.

INTRODUCTION

Recurrent pregnancy loss (RPL) is a condition characterised by the spontaneous loss of two or more pregnancies before 24 weeks of gestation, affecting 2-3% of couples of reproductive age.(1, 2) Over time, various risk factors for RPL have been identified and several diagnostic investigations are recommended by international guidelines, including screening for uterine anomalies, acquired thrombophilia, thyroid abnormalities and parental chromosomal translocations.(2) Despite the extensive diagnostic work-up being offered to couples with RPL, no underlying condition can be identified in 60-70% of cases.(3) For these unexplained cases, no evidence-based therapeutic options are available, which adds to the frustrating nature of this condition.(2) Indeed, multiple studies have shown that couples with RPL are more likely to deal with depression and anxiety.(4) It is considered important to offer supportive care to couples with RPL, consisting of intensive monitoring and care during early pregnancy as well as psychological support.(5, 6) Moreover, supportive care should certainly include reliable counselling regarding prognosis.

For couples with recurrent pregnancy loss (RPL) one question is vital: what is the chance of a future successful pregnancy? Even when aetiological mechanisms are not fully elucidated, well-developed and validated prediction models may provide adequate estimates of future pregnancy outcomes.(7) Currently, two prognostic tools are recommended by the ESHRE guideline on RPL.(2) Both models base their predictions on two factors: the number of preceding pregnancy losses and maternal age. Brigham et al.(8) predicted the chance of a subsequent ongoing pregnancy with fetal survival beyond 24 weeks of gestation, while Lund et al.(9) predicted pregnancy success rates at five, ten and fifteen years after referral. Yet, some important limitations must be kept in mind when using these prediction models.

First of all, as neither performance measures nor validation procedures were described for both models, their predictive performance remains unknown. Second, as these models were developed 21 and nine years ago, changing definitions and diagnostic investigations for RPL have most probably affected the reliability of the models in today's clinical practice. In addition, a limited number of candidate predictors were examined in both studies. Although it is indisputable that maternal age and previous number of losses are important predictors for future pregnancy outcome(2), it is likely that inclusion of other factors may improve accuracy of prediction. Lifestyle factors such as cigarette smoking have been associated with pregnancy loss in previous studies and may thus influence future pregnancy outcome.(10, 11) Moreover, although the focus has been on the female partner for many years, evidence is emerging that characteristics of the male partner also contribute to (recurrent) pregnancy loss.(12, 13)

The aim of this study was to explore whether predicting the chance of a subsequent ongoing pregnancy in couples with unexplained RPL could be improved by taking, besides maternal age and the number of previous pregnancy losses, additional candidate predictors into account. To the best of our knowledge, this is the first time that the predictive potential of both maternal and paternal factors was evaluated in this context.

MATERIAL AND METHODS

This study was conducted following the recommendations of the Transparent reporting of a multivariable prediction model for individual prognosis or diagnosis (TRIPOD) statement.(14) This study was approved by the Medical Research Ethics Committee of the Leiden University Medical Center (reference number P19.014).

Source of data

In this hospital-based cohort study, data from two specialised RPL units located in two Dutch academic hospitals (Erasmus MC, University Medical Center Rotterdam and Leiden University Medical Center) was obtained, covering the period between January 2012 and December 2019. Couples with RPL were referred to these clinics for diagnostic investigations, counselling, supportive care and/or intensive monitoring during the first trimester of a subsequent pregnancy. Baseline characteristics (described in more detail in the paragraph Candidate predictors) of all couples that visited the RPL clinics were registered in electronic patient records during the intake consultation, using a standardised template. Data on baseline characteristics and subsequent pregnancy outcome were extracted from the hospital database systems and entered in a study database, using a standardised template.

Eligibility criteria

Couples were included in the study database with at least two pregnancy losses before 24 weeks of gestation (following the definition of the ESHRE guideline on RPL) in the current relationship. Couples with pregnancy losses following oocyte or sperm donation and couples with an identified underlying condition for RPL (specified in the next paragraph) were excluded.

Diagnostic investigations for RPL

Diagnostic investigations considered for this study were based on recommendations of the current ESHRE guideline on RPL(2) and included screening for uterine anomalies, thyroid abnormalities (anti-thyroid peroxidase (TPO) and thyroid-stimulating hormone (TSH) levels), acquired thrombophilia (antiphospholipid antibodies(15)) and parental chromosomal translocations. Parental karyotyping was only performed in case of increased risk of abnormalities, following the risk table of Franssen et al.(16)

Outcome

We estimated the chance of a subsequent ongoing pregnancy, defined as fetal survival beyond 24 weeks of gestation(2) in the first pregnancy after intake consultation at the RPL clinic. All first pregnancy outcomes that occurred after intake consultation and before January 2021 were analysed. Pregnancies conceived by a new male partner (i.e.

a different partner than during the intake consultation) or conceived following oocyte or sperm donation were excluded from the analysis. Also, couples with no further pregnancy or with an unknown pregnancy outcome after intake consultation were also excluded from the present analysis.

Sample size calculation

For sample size considerations, we followed the recommendations as published by van Smeden et al.(17) An established rule of thumb for the required sample size to develop a prediction model is to ensure at least 10 events per candidate predictor parameter. However, van Smeden et al. stated this rule is insufficient to minimise the risk of model overfitting and to target precise model predictions. For binary outcomes, they showed that the number of candidate prediction parameters, the total sample size and the outcome proportion are the main drivers of the mean predictive accuracy of a prediction model. Therefore a sample size formula was presented, that aims to ensure that a new prediction model will on average have a small prediction error in the estimated outcome probabilities, as measured by the mean absolute prediction error (MAPE). An interactive calculation tool is available online and was used for this study: https://mvansmeden.shinyapps.io/BeyondEPV/. Before performing the present study, the number of available patients and predictors was known. For this situation, the calculation tool could be used to identify the maximum number of candidate predictors to be considered. With an anticipated outcome proportion of 70% couples with an ongoing pregnancy (8, 9, 18), a sample size of 526 (the number of couples available in our database) and a MAPE of 0.05 between observed and true outcome probabilities (as recommended by van Smeden et al.), the maximum number of candidate prediction parameters was determined a priori as 12.

Candidate predictors

The following candidate predictors were considered based on theoretical plausibility following previous research, expert opinion and availability: the number of previous pregnancy losses, primary or secondary RPL (with primary RPL being defined as no live birth in the current relationship), previous pregnancies conceived by *in vitro* fertilisation (IVF) or intracytoplasmic sperm injection (ICSI),maternal and paternal age, maternal and paternal body mass index (BMI) and maternal and paternal smoking status. All candidate predictor variables were collected during the intake consultation. The number of previous pregnancy losses, maternal and paternal ages were treated as continuous variables. Previous IVF or ICSI treatment and maternal and paternal smoking status were treated as dichotomous variables.

Statistical analysis

All analyses were performed in R studio version 1.3.9.50 and R version 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria).

Handling of missing data

To avoid a decrease of statistical power and selection bias, missing values were imputed. We assumed that missing values were missing at random. Based on the amount of missing data, missing values were imputed 30 times using multiple imputation with chained equations (MICE) with predictive mean matching.(19, 20) All candidate predictors and the outcome variable were included in the imputation model.(19) Rubin's rules were applied for pooling estimates across the imputed datasets.(21)

Model development

Initially we fitted univariable logistic regression models to assess the effect of individual predictors. Possible non-linearity in the associations between continuous predictors and the outcome were examined using the R studio package 'rcspline.plot'. Maternal age had a significant non-linear relation with the probability of a subsequent ongoing pregnancy and was modelled using a restricted cubic spline. For model development we used the R studio package 'pfmsi' which provides functions to apply pooling and variable selection in multiple imputed datasets. We performed multivariable logistic regression analysis with ongoing pregnancy as binary outcome. A backward selection process was used to determine the final multivariable logistic regression model, using the Akaike Information Criteria (AIC) as a stopping rule (corresponding to a *p*-value of 0.157).(20, 22) To assess the added value of additional predictors, we fitted smaller models including only a subset of the predictors derived from the backward selection.

Model performance

The resulting final model was internally validated using bootstrapping with 250 bootstrap samples, yielding estimates for the optimism in the performance for discrimination and calibration. The bootstrapping procedure was performed in combination with backward selection, as it is known that variable selection is a major reason for model overfitting.(20) Model calibration was ascertained by visual inspection of a calibration plot. Receiver operator characteristic (ROC) curve analysis was used as a measure for discrimination. Discrimination refers to the ability of a model to correctly assign higher probabilities to subjects with the outcome (ongoing pregnancy) compared to subjects without the outcome. An area under the ROC curve (AUC) of 0.5 indicates no discrimination and is comparable with tossing a coin: the ability of the model to assign a higher probability to a couple with ongoing pregnancy than to a couple without ongoing pregnancy is 50%. An AUC of 1.0 indicates perfected discrimination. The explained variance was described in terms of the Nagelkerke R^2. To prevent the model from overfitting, the calibration slope from the bootstrapping procedure was used to shrink the pooled regression coefficients and to determine a new intercept, being aligned with the shrunken coefficients.(20) Performance measures of the final model and smaller models including fewer predictors were compared.

RESULTS

After exclusions, the dataset included 526 couples with unexplained RPL and a subsequent pregnancy outcome after intake consultation at one of the two participating clinics. The flow of participants through the study is shown in Supplemental Figure 1. All included couples were in follow-up for at least one year after intake consultation. In 345 couples (66%) the first pregnancy after intake consultation was an ongoing pregnancy beyond 24 weeks of gestation. Of the remaining 181 couples (34%) without an ongoing pregnancy, 168 (93%) had a spontaneous pregnancy loss, eight (4%) had an ectopic pregnancy and five (3%) had a termination of pregnancy due to fetal abnormalities. Fifty-six pregnancy outcomes occurred in 2020, during the Covid-19 pandemic. None of these women were known to have had a SARS-CoV-2 infection during their pregnancy. Table 1 shows the characteristics of the total cohort and of couples with and without ongoing pregnancy separately. Percentages of missing values ranged from 0 to 22.8% per candidate predictor.

Table 1. Cohort characteristics

Characteristics	All couples (*n* = 526)	Ongoing pregnancy* (*n* = 345)	No ongoing pregnancy (*n* = 181)	Missing data *n* (%)
Mean age (SD), range				
Women	33.58 (4.67), 20-45	33.28 (4.42) 20-43	34.14 (5.08), 21-45	0 (0)
Men	35.50 (6.11), 20-67	35.10 (5.79) 20-67	36.28 (6.63), 21-55	26 (4.9)
Median number of pregnancy losses (IQR), range	3 (2-4), 2-11	3 (2-3), 2-10	3 (3-4), 2-11	0 (0)
Primary RPL, *n* (%)	308 (58.6)	202 (58.6)	106 (58.6)	0 (0)
History of IVF/ICSI treatment, *n* (%)	72 (13.7)	39 (11.3)	33 (18.2)	0 (0)
Mean BMI (SD), range				
Women	24.55 (4.59), 16.18-44.98	24.71 (4.83), 17.71-44.98	24.24 (4.08), 16.18-42.91	24 (4.6)
Men	25.51 (3.60), 18.26-41.77	25.36 (3.50), 18.26-41.77	25.79 (3.77), 19.27-40.75	120 (22.8)
Smoking, *n* (%)				
Women	65 (12.4)	37 (10.7)	28 (15.4)	6 (1.1)
Men	133 (25.3)	83 (24.1)	50 (27.6)	61 (11.6)

SD = standard deviation; IQR = interquartile range; RPL = recurrent pregnancy loss; IVF = in vitro fertilisation; ICSI = intracytoplasmic sperm injection; BMI = body mass index.
*Ongoing pregnancy defined as fetal survival beyond 24 weeks of gestation.

Predicting the chance of ongoing pregnancy

The number of previous pregnancy losses, maternal and paternal age and previous conceptions by IVF/ICSI treatment had statistically significant univariable associations with an ongoing pregnancy (Supplemental Table 1). Figure 1 shows the unadjusted relations between the predicted probability of an ongoing pregnancy and the continuous predictors

number of previous pregnancy losses, maternal and paternal age and maternal and paternal BMI. The probability of an ongoing pregnancy gradually declined with increasing number of previous pregnancy losses and increasing paternal age and sharply declined starting from maternal age 35. Although parental BMI effects were small, we observed a negative association between increasing paternal BMI and an ongoing pregnancy, while increasing maternal BMI slightly improved the chance of an ongoing pregnancy.

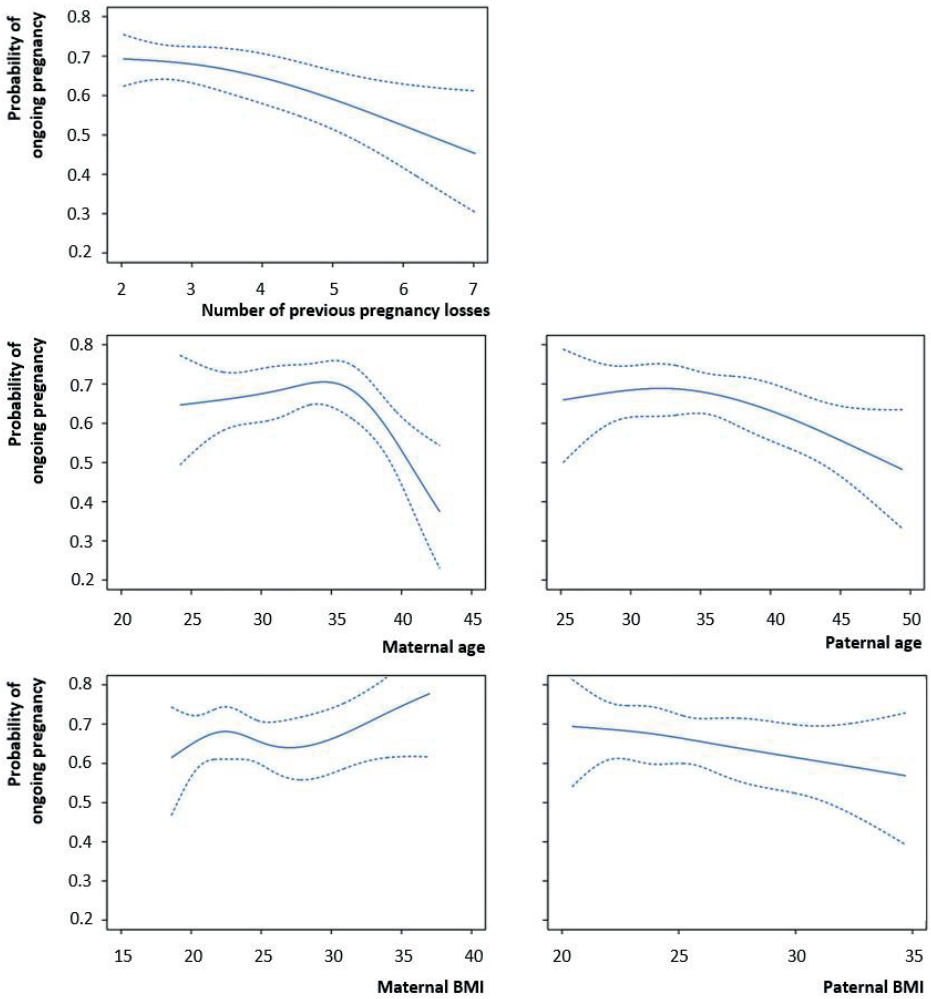


Figure 1. Univariable relations between continuous baseline variables and ongoing pregnancy

BMI = body mass index. Each panel depicts the probability of ongoing pregnancy (solid curve) with 95% confidence bands (dashed curves) as function of the baseline variable. Relations were characterised by restricted cubic spline functions. Only maternal age had a significant non-linear relation with the outcome.

The factors in the final multivariable model (Table 2) to predict the probability of having a subsequent ongoing pregnancy were the number of previous pregnancy losses, maternal age, paternal age, maternal BMI, paternal BMI, maternal smoking status and mode of conception (with or without history of IVF/ICSI treatment). The bootstrapping procedure yielded an adjusted calibration slope of 0.77, which was applied as a shrinkage factor to the intercept and coefficients of the final model. The odds of a subsequent ongoing pregnancy decreased with every increasing previous pregnancy loss. For example, the odds of an ongoing pregnancy after three pregnancy losses were 19% lower than the odds of an ongoing pregnancy after two pregnancy losses, and the odds after six pregnancy losses were 47% less than after three losses. A smoking woman had 38% lower odds of an ongoing pregnancy compared to a non-smoking woman. Couples with a history of IVF/ICSI treatment had a 46% reduced odds of an ongoing pregnancy compared to couples with spontaneous conceptions.

Table 2. Final logistic regression model for ongoing pregnancy

Intercept and predictors	β coefficient[a]	Odds ratio (95% CI)	*P*-value
Intercept	0.53		
Number of previous pregnancy losses	-0.16	0.81 (0.70-0.93)	0.004
Maternal age as restricted cubic spline[b]			
Maternal age	0.06	1.08 (0.92-1.25)	0.34
Maternal age'	-0.01	0.98 (0.71-1.38)	0.94
Maternal age''	-0.46	0.55 (0.12-2.46)	0.43
Maternal smoking	-0.36	0.62 (0.36-1.07)	0.09
Maternal BMI	0.03	1.04 (0.99-1.09)	0.09
Paternal age	-0.02	0.97 (0.93-1.01)	0.15
Paternal BMI	-0.04	0.95 (0.89-1.01)	0.11
History of IVF/ICSI treatment	-0.47	0.54 (0.312-0.92)	0.02

BMI = body mass index; 95% CI = 95% confidence interval; IVF = in vitro fertilisation; ICSI = intracytoplasmic sperm injection. *The predicted probability of a subsequent ongoing pregnancy can be calculated for individual couples using the formula shown in the Supplemental data. [a]Regression coefficients were multiplied with a shrinkage factor of 0.77 that was obtained from the bootstrapping procedure (described in Methods). β-values are expressed per 1-unit increase for continuous predictors and for the condition present (prediction value = 1) for dichotomous predictors. [b]Maternal age was fitted using a restricted cubic spline function with four knots placed at 25.27, 31.84, 35.94 and 40.53 years. The age variables with tick-marks (', '') represent the new variables created to allow for non-linear contributions from maternal age. These coefficients cannot be interpreted on their own; the partial effect plot for maternal age is shown in Supplemental Figure 3.

Model performance

The calibration plot of the final multivariable model indicated overall good calibration (Supplemental Figure 2). We compared the discrimination of the final model to that of smaller models including only a subset of the predictors. The optimism corrected AUCs ranged from 0.57 for a model only including the predictors maternal age (fitted as a linear variable) and number of previous pregnancy losses, to 0.63 for the final model including all predictors derived from the backward selection procedure. Performance measures for all models are shown in Supplemental Table 2.

Predicting ongoing pregnancy for specific couples

Figure 2 shows four couples with their respective characteristics and predicted chances of a subsequent ongoing pregnancy according to our final multivariable prediction model, including the number of previous pregnancy losses, maternal and paternal age, maternal and paternal BMI, maternal smoking status and mode of conception (with or without a history of IVF/ICSI treatment). We compared the predicted probabilities of our model with those provided by the commonly used prediction model of Brigham et al.(8), including only the number of previous pregnancy losses and maternal age fitted as a linear variable.

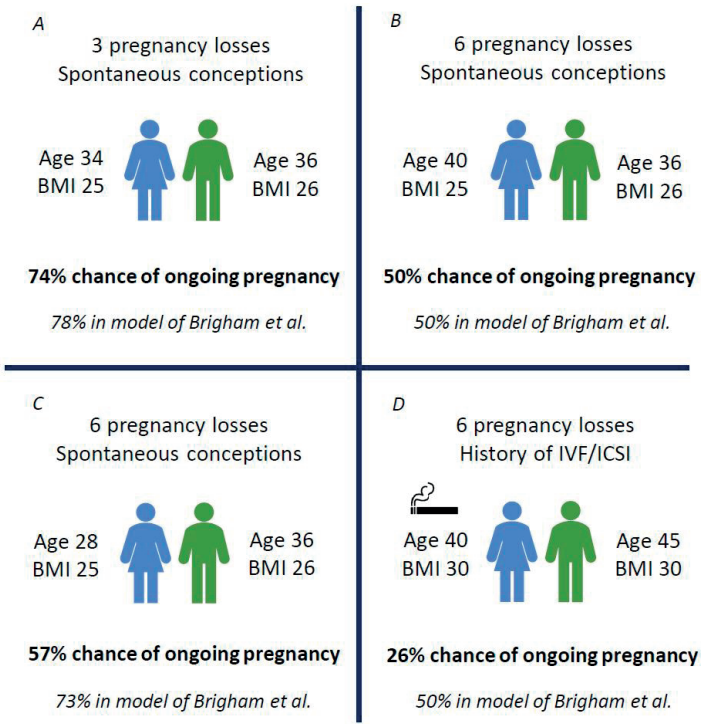


Figure 2. Predicting ongoing pregnancy: four scenarios

BMI = body mass index; IVF = in vitro fertilisation; ICSI = intracytoplasmic sperm injection. Chances of an ongoing pregnancy >24 weeks' gestation based on our final prediction model, including the following variables: number of previous pregnancy losses, maternal age (fitted as restricted cubic spline with four knots), paternal age, maternal BMI, paternal BMI, maternal smoking status and mode of conception. Predicted probabilities are shown for four couples and compared to the model of Brigham et al. Scenario A shows a couple with average characteristics based on our population statistics, i.e. with the median number of pregnancy losses, mean ages and BMIs as shown in Table 1. In scenario B, the number of previous pregnancy losses and maternal age are higher, while other characteristics are unchanged. Scenario C is similar to scenario B, except for a younger maternal age. In scenario D the number of pregnancy losses and the woman's age are similar to scenario B, but here the male partner is also of advanced age, the couple has a history of fertility treatment (IVF/ICSI), they are obese and the woman smokes.

For scenario A and B, the predicted chances of a subsequent ongoing pregnancy calculated with our model and with the model of Brigham et al. were similar (74% vs. 78% for scenario A and 50% both for scenario B). In scenario C our model provided a lower chance of an ongoing pregnancy compared to the model of Brigham et al. (57% vs. 73%). In scenario D the predicted probabilities resulting from both models were even more deviating. The estimate of our model was a 26% chance of an ongoing pregnancy, almost half the probability as calculated for scenario B. However, the model of Brigham et al. still estimated a 50% chance of an ongoing pregnancy, since this model is only based on the number of previous pregnancy losses and maternal age, being equal in scenarios B and D

DISCUSSION

We showed that predicting the chance of a subsequent ongoing pregnancy beyond 24 weeks of gestation in couples with RPL becomes more accurate when, besides the conventional predictors maternal age and the number of previous pregnancy losses, more variables are incorporated into the model. The additional predicting variables include both male and female characteristics, advocating a couple-focused rather than a female-focused approach in RPL. Still, the predictive ability of the current model remains limited and we emphasize that more research is needed in order to develop a model that can be used in clinical practice.

The apparent predictive performance of our final multivariable model in terms of the AUC was 0.66 (0.63 after internal validation with bootstrapping), compared to 0.57 for a model restricted to the conventional predictors maternal age and number of previous pregnancy losses. Although showing an improvement in predictive ability, an AUC between 0.60-0.70 is still considered as poor to moderate performance and indicates that the model will not successfully predict outcomes for many couples.(20) As Brigham et al.(8) and Lund et al.(9) did not mention any performance measures, it was not possible to make a direct comparison with their models. A recently published nationwide Danish cohort study that aimed to predict the chance of subsequent live birth in the general population based on maternal age and prior pregnancy events, reported an AUC of 0.60. Both this Danish cohort study and our study illustrate the difficulty of predicting future ongoing pregnancy. This may be due to the complex and largely unexplained multifactorial aetiology of (recurrent) pregnancy loss.

While we confirmed earlier findings showing that the number of previous pregnancy losses and woman's age are prognostic variables of great importance(2, 8, 9, 18), we also found that additional maternal variables (smoking status, BMI) as well as paternal parameters (age, BMI) increased predictive performance. Furthermore, we observed that previous IVF/ICSI treatment lowers the predicted chance of a subsequent ongoing pregnancy in couples with RPL. Our candidate predictors were chosen based on previous epidemiologic and basis research and although one should be cautious with interpreting the results of a prediction study aetiologically(7), it is likely that some of the predictors have a causal relation with the outcome.

Maternal age is strongly associated with a higher risk of fetal aneuploidy, an established cause of pregnancy loss.(23) Advanced paternal age has been linked to increased levels of sperm DNA fragmentation, which is associated with (recurrent) pregnancy loss.(13, 24, 25) Likewise, paternal obesity may cause excessive oxidative stress and affect pregnancy outcome by damaging DNA integrity of the spermatozoa.(26) Maternal smoking is well-

known to increase the risk of pregnancy complications, including pregnancy loss.(10) On the other hand, the relation between assisted reproductive techniques, including IVF/ICSI treatment, and an increased risk of pregnancy loss is less straight-forward. It is complex to determine whether this increased risk can be attributed to the treatment itself, whether it is a proxy for underlying (unidentified) patient characteristics, or whether it is due to the fact that ART pregnancies are closely monitored and subsequent (early) pregnancy loss is more often detected compared to couples who conceived naturally.(27) Furthermore, we observed a positive association between increasing maternal BMI and the chance of an ongoing pregnancy in our cohort. A previous study in couples with unexplained RPL demonstrated a U-shaped relationship between miscarriage rate in the subsequent pregnancy and maternal pre-pregnancy BMI, with the highest risk of miscarriage in underweight women, followed by obese women (BMI >30 kg/m^2).(28) Although we observed similar high risks of pregnancy loss in underweight women with BMI <20 kg/m^2, in our population the highest chance of an ongoing pregnancy was found in obese women. However, it should be noted that the number of obese women in our sample was limited and the observed BMI effect was relatively weak and uncertain.

When developing a prediction model it is important to assess the presence of non-linear patterns between continuous predictors and the outcome of interest.(29) We found that maternal age had a non-linear relationship with the chance of an ongoing pregnancy, with a negative effect starting around 35 years and we estimated this relationship using a restricted cubic spline. A similar pattern for the maternal age effect was observed in two prior studies(30, 31) predicting chances of live birth in other (large) populations, not restricted to RPL patients; these studies also fitted maternal age as restricted cubic spline in their models. However, previous prediction models for RPL handled maternal age as a linear term, which probably differs substantially from the “true” predictor-outcome relationship, as it assumes that the effect is the same at each part of the range of maternal age.

We believe that our study holds several strengths compared to other prediction studies on unexplained RPL. We followed TRIPOD recommendations for model development and reporting.(14) To prevent overfitting, we determined the maximum number of candidate predictors a priori.(17, 32) Furthermore, we selected candidate predictors based on theoretical plausibility instead of choosing predictors on the basis of the strength of their unadjusted univariable associations with the outcome. The last strategy is undesired as this most often leads to substantial uncertainty in model structure and important predictors may be rejected because of nuances in the study data.(29, 33, 34) We used backward elimination with AIC for predictor selection, being a preferred method, especially in smaller data sets.(20) In addition, we performed internal bootstrap validation and used the shrinkage factor to adjust the regression

coefficients and apparent performance for optimism(20), which was not done in any of the previously published prediction models for RPL. Besides these methodological assets, we used data of a strictly defined population of couples with unexplained RPL, containing information on both partners, being systematically collected during intake consultations. Still, some missing data existed, mainly on paternal variables. However, it was possible to impute these data using multiple imputation. This technique takes into account statistical uncertainty in the imputed values and, if data are missing at random, provides less biased results compared to complete case analysis.

The aim of this study was to identify predictors for a subsequent ongoing pregnancy beyond 24 weeks of gestation, after referral to the clinic. This outcome was available for the vast majority of couples in our database, while the outcome of a subsequent live birth as well as outcomes of later occurring pregnancies were more often missing (due to the fact that many women were referred back to their local hospital or midwifery practice). Ideally, patients would like to know their overall chances of having a future live birth. Therefore, the ultimate model should predict the cumulative chances of live birth within a certain time period, for instance within five years after referral. This would require a prospective cohort study with structural follow-up of couples with RPL for at least five years after first consultation. Furthermore, in future research, the effects of more potential predictors such as alcohol consumption of both partners and level of sperm DNA fragmentation should be evaluated, which have previously been associated with pregnancy loss but were unavailable in this study. In a sufficiently large cohort including couples with both explained and unexplained RPL, it may also be considered to assess identified risk factors (for instance presence of anti-TPO antibodies or APS) as predicting variables and to assess meaningful interactions between different predictors.

Conclusions

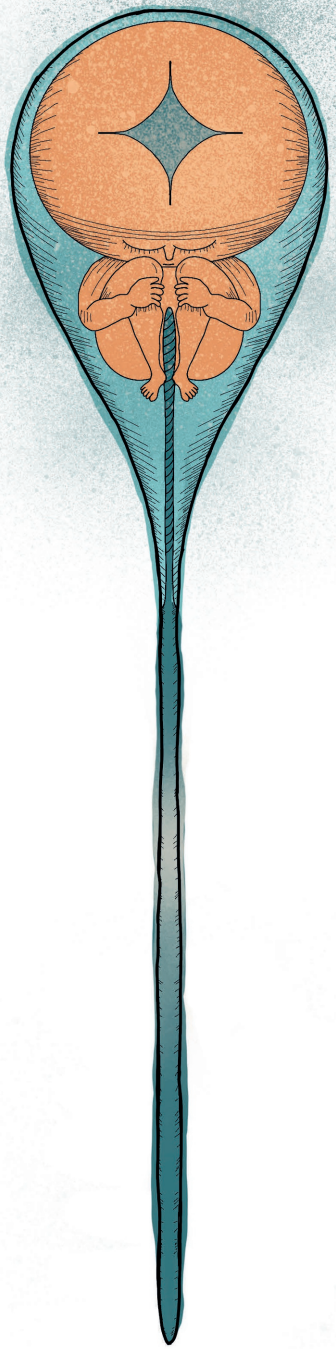
Couples with RPL need something to hold on to, that helps to shape their expectations and assists in making decisions regarding new pregnancy attempts. In addition, stratification of couples into risk groups can be used for further in-depth personalised research, for instance on interventions. To facilitate this, an accurate well-developed and validated prediction model is needed. To date, such a model is not yet within reach. Although we showed in this study that we should look beyond the number of previous pregnancy losses and maternal age and we should also consider additional predictors including male factors and lifestyle factors, the predictive ability - and therefore the clinical applicability- of the model is still insufficient. However, our findings serve as an important starting point for the development of a new prediction tool to use in clinical practice.

REFERENCES

1. Rai R, Regan L. Recurrent miscarriage. Lancet 2006;368:601-11.
2. 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.
3. Jaslow CR, Carney JL, Kutteh WH. Diagnostic factors identified in 1020 women with two versus three or more recurrent pregnancy losses. Fertil Steril. 2010;93:1234-43.
4. 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;114:1288-96.
5. 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;26:873-7.
6. 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;28:398-405.
7. 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:ii1-ii5.
8. Brigham SA, Conlon C, Farquharson RG. A longitudinal study of pregnancy outcome following idiopathic recurrent miscarriage. Hum Reprod. 1999;14:2868-71.
9. Lund M, Kamper-Jørgensen M, Nielsen HS, Lidegaard Ø, Andersen AM, Christiansen OB. Prognosis for live birth in women with recurrent miscarriage: what is the best measure of success? Obstet Gynecol 2012;119:37-43.
10. Pineles BL, Park E, Samet JM. Systematic review and meta-analysis of miscarriage and maternal exposure to tobacco smoke during pregnancy. Am J Epidemiol 2014;179:807-23.
11. 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 Epidemol Community Health 2018;72:783.
12. du Fossé NA, van der Hoorn MP, van Lith JMM, le Cessie S, Lashley E. Advanced paternal age is associated with an increased risk of spontaneous miscarriage: a systematic review and meta-analysis. Hum Reprod Update 2020;26:650-69.
13. McQueen DB, Zhang J, Robins JC. Sperm DNA fragmentation and recurrent pregnancy loss: a systematic review and meta-analysis. Fertil Steril. 2019;112:54-60.e3.
14. 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;162:55-63.
15. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemosat. 2006;4:295-306.
16. Franssen MT, Korevaar JC, Leschot NJ, Bossuyt PM, Knegt AC, Gerssen-Schoorl KB et al. Selective chromosome analysis in couples with two or more miscarriages: case-control study. BMJ 2005;331:137-41.
17. van Smeden M, Moons KG, de Groot JA, Collins GS, Altman DG, Eijkemans MJ et al. Sample size for binary

logistic prediction models: Beyond events per variable criteria. Statistical Methods in Medical Research 2019;28:2455-74.

18. Sugiura-Ogasawara M, Ozaki Y, Kitaori T, Suzumori N, Obayashi S, Suzuki S. Live birth rate according to maternal age and previous number of recurrent miscarriages. Am J Reprod Immunol. 2009;62:314-9.
19. White IR, Royston P, Wood AM. Multiple imputation using chained equations: Issues and guidance for practice. Stat Med. 2011;30:377-99.
20. Moons KG, Altman DG, Reitsma JB, Ioannidis JP, Macaskill P, Steyerberg EW et al. Transparent Reporting of a multivariable prediction model for Individual Prognosis or Diagnosis (TRIPOD): explanation and elaboration. Ann Intern Med. 2015;162:W1-73.
21. Rubin DB. Inference and Missing Data. Biometrika 1976;63:581-92.
22. Steyerberg EW, Eijkemans MJ, Harrell FE, Jr., Habbema JD. Prognostic modeling with logistic regression analysis: in search of a sensible strategy in small data sets. Med Decis Making 2001;21:45-56.
23. Grande M, Borrell A, Garcia-Posada R, Borobio V, Muñoz M, Creus M et al. The effect of maternal age on chromosomal anomaly rate and spectrum in recurrent miscarriage. Hum Reprod. 2012;27:3109-17.
24. Wyrobek AJ, Eskenazi B, Young S, Arnheim N, Tiemann-Boege I, Jabs EW et al. Advancing age has differential effects on DNA damage, chromatin integrity, gene mutations, and aneuploidies in sperm. Proc Natl Acad Sci. 2006;103:9601.
25. Schmid TE, Grant PG, Marchetti F, Weldon RH, Eskenazi B, Wyrobek AJ. Elemental composition of human semen is associated with motility and genomic sperm defects among older men. Hum Reprod. 2013;28:274-82.
26. Wright C, Milne S, Leeson H. Sperm DNA damage caused by oxidative stress: modifiable clinical, lifestyle and nutritional factors in male infertility. Reprod Biomed Online 2014;28:684-703.
27. Wang JX, Norman RJ, Wilcox AJ. Incidence of spontaneous abortion among pregnancies produced by assisted reproductive technology. Hum Reprod. 2004;19:272-7.
28. Metwally M, Saravelos SH, Ledger WL, Li TC. Body mass index and risk of miscarriage in women with recurrent miscarriage. Fertil Steril. 2010;94:290-5.
29. Harrell FE, Jr. Regression Modeling Strategies. With Applications to Linear Models, Logistic Regression, and Survival Analysis. 1 ed: Springer, New York, NY, 2001.
30. McLernon DJ, Steyerberg EW, te Velde ER, Lee AJ, Bhattacharya S. Predicting the chances of a live birth after one or more complete cycles of in vitro fertilisation: population based study of linked cycle data from 113873 women. BMJ 2016;355:i5735.
31. Kolte AM, Westergaard D, Lidegaard Ø, Brunak S, Nielsen HS. Chance of live birth: a nationwide, registry-based cohort study. Hum Reprod. 2021;36(4):1065-1073.
32. Riley RD, Ensor J, Snell KIE, Harrell FE, Jr., Martin GP, Reitsma JB et al. Calculating the sample size required for developing a clinical prediction model. BMJ 2020;368:m441.
33. Steyerberg EW. Clinical Prediction Models. A Practical Approach to Development, Validation, and Updating. 1 ed: Springer-Verlag New York, 2009.
34. Sauerbrei W, Boulesteix A-L, Binder H. Stability Investigations of Multivariable Regression Models Derived from Low- and High-Dimensional Data. J Biopharm Stat. 2011;21:1206-31.



CHAPTER 6

Identification of distinct seminal plasma cytokine profiles associated with male age and lifestyle characteristics in unexplained recurrent pregnancy loss

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ABSTRACT

Background

Seminal plasma contains a wide range of cytokines, chemokines and growth factors. Part of these signalling molecules assist in inducing a state of active maternal immune tolerance towards the fetus. Disbalances in seminal plasma content may contribute to pregnancy loss. This study investigated cytokine expression profiles in seminal plasma of male partners of couples with unexplained recurrent pregnancy loss (RPL) and the association with clinical and lifestyle characteristics, including smoking, alcohol consumption and body mass index (BMI).

Methods

In the seminal plasma of 52 men who visited a specialised RPL clinic the levels of 25 pre-selected cytokines, chemokines and growth factors were measured by Bio-Plex assay or ELISA. Two-way hierarchical cluster analysis was performed. Identified patient clusters were compared on clinical and lifestyle characteristics.

Results

Two distinct cytokine expression profiles in the seminal plasma were revealed by cluster analysis. Patient cluster I showed relatively higher levels of pro-inflammatory cytokines, including IL-1α, IL-1β, IL-6, IL-8, IL-12, IL-18 and TNF-α, compared to Patient cluster II. Men belonging to Patient cluster I were significantly older and had significantly more lifestyle risk factors compared to men in Patient cluster II.

Conclusion

Cluster analysis suggested the existence of a less favourable pro-inflammatory cytokine expression profile, being present in part of men affected by RPL and associated with advanced male age and lifestyle risk factors. These findings may serve as a starting point for further research into underlying mechanisms and ultimately lead to novel diagnostic and therapeutic approaches for couples with RPL.

INTRODUCTION

Previous research indicated that seminal plasma is not only a transporter medium that protects and nourishes the spermatozoa. It also primes the maternal immune system by carrying a multitude of cytokines, chemokines, paternal antigens and other immunological factors.(1-3) These signalling molecules have direct effects on the cervix and endometrium and help to induce a state of active maternal immune tolerance, important for normal development of human pregnancy.(4-6) A balance between pro-inflammatory- and immunoregulatory factors in the seminal plasma seems necessary to optimally support the female reproductive tract in developing tolerance and enabling implantation.(7) Disbalances in seminal plasma composites may contribute to pregnancy loss or complications later in gestation.(8, 9)

Recurrent pregnancy loss (RPL) is defined as a minimum of two pregnancy losses before the fetus reaches viability. Multiple risk factors for RPL have been established, including parental chromosomal translocations, uterine anomalies, maternal thrombophilia and thyroid auto-immunity)(10). Despite this, RPL remains unexplained in 60-70% of cases.(11) Advanced maternal age, smoking and obesity are established risk factors for pregnancy loss.(12-14) More recently, a number of studies showed that advanced paternal age and lifestyle factors may contribute to pregnancy loss as well.(15-17) This implicates that the male role in achieving a successful pregnancy involves more than just the conception. We hypothesise that advanced paternal age and lifestyle factors such as smoking or obesity, result in disbalances in seminal plasma components that may contribute to pregnancy loss.

Prior studies reported reference values for several immunological factors in the seminal plasma of healthy fertile men, to provide a foundation for further studies in pathologic conditions.(2, 3) The current exploratory study is the first to investigate the levels of pre-selected seminal plasma cytokines and other signalling molecules in male partners of couples with unexplained RPL. Further insight in the seminal plasma content of these men may lead to a better understanding of the complex aetiology of RPL. We used cluster analysis to analyse multiplex cytokine measurements in seminal plasma and to identify different subgroups of patients. In addition, we evaluated associations between cytokine expression profiles in the seminal plasma and clinical and lifestyle factors.

MATERIALS AND METHODS

Ethics approval

This research was approved by the Medical Research Ethics Committee of the Leiden University Medical Center (reference number P11.196, year of approval 2012). All participants provided informed consent for participation.

Study participants

Patients with RPL

Subjects were 52 male partners of couples with RPL who visited the RPL outpatient clinic of the Leiden University Medical Center between 2012 and 2019. Inclusion criteria were (i) a minimum of three consecutive pregnancy losses before 20 weeks of gestation (definition for RPL used at our centre when the study was initiated) and (ii) unexplained RPL (i.e. no evidence of antiphospholipid syndrome (APS), uterus anomalies, anti-thyroid peroxidase (TPO) antibodies or parental chromosomal translocations, following the ESHRE guideline for RPL(10)). Exclusion criteria were (i) symptoms of urinary or genital tract infection and (ii) use of antibiotics or immune-modifying medications. Couples received no treatment other than supportive care (including frequent ultrasound monitoring during early pregnancy). Two out of 52 included men had one or more children from a previous relationship. One participant had a brother who also suffered from RPL with his partner.

Control group

To provide an indication of normal ranges of cytokines and other immunological factors in the seminal plasma, 11 proven fertile men were included as a control group. They had at least one live birth with their partner and no history of pregnancy loss. They were non-smoking, had a BMI <25 kg/m^2 and were aged ≤40 years.

Clinical data collection

Baseline characteristics were collected during intake consultation at the RPL outpatient clinic. The following data were extracted from medical records: male age, height, weight, smoking behaviour and alcohol consumption; detailed obstetric history of the couple; date of semen collection; outcome of the subsequent pregnancy after semen collection.

Semen collection and storage

Semen samples were collected by masturbation, following 48-72 h of sexual abstinence. Complete ejaculates were collected directly in a plastic-free container and processed within 120 min after collection. Samples were centrifuged at 600 g for 10 min to remove sperm and cellular debris and the supernatant (seminal plasma) was immediately aliquoted into 100 µL volumes and stored at -20°C until analysis. Median time between last pregnancy loss and semen collection was 5 months (interquartile range 3-7).

Detection of cytokines and other immunological factors in the seminal plasma

Key signalling molecules in the seminal plasma were selected based on previous literature (shown in Supplementary Table 1). The levels of the following cytokines, chemokines, growth factors and regulatory factors present in the seminal plasma were assessed by the Bio-Plex Luminex™ system assay (Bio-Rad, Veenendaal, the Netherlands): IL-1α, IL-1β, IL-2, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-16, IL-18, TNF-α, IFN-γ, SDF-1α, MCP-1, FGF, VEGF, GM-CSF, M-CSF, G-CSF, sHLA class I and TGF-β1, TGF-β2, TGF-β3 in latent and active form. Bio-plex Luminex™ system (Bio-Rad) was used for read-outs. Methods for detection of cytokines were similar as those earlier described by Meuleman et al.(18) To measure total TGF-β1, TGF-β2 and TGF-β3, tests were performed with and without prior acid activation of the seminal plasma to release biologically active protein from the latent precursor form. An enzyme-linked immunosorbent assay (ELISA) was performed to detect Prostaglandin E2 (PGE2) and soluble HLA-G (sHLA-G). Characteristics of assays used for detection of cytokines, with individual limits of detection and lower and upper limits of quantification for each measured factor are shown in Supplementary Table 2. The manufacturer's instructions were followed. To measure sHLA class I, monoclonal antibody to HLA-class I purified antibody W6/32 (Department of Immunology, LUMC, the Netherlands) was coupled via carboxyl groups on the surface of polystyrene beads (COOH bead: Bio-Rad) according to the procedure of the Bio-Plex Amine Coupling kit (Bio-Rad). Concentrations were expressed in picograms per millilitre (pg/mL), or nanograms per millilitre (ng/mL) when indicated.

Data analysis and statistics

Analyses were performed in SPSS version 25 (IBM SPSS Statistics, IBM Corp., Armonk, NY, USA) and in R studio version 1.3.9.50 (R Foundation for Statistical Computing, Vienna, Austria). For calculations, measurement values below the detection limit were set to one half the detection limit and measurement values exceeding the upper limit of quantification were replaced by the highest concentration measured for that particular factor (similar to the study in fertile men of Politch et al.(3).

To explore correlations between individual cytokines, Spearman's rank correlation coefficients (ρ) were calculated . Since advanced age and obesity have been associated with a tendency toward a more pro-inflammatory systemic cytokine phenotype(19, 20), we assessed whether this is also reflected in the seminal plasma by calculating Spearman's rank correlations between pro-inflammatory cytokines and male age and BMI.

Agglomerative hierarchical cluster analysis was performed using R studio packages gplots and heatmap.2. Before clustering, all values were log transformed and scaled using the mean centering with standard deviation (Z-scores), so that each cytokine would contribute in similar manner to the final classification. Four agglomerative clustering

methods were assessed by calculating the agglomerative coefficient, which measures the amount of clustering structure found in the data.(21) As Ward's minimum variance clustering (with Euclidean distance) showed the highest agglomerative coefficient (0.80), dendrograms were generated using this method.

Population characteristics were described using means or medians and percentages. Independent Samples T tests were used to compare means between clusters and Chi-square tests or Fisher's Exact Tests were used to compare categorical data. Correction for multiple comparisons was performed when indicated using the step-up Benjamini-Hochberg procedure(22), with adjusted *P*-values reported. Statistical significance was inferred when (adjusted) $P < 0.05$.

Since previous studies showed that the risk of (recurrent) pregnancy loss significantly increases with advanced paternal age and when multiple lifestyle risk factors are present simultaneously (15, 17), we developed a score to combine these factors: the Age Lifestyle Index. The Age Lifestyle Index was calculated for each patient based on the following factors: age, BMI, smoking behaviour and alcohol consumption. Cut-off points for the scoring method were based on previous studies and established classification systems: male age <40 years: score 0, 40-44: score 1, ≥45: score 2(23); BMI <25 kg/m^2: score 0, 25-29: score 1, ≥30: score 2(24); non-smoking: score 0, smoking occasionally: score 1, smoking daily: score 2(25); alcohol consumption <2 units per day: score 1 and alcohol consumption ≥2 units per day: score 1(26). Median total scores were compared between the clusters with a Mann-Whitney *U* test.

RESULTS

Concentrations of cytokines, chemokines and growth factors in the seminal plasma

Seminal plasma samples of 52 male partner of women with RPL were analysed. As the data did not satisfy the assumptions of normal distribution, concentrations of seminal plasma components were described in medians with interquartile ranges. Descriptive statistics for all measured factors are shown in Table 1.

Table 1. Descriptive statistics of cytokines, chemokines and growth factors in the seminal plasma of male partners of women with RPL

Factor	% Detectable	Percentiles			Range
		25th	Median	75th	
IL-1α	94	15.8	25.4	30.1	ND – 176.7
IL-1β	98	0.46	0.63	1.1	ND – 16.3
IL-6	98	3.2	5.5	12.3	ND – 300.3
IL-8	100	298.4	496.2	697.9	136.8 – 5770.7
IL-12 (p70)	100	2.5	3.0	3.5	0.8 – 6.9
IL-18	98	1.6	2.5	3.8	ND – 22.5
MCP-1	100	532.9	915.7	1172	143.5 – 3331
SDF-1α	98	3773	4577	5340	ND – 6278
TNF-α	100	23.3	28.0	50.0	4.8 – 275.2
IL-10	98	8.9	12.0	15.0	ND – 56.4
PGE2*	100	2374	5186	10029	559 – 35167
TGF-β1 active	100	759.7	1486	2214	88.9 – 8999
TGF-β1 latent	100	89574	157763	225494	290.4 – 396013
TGF-β2 active	100	285.2	523.7	818.8	156.1 – 1871
TGF-β2 latent	98	9204	11096	12747	ND – 17030
TGF-β3 active	100	1575	3357	4927	138.0 – 9346
TGF-β3 latent	100	49741	106504	188443	123.5 – 518739
sHLA-G*	100	29.6	50.3	107.6	4.7 – 1593
sHLA class I*	100	275.2	374.9	523.0	116.3 – 2999
IL-7	100	710.8	1267	1995	202.3 – 3542
IL-2	98	2.1	2.7	3.7	ND – 8.5
IL-16	98	4.8	8.0	15.6	ND – 174.2
IFN-γ	98	25.5	43.2	91.2	ND – 293.1
VEGF*	100	18.6	7.7	121.0	5.4 – 326.1
FGF	100	9.1	11.5	14.0	4.1 – 25.1
G-CSF	98	39.3	55.1	76.6	27.1 – 576.1
GM-CSF	79	0.23	0.53	0.88	0.10 – 3.31
M-CSF	100	157.7	182.1	226.5	35.2 – 854.6

Concentrations in pg/ml, except for * (ng/ml); ND = Non detectable

Correlations between cytokines, chemokines and growth factors

Spearman's rank correlation coefficients between individual seminal plasma components are shown in Fig. 1. Significant positive correlations were mainly found between pro-inflammatory cytokines: IL-6, IL-8, IL-1β, TNF-α and IL-16 were all highly correlated

(ρ >0.57; adjusted *P*-values <0.05). Strong negative correlations were found between IL-7 and sHLA-G (ρ =-0.57), IL-7 and TGF-β1 (ρ =-0.49), VEGF and PGE2 (ρ =-0.43), IL-16 and TGF-β3 (ρ =-0.39) and IL-1α and TGF-β1 (ρ =-0.38), with adjusted *P*-values all being < 0.05.

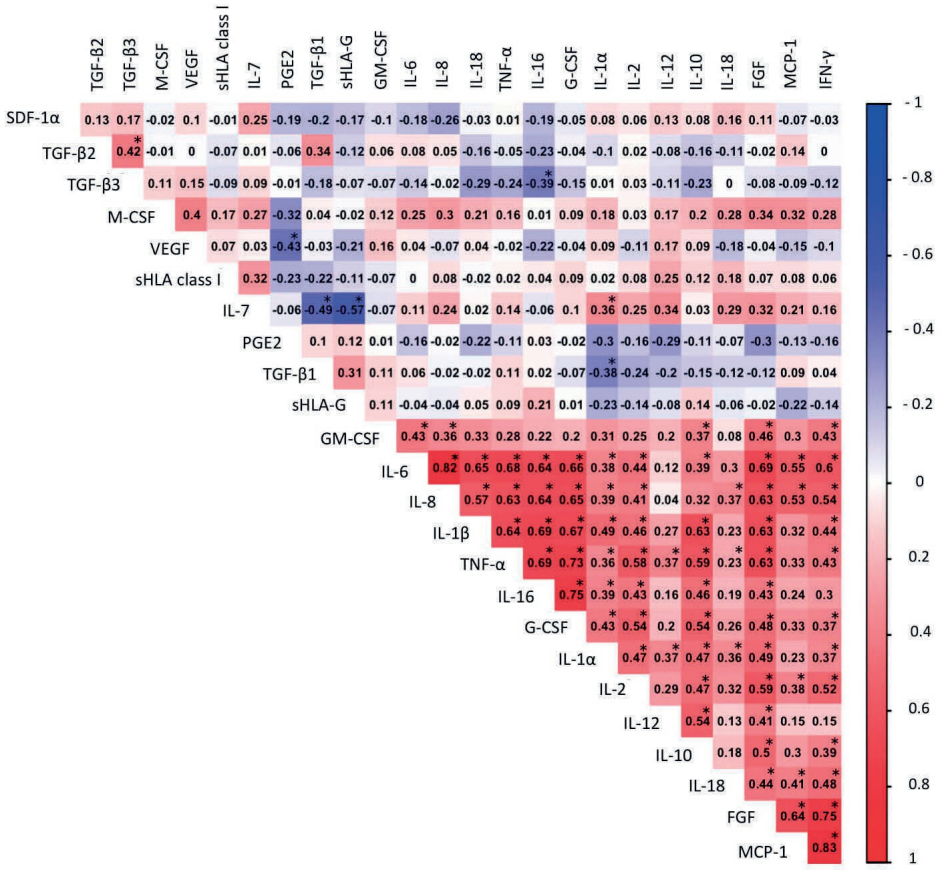


Figure 1. Correlation matrix

Spearman's rank correlations (ρ) between individual seminal plasma signalling molecules. Red colour indicates a positive correlation, white colour indicates no correlation and blue colour indicates a negative correlation. *Benjamini-Hochberg adjusted P-value <0.05 (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Correlations between pro-inflammatory cytokines and age and BMI

Significant positive correlations were found between male age and IL-6 (ρ = 0.339) , IL-8 (ρ = 0.384) and IL-16 (ρ = 0.333), with adjusted *P*-values all <0.05. Correlations between BMI and pro-inflammatory cytokines did not reach significance after Benjamini-Hochberg adjustment.

Cluster analysis

The results of the cluster analysis were visualized in a heat map with two dendrograms (Fig. 2), representing the degrees of relatedness between patients (Patient cluster I and II) and between cytokines (Cytokine cluster I and II).

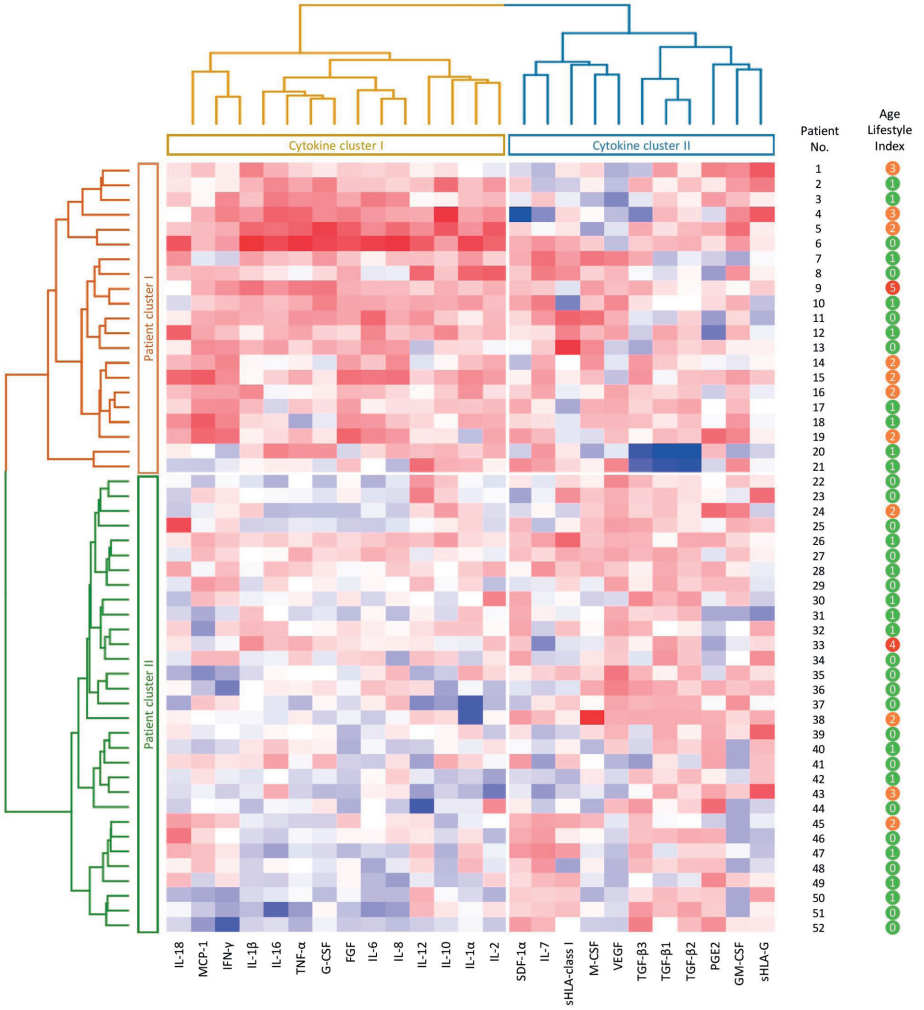


Figure 2. Cluster analysis

Two-way hierarchical cluster analysis with Euclidean distance and Ward's aggregation method. Columns represent cytokines and other immunological factors present in the seminal plasma. Rows represent 52 patient samples (seminal plasma from male partners of women with unexplained RPL). Cytokine concentrations are indicated using a colour scale, ranging from blue (low) to red (high). Both patients and cytokines were separated by the algorithm into two main clusters, indicated as Patient clusters I and II and Cytokine clusters I and II. Patient cluster I contains patients that showed higher expression of pro-inflammatory factors in the seminal plasma compared to Patient cluster II. The Age Lifestyle Index (scoring method explained in paragraph Data analysis and statistics) for each patient is shown on the right, ranging from green (low) to red (high) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Clustering of seminal plasma components relates the measured cytokines and other immunological factors to each other based on their expression in the samples. Cytokine cluster I contained mainly pro-inflammatory factors (IL-18, MCP-1, IL-1β, IL-16, TNF-α, IL-6, IL-8, IL-12, IL-1α, IL-2). Cytokine cluster II primarily contained immunoregulatory factors (TGF-β1, TGF-β2, TGF-β3, IL-7, sHLA class I, sHLA-G, PGE2, SDF-1a).

The patient clustering showed a separation into two main clusters, containing 21 and 31 patients, respectively. The two patient clusters mostly differed with respect to the expression of seminal plasma signalling molecules belonging to Cytokine cluster I. Patient cluster I contained patients that showed high expression of pro-inflammatory factors in the seminal plasma. Less pronounced differences were observed between the patient clusters with regard to Cytokine cluster II (TGF-β, SDF-1α, IL-7, sHLA class I, M-CSF, VEGF, PGE2, sHLA-G). Median concentrations of cytokines and other immunological factors were compared between the two Patient clusters and the control group (shown in Supplementary Figure 1). For pro-inflammatory cytokines IFN-γ, IL-1β, IL-6, IL-8, IL-18 and MCP-1 the seminal plasma concentrations were markedly higher in Patient cluster I compared to the control group, while for these factors the concentrations of Patient cluster II overlapped with concentrations of the control group.

In Table 2, clinical parameters and characteristics of patients belonging to Patient cluster I and II are shown. The 21 patients in Patient cluster I were significantly older compared to Patient cluster II (mean ages of 37.8 and 34.4, respectively, P = 0.002). In Patient cluster I, 33% of men was beyond 40 years of age, compared to 6% in Patient cluster II (P = 0.012). The proportion of alcohol consumers (≥2 units per day) was also significantly higher in Patient cluster I (19% versus 0%, P = 0.022). No significant difference was found in mean BMI between the clusters, Patient cluster I comprised significantly more men with BMI ≥25 (52% versus 29%, P = 0.043). Male age, BMI, smoking behaviour and alcohol consumption were also evaluated as a combined variable, the Age Lifestyle Index. Individual scores are shown in Fig. 2. The median Age Lifestyle Index was significantly higher for Patient cluster I compared to Patient cluster II: medians and interquartile ranges 1 (1-2) and 0 (0-1), P = 0.010.

The live birth rate (for the first pregnancy after semen collection) was 71% in Patient cluster I, compared to 81% in Patient cluster II (P = 0.355). The miscarriage rate was 24% in Patient cluster I and 13% in Patient cluster II (P = 0.231).

Table 2. Clinical parameters and male age and lifestyle characteristics compared between Patient clusters I and II

	Patient cluster I *n* = 21	Patient cluster II *n* = 31	*P*-value
Reproductive details of couples:			
Number of pregnancy losses *median (interquartile range)*	3 (3-4)	3 (3-4)	
History of fertility treatment	4 (19)	1 (3)	0.079
IVF	2 (10)	0 (0)	
IUI only	2 (10)	1 (3)	
Outcome of subsequent pregnancy			
Live birth	15 (71)	25 (81)	0.355
Miscarriage	5 (24)	4 (13)	0.231
Termination of pregnancy	0 (0)	1 (3)	
missing	1 (5)	1 (3)	
Male characteristics:			
Age *mean (SD)*	37.76 (3.91)	34.36 (3.66)	0.002*
Age ≥40 years *n* (%)	7 (33)	2 (6)	0.012*
BMI *mean (SD)*	26.17 (3.82)	24.7 (3.02)	0.133
BMI ≥25 *n* (%)	11 (52)	9 (29)	0.043*
Smoking			
Occasionally	-	4 (13)	0.138
Daily	3 (14)	3 (10)	0.675
missing	1 (5)	0 (0)	
Alcohol consumption ≥ 2 units per day *n* (%)	4 (19)	0 (0)	0.022*
missing	2 (10)	3 (10)	
Age Lifestyle Index *median (interquartile range)*	1 (1-2)	0 (0-1)	0.015*

IVF = in vitro fertilisation; IUI = intrauterine insemination; BMI = body mass index
*$P < 0.05$

DISCUSSION

This is the first study that evaluated the content of cytokines, chemokines and growth factors in the seminal plasma of male partners of couples with RPL. Hierarchical cluster analysis revealed two distinct patient clusters. Patient cluster I showed a trend towards more unfavourable characteristics, both with regard to cytokine expression in the seminal plasma and clinical parameters. Levels of pro-inflammatory cytokines such as IL-6, IL-8, IL-12, IL-16, IL-18 and TNF-α were relatively high. Previous studies associated abundance of these agents in the seminal plasma with silent male reproductive tract infection, inflammation and infertility.(1) A profile with high levels of pro-inflammatory cytokines might induce an inflammatory maternal immune response eventually leading to pregnancy loss.(27)

Some remarkable differences were found with respect to clinical and lifestyle parameters between the two patient clusters. Patients of cluster I were significantly older and included significantly more moderate to heavy alcohol consumers. In addition, Patient cluster I scored significantly higher on the Age Lifestyle Index, reflecting the combined factors of age, BMI, smoking and alcohol consumption. There is solid evidence that these (lifestyle) risk factors may lead to oxidative stress, caused by accumulation of reactive oxygen species (ROS).(28, 29) Therefore, what could potentially be underlying our observations, is the complex interplay between ROS and seminal plasma cytokines. Positive correlations have been observed between ROS production and pro-inflammatory cytokines in the seminal plasma, including TNF-α, IL-6, IL-8 and IL-16.(28, 30) The mechanisms behind this are not fully elucidated. Some cytokines may stimulate the generation of ROS.(31, 32) On the other hand, ROS can promote production of cytokines. (32, 33) Excessive ROS in the seminal plasma may negatively affect sperm DNA integrity. (34, 35) This suggests that pro-inflammatory cytokines in the seminal plasma may not only contribute to pregnancy loss by interfering with the maternal immune response, but also through (either directly or indirectly) damaging the DNA of the male gamete. While conventional sperm parameters including volume, morphology and motility have no clear link with pregnancy loss and are poor predictors of future pregnancy outcomes, increased levels of sperm DNA fragmentation are strongly associated with RPL.(36, 37)

When comparing seminal plasma concentrations of our RPL patients to the results in the control group and the results earlier reported in healthy fertile men by Politch et al.(3), we found remarkably higher levels of pro-inflammatory cytokines including IL-1β, IL-6, IL-8, IL-10 and IFN-γ, suggesting a potential role for these cytokines in the development of RPL. However, as cytokines do not act in isolation, but function in a network, it seems more appropriate to study these signalling molecules as a system and not as individual factors. Therefore, cluster analysis was applied to find patterns in seminal plasma

cytokine profiles. We showed that this is a helpful tool to visualize clusters that do not have to be pre-defined and can therefore be used to find subgroups of patients with potentially similar disease-related mechanisms. Another strength of this study is the well-defined population of male partners of couples with unexplained RPL. Since no female risk factors for RPL could be identified in these couples, it is plausible that male (sperm) factors may play a role here.

A next step would be to validate the identified patient clusters and their associations with clinical factors within larger datasets. This would also allow for multivariable regression analysis, to assess the effect of individual clinical factors adjusted for other potentially confounding factors. Furthermore, since cytokine networks are dynamic, it would be valuable to examine the robustness of these clusters over time. Two studies indicated variation over time in seminal plasma cytokine content, particularly for IFN-γ and to a lesser extent for IL-8. The authors mentioned lifestyle factors as potentially contributing to the regulation of cytokine fluctuations.(38, 39) It seems promising to investigate to what extent any lifestyle modifications are reflected in the content of the seminal plasma. Additionally, the link between the seminal plasma microbiome, cytokine expression profiles and RPL may be a subject of future research.(Tomaiuolo et al. 2020)

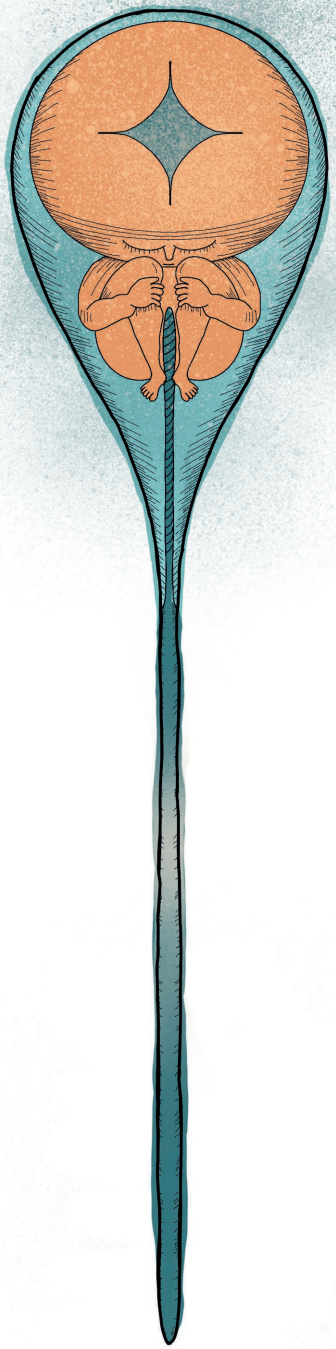
The ultimate goal is to develop a panel consisting of cytokines and other seminal biomarkers such as DNA fragmentation level, which can be used for diagnostic and prognostic purposes in clinical practice. Our results suggest that there might be a potential difference in outcome of the subsequent pregnancy based on the two identified cytokine expression profiles, however, potentially due to the moderate sample size of this exploratory study, differences in live birth and miscarriages rate did not reach statistical significance. Larger studies are needed to further investigate the potential of cytokines and other semen factors to predict future pregnancy outcome. This will contribute to providing answers to couples with unexplained RPL and may also serve as a starting point for therapeutic interventions.

REFERENCES

1. Fraczek M, Kurpisz M. Cytokines in the male reproductive tract and their role in infertility disorders. J Reprod Immunol. 2015;108:98-104.
2. 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.
3. 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.
4. Robertson SA, Mau VJ, Hudson SN, Tremellen KP. Cytokine-leukocyte networks and the establishment of pregnancy. Am J Reprod Immunol. 1997;37(6):438-42.
5. Bromfield JJ. Seminal fluid and reproduction: much more than previously thought. J Assist Reprod Genet. 2014;31(6):627-36.
6. Robertson SA, Prins JR, Sharkey DJ, Moldenhauer LM. Seminal fluid and the generation of regulatory T cells for embryo implantation. Am J Reprod Immunol. 2013;69(4):315-30.
7. Nederlof I, Meuleman T, van der Hoorn MLP, Claas FHJ, Eikmans M. The seed to success: The role of seminal plasma in pregnancy. J Reprod Immunol. 2017;123:24-8.
8. Schjenken JE, Robertson SA. Seminal Fluid Signalling in the Female Reproductive Tract: Implications for Reproductive Success and Offspring Health. Adv Exp Med Biol. 2015;868:127-58.
9. Dekker GA, Robillard PY, Hulsey TC. Immune maladaptation in the etiology of preeclampsia: a review of corroborative epidemiologic studies. Obstet Gynecol Surv. 1998;53(6):377-82.
10. 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).
11. Jaslow CR, Carney JL, Kutteh WH. Diagnostic factors identified in 1020 women with two versus three or more recurrent pregnancy losses. Fertil Steril. 2010;93(4):1234-43.
12. Nybo Andersen AM, Wohlfahrt J, Christens P, Olsen J, Melbye M. Maternal age and fetal loss: population based register linkage study. BMJ. 2000;320(7251):1708-12.
13. Pineles BL, Park E, Samet JM. Systematic review and meta-analysis of miscarriage and maternal exposure to tobacco smoke during pregnancy. Am J Epidemiol. 2014;179(7):807-23.
14. Metwally M, Ong KJ, Ledger WL, Li TC. Does high body mass index increase the risk of miscarriage after spontaneous and assisted conception? A meta-analysis of the evidence. Fertil Steril. 2008;90(3):714-26.
15. du Fossé N, van der Hoorn M-L, Eikmans M, Heidt S, le Cessie S, Mulders A, et al. Evaluating the role of paternal factors in aetiology and prognosis of recurrent pregnancy loss: study protocol for a hospital-based multicentre case–control study and cohort study (REMI III project). BMJ Open 2019;9(11):e033095.
16. Venners SA, Wang X, Chen C, Wang L, Chen D, Guang W, et al. Paternal smoking and pregnancy loss: a prospective study using a biomarker of pregnancy. Am J Epidemiol. 2004;159(10):993-1001.
17. Ruixue W, Hongli Z, Zhihong Z, Rulin D, Dongfeng G, Ruizhi L. The impact of semen quality, occupational exposure to environmental factors and lifestyle on recurrent pregnancy loss. J Assist Reprod Genet. 2013;30(11):1513-8.
18. Meuleman T, Snaterse G, van Beelen E, Anholts JDH, Pilgram GSK, van der Westerlaken LAJ, et al. The immunomodulating effect of seminal plasma on T cells. J Reprod Immunol. 2015;110:109-16.

19. Rea IM, Gibson DS, McGilligan V, McNerlan SE, Alexander HD, Ross OA. Age and Age-Related Diseases: Role of Inflammation Triggers and Cytokines. Front Immunol. 2018;9:586-.
20. Park HS, Park JY, Yu R. Relationship of obesity and visceral adiposity with serum concentrations of CRP, TNF-alpha and IL-6. Diabetes Res Clin Pract. 2005;69(1):29-35.
21. Kaufman L, Rousseeuw P. Finding Groups in Data: An Introduction to Cluster Analysis2009.
22. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society: Series B (Methodological). 1995;57(1):289-300.
23. du Fossé NA, van der Hoorn M-LP, van Lith JMM, le Cessie S, Lashley EELO. Advanced paternal age is associated with an increased risk of spontaneous miscarriage: a systematic review and meta-analysis. Hum Reprod Update. 2020;26(5):650-69.
24. World Health Organisation. Body mass index - BMI 2020 [Available from: https://www.euro.who.int/en/health-topics/disease-prevention/nutrition/a-healthy-lifestyle/body-mass-index-bmi.
25. World Health Organisation. WHO Policy on non-recruitment of smokers or other tobacco users: frequently asked questions 2018 [Available from: https://www.who.int/employment/FAQs_smoking_English.pdf].
26. National Health Service. Alcohol units 2018 [Available from: https://www.nhs.uk/live-well/alcohol-support/calculating-alcohol-units].
27. 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.
28. Tremellen K. Oxidative stress and male infertility—a clinical perspective. Hum Reprod Update. 2008;14(3):243-58.
29. Vaughan DA, Tirado E, Garcia D, Datta V, Sakkas D. DNA fragmentation of sperm: a radical examination of the contribution of oxidative stress and age in 16945 semen samples. Hum Reprod. 2020.
30. Jiang L, Zheng T, Huang J, Mo J, Zhou H, Liu M, et al. Association of semen cytokines with reactive oxygen species and histone transition abnormalities. J Assist Reprod Genet. 2016;33(9):1239-46.
31. 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.
32. 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.
33. Rajasekaran M, Hellstrom WJ, Naz RK, Sikka SC. Oxidative stress and interleukins in seminal plasma during leukocytospermia. Fertil Steril. 1995;64(1):166-71.
34. Moustafa MH, Sharma RK, Thornton J, Mascha E, Abdel-Hafez MA, Thomas AJ, et al. Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. Hum Reprod. 2004;19(1):129-38.
35. Aitken RJ, De Iuliis GN. On the possible origins of DNA damage in human spermatozoa. Mol Hum Reprod. 2010;16(1):3-13.
36. McQueen DB, Zhang J, Robins JC. Sperm DNA fragmentation and recurrent pregnancy loss: a systematic review and meta-analysis. Fertil Steril. 2019;112(1):54-60.e3.
37. 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.

38. Sharkey DJ, Tremellen KP, Briggs NE, Dekker GA, Robertson SA. Seminal plasma pro-inflammatory cytokines interferon-γ (IFNG) and C-X-C motif chemokine ligand 8 (CXCL8) fluctuate over time within men. Hum Reprod. 2017;32(7):1373-81.
39. Sharkey DJ, Tremellen KP, Jasper MJ, Gemzell-Danielsson K, Robertson SA. Seminal fluid induces leukocyte recruitment and cytokine and chemokine mRNA expression in the human cervix after coitus. J Immunol. 2012;188(5):2445-54.
40. Tomaiuolo, R., et al., Microbiota and Human Reproduction: The Case of Male Infertility. High-Throughput, 2020.9(2): p. 10.



CHAPTER 7

Impaired immunomodulatory effects of seminal plasma may play a role in unexplained recurrent pregnancy loss: results of an in vitro study

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ABSTRACT

Background

Seminal plasma contains signalling molecules capable of modulating the maternal immune environment to support implantation and pregnancy. Prior studies indicated that seminal plasma induces changes in gene transcription of maternal immune cells. Reduced immune suppressive capacity may lead to pregnancy loss. The aim of this study was to investigate the immunomodulating effects of seminal plasma on T cells and monocytes in the context of recurrent pregnancy loss (RPL).

Methods

Female T cells and monocytes were incubated with seminal plasma of 20 males in unexplained RPL couples (RPL males) and of 11 males whose partners had ongoing pregnancies (control males). The effect of seminal plasma on messenger RNA (mRNA) expression of immune cells was measured. Levels of mRNA expression were related to key signalling molecules present in the seminal plasma. Agglomerative hierarchical cluster analysis was performed on seminal plasma expression profiles and on mRNA expression profiles.

Results

Expression of CD25 and anti-inflammatory IL-10 by female T cells was significantly lower after stimulation with seminal plasma of RPL males compared to control males. Female monocytes treated with seminal plasma of RPL males showed an immune activation signature of relatively elevated HLA-DR expression. Expression of these T cell and monocyte components was particularly correlated with the amounts of TGF-β and VEGF in the seminal plasma.

Conclusion

Our findings indicate that seminal plasma has immunomodulating properties on female immune cells compatible with the induction of a more regulatory phenotype, which may be impaired in cases of unexplained RPL.

INTRODUCTION

Recurrent pregnancy loss (RPL) is a condition defined as the demise of two or more pregnancies before the fetus reaches viability.(1) Although multiple risk factors for RPL have been identified, including parental chromosomal translocations, uterine anomalies and several other maternal conditions, no explanation can be found in 60-70% of affected couples who undergo diagnostic investigations.(1, 2) Emerging evidence suggests that maternal immune response towards the embryo plays a pivotal role in at least part of the unexplained cases of RPL.(3-5) In fact, the phenomenon of a successful pregnancy involves the tolerance of a semi-foreign body by the maternal immune system. One potentially important player in attaining this state of immune tolerance towards the embryo is the seminal plasma.

The spermatozoa, carriers of the paternal genome, are surrounded by a nourishing and protecting fluid: the seminal plasma. The seminal plasma contains a wide variety of signalling molecules that are thought to exert their effects on female tissues directly after ejaculation.(6-8) For instance, TGF-β, prostaglandin E2 (PGE2) and soluble HLA-G (sHLA-G) are major tolerance-inducing agents present in high concentrations in the seminal plasma, while IFN-γ is a potent inhibitor of TGF-β and was found to be increased in seminal plasma of males in subfertile and RPL couples.(5, 9-11) Signalling molecules in the seminal plasma are thought to induce gene expression and recruitment of immune cells in the female reproductive tract.(12) Regulatory T cells (Tregs) are considered essential in the fetal-maternal interface because of their suppressive capacity.(13) Studies in female mice showed expansion of CD4+CD25+Foxp3+ Tregs in uterus-draining lymph nodes after exposure to seminal plasma.(14) Moreover, in a human in vitro setting, incubation of peripheral blood T cells with seminal plasma led to increased messenger RNA (mRNA) expression of CD25, IL-10 and Foxp3, suggesting the induction of a Treg cell pool.(15) Besides activation of the adaptive immune system, it has been shown that seminal plasma has immunomodulating effects on cells of the innate immune system as well. In a previous study, culturing monocytes in the presence of seminal plasma led to a change in gene expression, compatible with a diminished extent of maturation and immune activating capacity of these cells.(16)

Although prior studies have indicated that seminal plasma exerts stimulatory effects on female immune cells and promotes suppressive activity, little is known on the role of seminal plasma in the specific context of RPL. Key questions include whether perturbations in the stimulatory capacity of seminal plasma may contribute to RPL, and if these perturbations exist, whether these could be attributed to disbalances in seminal plasma content. The aim of the current study was to investigate the effect of seminal plasma on mRNA expression of important activation markers in human T cells and

monocytes in an in vitro model. The stimulatory capacity of seminal plasma of males in RPL couples was compared with that of males whose partners had ongoing pregnancies. Furthermore, correlations between mRNA expression of immune cells after seminal plasma stimulation and signalling molecules present in the seminal plasma were studied.

MATERIALS AND METHODS

Ethics approval

This study was approved by the Medical Research Ethics Committee of the Leiden University Medical Center (reference numbers P11.196 and P19.014). All participants provided informed consent to take part in the study.

Study participants

RPL group

Seminal plasma samples of 20 male partners in couples with unexplained RPL were used for this study (RPL males). These couples visited the specialized RPL clinic of the Leiden University Medical Center between 2012 and 2019. They had a minimum of three pregnancy losses before 20 weeks of gestation. No underlying condition for RPL was identified, i.e. there was no evidence of maternal antiphospholipid syndrome, uterine anomalies, anti-thyroid peroxidase (TPO) antibodies or parental chromosomal translocations (following the recommended diagnostic investigations of the ESHRE guideline on RPL(1)). They had no anamnestic symptoms of genital tract infection and did not use any immune-modifying medications.

Control group

Seminal plasma samples of 11 healthy fertile males who had one or more live births with their partner and no history of pregnancy loss were used as a control group (control males). They had no anamnestic symptoms of genital tract infection and did not use any immune-modifying medications.

Semen collection

After 48-72 hours of sexual abstinence, semen samples were collected by masturbation. Complete ejaculates were collected directly in a plastic-free container and processed within 120 min after collection. To remove sperm and cellular debris, samples were centrifuged at 600 g for 10 min and the supernatant (seminal plasma) was aliquoted in 100 µL volumes and stored at-20°C until analysis.

Detection of signalling molecules in the seminal plasma

Key seminal plasma signalling molecules were selected based on previous studies.(7, 11, 12, 17) Levels of IL-1β, IL-6, IL-8, IL-10, IL-12, IL-16, IL-18, TNF-α, IFN-γ, VEGF, sHLA class I and TGF-β1, TGF-β2 and TGF-β3 were measured using the Bio-Plex Luminex™ system assay (Bio-Rad, Veenendaal, the Netherlands), following the manufacturer's instructions and as previously described by Meuleman et al.(15) To measure total levels of TGF-β isoforms including the latent precursor form, tests were performed with and without prior acid activation. Prostaglandin E2 (PGE2) and sHLA-G were detected using

an enzyme-linked immunosorbent assay (ELISA). To measure sHLA class I, an earlier described in-house developed assay was used(18). All assay specifications are shown in Supplementary Table 1. Concentrations are expressed in picograms per milliliter (pg/ml) unless indicated otherwise.

Stimulation of T cells and monocytes with seminal plasma

Human peripheral blood mononuclear cells (PBMCs) were isolated by means of density gradient centrifugation (Ficoll separation solution, pharmacy Leiden University Medical Center, the Netherlands) from a single buffy coat obtained from one anonymous healthy female donor (Sanquin Blood Supply, Amsterdam, the Netherlands) after informed consent. PBMCs were purified by the depletion of either non-T cells or non-monocytes using magnetic cell sorting (EasySep Human T Cell Enrichment Kit and EasySep Human Monocyte Enrichment Kit, STEMCELL Technologies, Köln, Germany) following the manufacturer's protocol. PBMC enriched T cells (CD3+ fraction) and monocytes (CD14+ fraction) were separately incubated in flat-bottom 48-well plates (Costar) at a density of 0.5 × 106 cells per well for 24 hours. Seminal plasma was added at the beginning of the culture at a concentration of 1:500 in 500 µl culture medium containing RPMI-1640 with 10% human serum and 1% L-glutamine (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, United States). The 1:500 seminal plasma concentration was based on previous (unpublished) pilot studies, as this concentration induced the highest cell responses, without being toxic to immune cells. As a negative control, cells were cultured with culture medium alone (without seminal plasma). After 24 hours, cells were harvested and stored in 300 µl of RNAlater (RNA stabilization buffer, Qiagen, Venlo, the Netherlands) at -20°C.

Messenger RNA transcript analysis

RNA extraction was performed with NucleoSpin® columns (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Quantity of RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Complement DNA (cDNA) synthesis and real-time quantitative PCR were performed as described in more detail elsewhere.(15) Briefly, to synthesize cDNA, RNA was combined with oligo dT (Invitrogen; 0.25 mg) and random nucleotide hexamers (Invitrogen; 0.25 mg). Quantitative PCR was performed on a ViiA7 (Life Technologies, Carlsbad, California, USA) using specific primers and SYBR Green (BioRad) for general fluorescence detection. For each sample, levels of target mRNA transcripts were standardized to beta-actin (ACTB) and glyceraldehyde-3-phoseminal plasmahate dehydrogenase (GADPH) reference genes using the ΔCq method and the formula $\Delta Cq = 2^{-(Cq\ [transcript] - AVG\ Cq\ [references])}$. Cq values for GADPH and ACTB were stable across all samples and highly correlated with each other (coefficient of variation = 0.05 for both factors; r = 0.96). Primer sequences for the selected mRNA transcripts are shown in Supplementary Table 2.

Data analysis and statistics

Analyses were performed in R studio version 1.3.9.50 (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism version 8.4.2 (GraphPad Software, San Diego, California USA). For calculations, measurement levels of seminal plasma factors below the detection limit were set to one half the detection limit and measurement values that were marked as out of range were replaced by the highest extrapolated value for that particular factor (similar to methods used in previous studies(7, 17)). To visualize seminal plasma expression profiles and mRNA expression profiles of female T cells and monocytes, heatmaps were created using R studio packages gplots and ComplexHeatmap. First, all values were log transformed and subsequently scaled using Z-scores. To identify expression patterns, both rows and columns were clustered using unsupervised agglomerative hierarchical cluster analysis with Ward's minimum variance method and 1-Spearman's rank correlation coefficient (ρ) as distance metric.(19).

Standardized signals of mRNA expression were statistically compared between the RPL group, the control group and the negative control group with a Kruskal Wallis test for unpaired non-parametric data. To explore correlations between individual seminal plasma factors and standardized signals of mRNA expression, Spearman's rank correlation coefficients were calculated and shown in a correlation matrix. These correlations were only assessed in the selection of seminal plasma factors and mRNA transcripts that significantly differed between the RPL group and the control group. Correction for multiple comparisons was performed using the Benjamini-Hochberg method. Statistical significance was inferred when (adjusted) $P<0.05$.

RESULTS

Characteristics of study participants

Clinical characteristics of RPL males (*n* = 20) and control males (*n* = 11) are shown in Table 1. No significant differences were found between the two groups for age, body mass index, smoking and history of fertility treatment.

Table 1. Characteristics of study participants

	RPL males (*n* = 20)	Control males (*n* = 11)	*P*-value
Age (years) mean (SD), range	36.07 (4.09), 29-45	32.9 (3.88), 29-40	0.06
BMI (kg/m²) Mean (SD), range	24.56 (2.73), 18-29	22.38 (3.29), 19-30	0.08
Smoking *n* (%)	3 (15)	0 (0)	0.18
History of fertility treatment* *n* (%)	3 (15)	0 (0)	0.18
Number of pregnancy losses median (interquartile range)	3 (3-4)	-	-

BMI = body mass index, RPL = recurrent pregnancy loss, SD = standard deviation.
Control males: males whose partners had ongoing pregnancies.
*Fertility treatment: intrauterine insemination or in vitro fertilisation

Seminal plasma expression profiles of immunological factors differ between RPL males and control males

In Table 2, descriptive statistics for all factors measured in the seminal plasma are shown for RPL males and control males. Concentrations of TGF-β1, TGF-β2, VEGF and sHLA-G were significantly lower in RPL males compared to control males. In Figure 1, the seminal plasma expression profiles of RPL males and control males are shown in a heatmap. The clustering algorithm separated the samples in three subgroups. Two subgroups mainly contained semen samples of RPL males (in orange and yellow), while one subgroup mainly contained samples of control males (in green). One subgroup of RPL samples (in orange) showed relatively high concentrations of pro-inflammatory cytokines, including IL-12, IL-18, IL-16, IL-8, IL-16, IL-1β, IFN-γ and TNF-α. In contrast, in the majority of the control samples, levels of pro-inflammatory cytokines were low, while levels of TGF-β, VEGF, sHLA-G and sHLA class I were relatively high compared to the RPL samples.

Table 2. Key seminal plasma immunological factors compared between RPL males and control males

Seminal plasma factor	RPL males (*n* = 20) Median (IQR)	Control males (*n* = 11) Median (IQR)	*P*-value
IL-1β	1.40 (0.64-2.52)	0.58 (0.37-0.91)	0.023
IL-6	10.3 (2.92-36.8)	5.56 (0.17-11.9)	0.086
IL-8	545.5 (284.7-888.4)	275.4 (57.0-551.7)	0.054
IL-10	4.00 (0.35-18.8)	0.35 (0.35-11.3)	0.359
IL-12 (p70)	3.60 (3.41-5.20)	4.55 (1.82-5.29)	0.640
IL-16	14.0 (5.36-44.4)	10.7 (4.43-43.6)	0.583
IL-18	2.70 (1.81-3.63)	2.23 (0.57-2.66)	0.169
IFN-γ	32.0 (19.9-104.0)	13.9 (2.15-40.3)	0.025
TNF-α	66.7 (36.1-155.7)	34.3 (0.57-80.9)	0.023
TGF-β1 (total)	122174 (80163-200642)	76297 (530961-773132)	<0.001*
TGF-β2 (total)	1116 (9475-13111)	28236 (22439-33229)	<0.001*
TGF-β3 (total)	127494 (51864-194999)	72433 (28869-528085)	0.984
PGE2‡	6008 (3758-10834)	7269 (3073-14541)	0.823
VEGF	18568 (13770-104158)	446075 (375250-528775)	<0.001*
sHLA-G‡	51.7 (26.7-80.2)	165.9 (70.2-1593)	0.007*
sHLA class I‡	434.4 (247.0-604.3)	1108 (358.7-2083)	0.044

IQR = interquartile range, RPL = recurrent pregnancy loss.
Control males: males whose partners had ongoing pregnancies.
These data are visualized in Figure 1.
* Significant after correction for multiple comparisons with Benjamini-Hochberg
Concentrations are shown in pg/ml, unless indicated with ‡ (ng/ml)

Stimulation with seminal plasma of RPL males induces different alterations in mRNA expression of female immune cells compared to stimulation with seminal plasma of control males

Figure 2 shows mRNA expression by female T cells and monocytes after stimulation with seminal plasma of RPL males, seminal plasma of control males and in the negative control group (cells incubated without seminal plasma). Stimulation with seminal plasma of control males induced several significant increases in mRNA expression by T cells: fold changes were 3.4 for CD25, 3.5 for IL-10 and 7.1 for Foxp3 (all fold changes reported in this paragraph are relative to mRNA expression in the negative control group). Stimulation with seminal plasma of RPL males also led to significantly increased mRNA expression of Foxp3 by T cells (fold change 7.1), but did not induce significant differences in IL-10 and CD25 mRNA expression. In monocytes, stimulation with seminal plasma of both control males and RPL males led to significantly decreased mRNA expression of HLA-DR, but HLA-DR expression was significantly less downregulated by seminal plasma of RPL males (fold changes-6.0 for control males and-3.2 for RPL males).

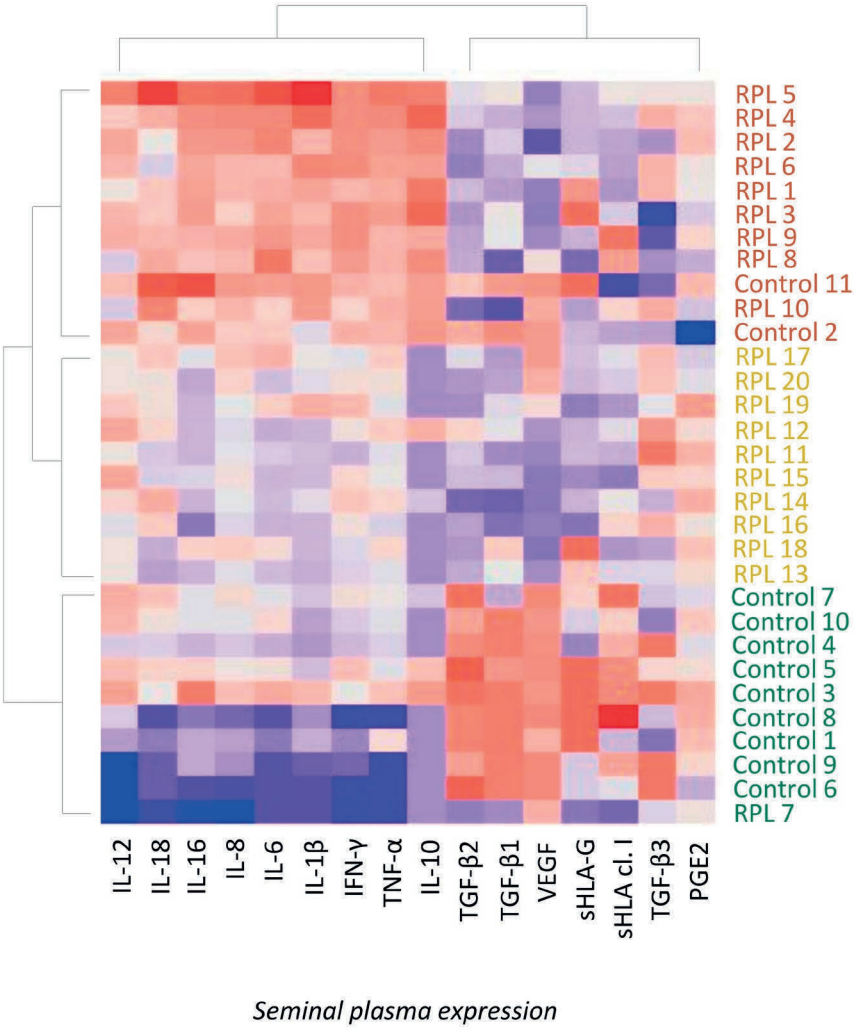


Figure 1. Cluster analyses of seminal plasma expression profiles and mRNA expression profiles of immune cells after stimulation with seminal plasma

Rows represent seminal plasma samples of males in couples with RPL (RPL males) and males whose partners had ongoing pregnancies (control males), and columns represent immunological factors present in the seminal plasma. Standardized concentrations are indicated in colours ranging from blue (low) to red (high). Both seminal plasma samples and seminal plasma factors were clustered with 1-Spearman's rank correlation distance and Ward's aggregation method. Dendrograms generated with this clustering method are shown in grey. Rows belonging to each cluster are labelled with colours orange, yellow and green, respectively.

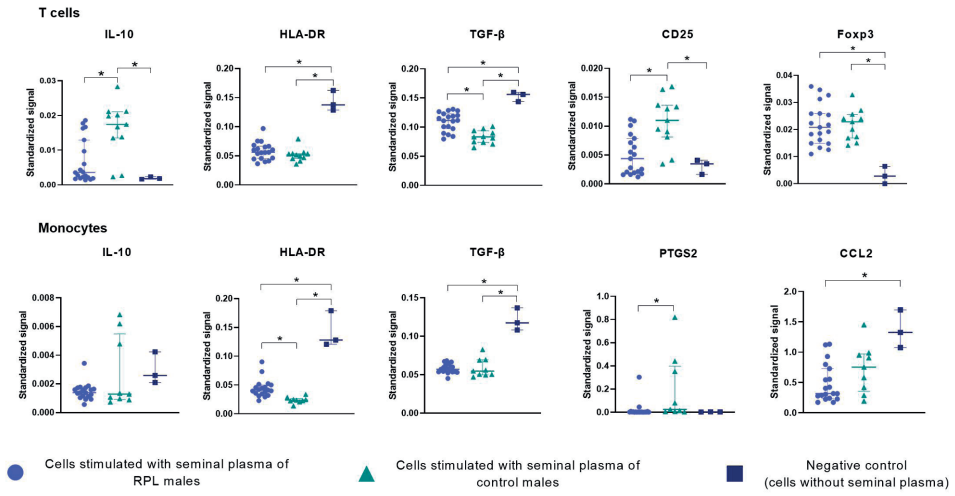
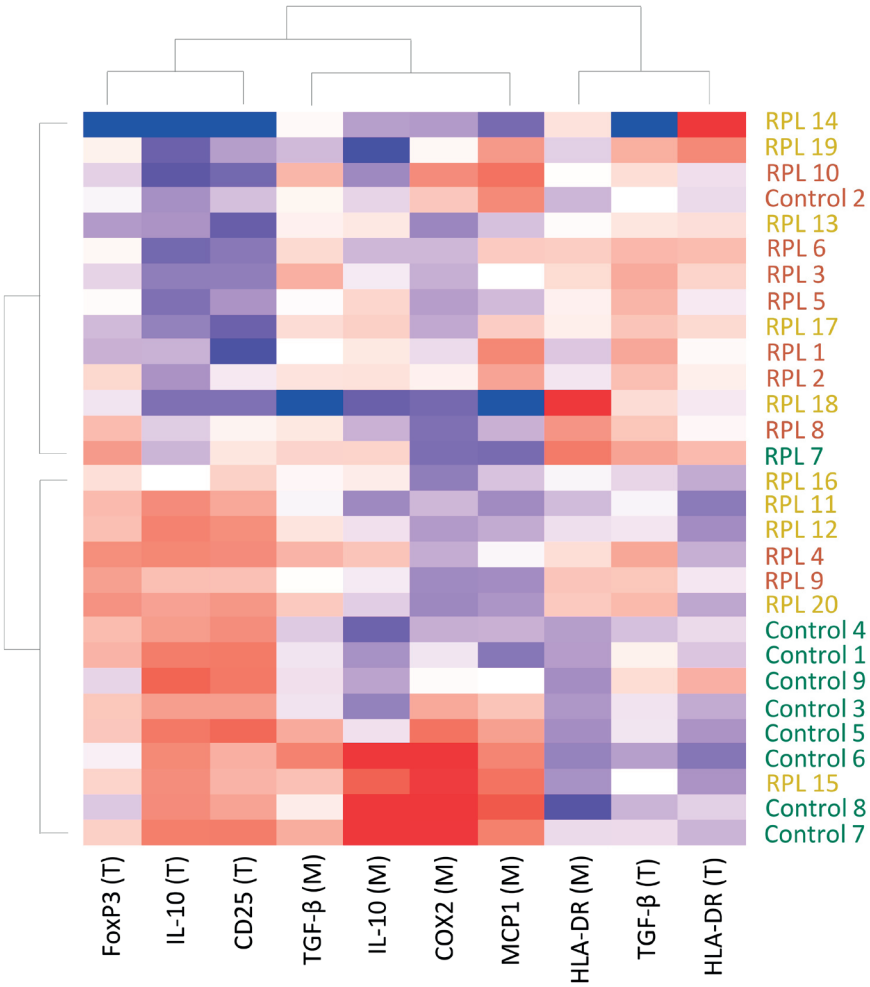


Figure 2. Messenger RNA expression of female T cells and monocytes after stimulation with seminal plasma of RPL males, seminal plasma of control males and without seminal plasma stimulation

Messenger RNA expression (standardized signals) was compared between three groups: after stimulation with seminal plasma of males in RPL couples (RPL males), after stimulation with seminal plasma of males whose partners had ongoing pregnancies (control males) and without seminal plasma stimulation (negative controls). For each sample, levels of mRNA expression were standardized to the average expression of beta-actin (ACTB) and glyceraldehyde-3-phoseminal plasmahate dehydrogenase (GADPH) reference genes using the ΔCq method and the formula $2^{-(Cq\ [transcript]\ -\ AVG\ Cq\ [references])}$. Significant differences in mRNA expression between groups are indicated with * (after Benjamini-Hochberg adjustment for multiple comparisons).

Figure 3 shows a cluster analysis of mRNA expression profiles of T cells and monocytes after stimulation with seminal plasma of either RPL males or control males. Based on the mRNA expression profiles, the clustering algorithm was able to separate the two groups to a great extent. The cluster analysis showed high correlation between expression of Foxp3, IL-10 and CD25 by T cells and between expression of IL-10, PTGS2 and CCL2 by monocytes. Also the mRNA expression of TGF-β by T cells and HLA-DR by monocytes and T cells were correlated. Furthermore, the linkage between seminal plasma expression of individual samples (Figure 1) and induction of T cell and monocyte responses (Figure 3) was visualized by the corresponding sample numbers and colour labels used in both Figures. Five out of ten RPL seminal plasma samples with an expression profile more similar to controls (in yellow) belong to the lower cluster of Figure 3, representing mRNA expression of immune cells more similar to controls. Seven out of nine RPL seminal plasma samples with a pro-inflammatory profile (in orange) belong to the upper cluster of Figure 3, with deviating responses of immune cells.



mRNA expression of T cells and monocytes after stimulation with seminal plasma

Figure 3. Messenger RNA expression profiles of female T cells and monocytes after stimulation with seminal plasma of RPL males and of control males

Rows represent stimulation with either seminal plasma of RPL males or control males. Columns represent mRNA expression of activation markers in T cells (T) and monocytes (M) after stimulation with seminal plasma. Standardized signals of mRNA expression are indicated in colours ranging from blue (low) to red (high). Both rows and columns were clustered with 1-Spearman's rank correlation distance and Ward's aggregation method. Dendrograms generated with this clustering method are shown in grey. Rows were labelled with colours orange, yellow and green based on their clustering in Figure 1. During the experiment, wells containing seminal plasma of two control males and monocytes were coincidentally mixed and could not be included in this part of the analysis. For this reason, nine instead of 11 controls are shown in this heatmap.

Messenger RNA expression of female T cells and monocytes after stimulation with seminal plasma is correlated with immunological factors present in the seminal plasma

Figure 4 shows correlations between seminal plasma factors and mRNA expression of T cells and monocytes. Messenger RNA expression of IL-10 and CD25 by T cells was positively correlated with TGF-β2 and VEGF. Messenger RNA expression of TGF-β by T cells was positively correlated with the amount of pro-inflammatory cytokines IL-1β, IFN-γ and TNF-α and negatively correlated with TGF-β1, TGF-β2 and sHLA class I in the seminal plasma. PTGS2 expression by monocytes was positively correlated with TGF-β2 and VEGF. Messenger RNA expression of HLA-DR by monocytes was negatively correlated with the amounts of TGF-β1, TGF-β2 and VEGF in the seminal plasma. The correlations shown were calculated within the total group of males. Correlations calculated within each group (RPL males and control males) separately were not significant.

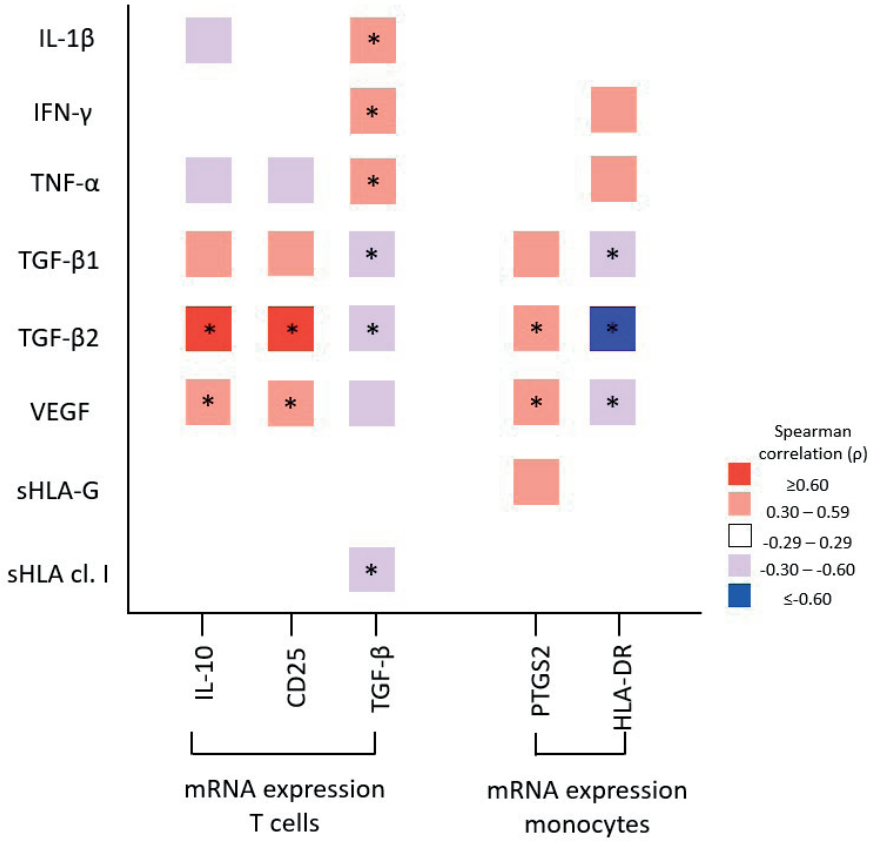


Figure 4. Correlations between mRNA expression of T cells and monocytes after seminal plasma stimulation and individual factors in seminal plasma

Only Spearman's rank correlation coefficients (ρ) ≥0.30 or ≤-0.30 are shown. Shown correlations were calculated within the total group of males. Significant correlations (after Benjamini-Hochberg adjustment for multiple comparisons) are indicated with *.

DISCUSSION

In this study we demonstrated that contact with seminal plasma leads to a change in gene expression of female T cells and monocytes. We showed for the first time that the stimulatory capacity of seminal plasma of males in RPL couples deviate from that of seminal plasma of control males whose partners had ongoing pregnancies. Our findings clearly suggest that impaired immunomodulatory effects due to disbalances in seminal plasma content play a role in the pathophysiology of unexplained RPL.

Previous *in vitro* studies showed that seminal plasma exposure has impact on female T cells and monocytes, compatible with the differentiation toward a more immune regulatory phenotype.(3, 15) We observed increased mRNA expression of IL-10, CD25 and Foxp3 by T cells after interaction with seminal plasma. IL-10 is known for its properties to sustain and amplify a suppressive immune response, while CD25 and Foxp3 may be indicators of the induction or development of a Tregs subset, which is considered vital for immunotolerance toward the semi-allogeneic fetus.(20, 21) Remarkably, Meuleman et al. only found changes in IL-10 and CD25 mRNA expression by purified T cells in the presence of antigen presenting cells (APCs), while they observed increased mRNA expression of Foxp3 also in absence of APCs. Differences with our study may be attributable to the fact that Meuleman et al. only used seminal plasma samples collected at an infertility clinic and did not include a healthy fertile control group. In our study, we only found significant increases in IL-10 and CD25 expression after stimulation with seminal plasma of control males and not after stimulation with samples of RPL males. Possibly, certain effects of seminal plasma on T cells do not essentially depend on the presence of APCs but also on the composition of the seminal plasma. Nevertheless, it is likely that the effects that we found would be amplified in the presence of APCs.

Differences were observed between the stimulatory capacity of seminal plasma of RPL males and seminal plasma of control males. After incubation with seminal plasma of RPL males, we observed no significant change in mRNA expression of CD25 and IL-10 by T cells. Prior studies that investigated the prevalence of CD25+ T cell subsets in normal pregnancy and in unexplained (recurrent) pregnancy loss found significantly lower proportions in the peripheral blood and decidua of females with pregnancy loss, suggesting that these cells might be important for maintenance of the pregnancy.(22-26) In contrast, we found relatively higher mRNA expression of HLA-DR by monocytes after incubation with seminal plasma samples of the RPL group. Multiple previous studies showed upregulation of HLA-DR on CD3+ and CD8+ T cells in females with unexplained RPL and another study found significantly increased HLA-DR+ monocyte subsets in females with preeclampsia.(25, 27-29) HLA-DR, which is a surface activation marker involved in antigen presentation, is capable of both inducing and intensifying an

immune reaction.(30) It has been postulated that an excess of HLA-DR+ cells may lead to a reduced immune regulatory environment, ultimately resulting in pregnancy failure. (29)

Our results suggest that the observed altered maternal immune response towards seminal plasma in RPL cases may be related to perturbations in seminal plasma content. Most striking were the highly positive correlations between seminal plasma TGF-β and mRNA expression of IL-10 and CD25 by T cells, and the negative correlation between seminal plasma TGF-β and mRNA expression of HLA-DR by monocytes. Normally, the seminal plasma is a rich source of TGF-β, which is known for its ability to induce anti-inflammatory and immunosuppressive effects.(12) We found significantly lower concentrations of TGF-β in the seminal plasma of males in RPL couples compared to seminal plasma samples of the control group. No significant correlations were found between seminal plasma factors and mRNA expression of immune cells when calculated within each group (RPL males or control males) separately. This may be explained by the moderate sizes of the individual groups as well as by the fact that differences in seminal plasma concentrations were much larger between groups than within groups.

Our study holds several strengths. First, we compared the immunomodulating effects of seminal plasma between cases with a pathological condition (RPL) and a carefully selected control group, while previous studies were limited to effects of seminal plasma stimulation in general. In addition, we included only couples with unexplained RPL as case group. These cases were diagnosed after a complete diagnostic work-up following recommendations of the clinical ESHRE guideline (which mainly focusses on maternal risk factors for RPL).(1) This makes it more plausible that seminal plasma factors may contribute to the pathophysiology of RPL in this selection of patients. However, RPL is a multifactorial condition and it is unlikely that insufficient immune suppression due to seminal plasma disbalances was involved in all of the included cases. This was reflected in our cluster analysis performed on seminal plasma samples, showing one subgroup consisting of RPL samples with expression profiles deviating from the control group and one subgroup resembling the expression profiles of the control samples. We showed that, in some but not all RPL males, the presence of a seminal plasma expression profile more similar to control males was related to more a normal induction pattern of immune cells. However, the current data are not yet sufficient to accurately predict individuals who may have male contributions to RPL and those who do not, which is an important goal for the future. As we showed in a previous study, seminal plasma expression profiles may be linked to male age and lifestyle characteristics.(17) As these factors are assumed to be part of the causal pathway instead of being confounders, we did not adjust for or matched on these factors in this study.(31) In larger studies, it would be interesting to evaluate associations between male characteristics and the immunomodulating

effect of seminal plasma. Furthermore, as a limitation it should be mentioned that this exploratory study used an *in vitro* model with peripheral immune cells. Several studies showed the existence of substantial T cell and monocyte populations in the human female reproductive tract of premenopausal women, which may come into direct contact with (soluble components of) seminal plasma after ejaculation(32-35). Modest numbers of CD4+ T cells have been shown to be present in the normal vaginal and ectocervical epithelium, while the lamina propria and the luminal and glandular epithelium of the endocervix contain higher numbers of CD4+ T cells.(35) It is possible that, *in vivo*, these cells are directly primed by seminal plasma to expand into Treg cells. However, we acknowledge that this study could not fully capture the complex interactions between all cells and signalling molecules present in the female reproductive tract. Although direct interactions between T cells and seminal plasma components seem possible *in vivo*, APCs are probably also a major contributor to seminal plasma mediated induction of T cells, and these were not included in the current model. Furthermore, it would be an interesting next step to investigate the effect of seminal plasma on female epithelial tract cells. Another point to consider is that gene expression of immune cells was investigated at a specific, short-term moment in time (after 24 hours of incubation with seminal plasma). However, we expected the first signs of cell activation to be visible by then, since a quick induction of tolerance towards the fetus at the implantation site is crucial.(12)

To conclude, our findings support the immunoregulatory potential of seminal plasma constituents and indicate that perturbations in seminal plasma priming may be involved in cases of unexplained RPL, advocating a male contribution to this condition. Our study serves as an important starting point for future studies to examine interactions between seminal plasma and the immune environment in the female reproductive tract in greater detail. Ultimately, defining the pathways and mechanisms underlying a state of active immune tolerance in pregnancy could lead to novel therapeutic strategies for couples with RPL.

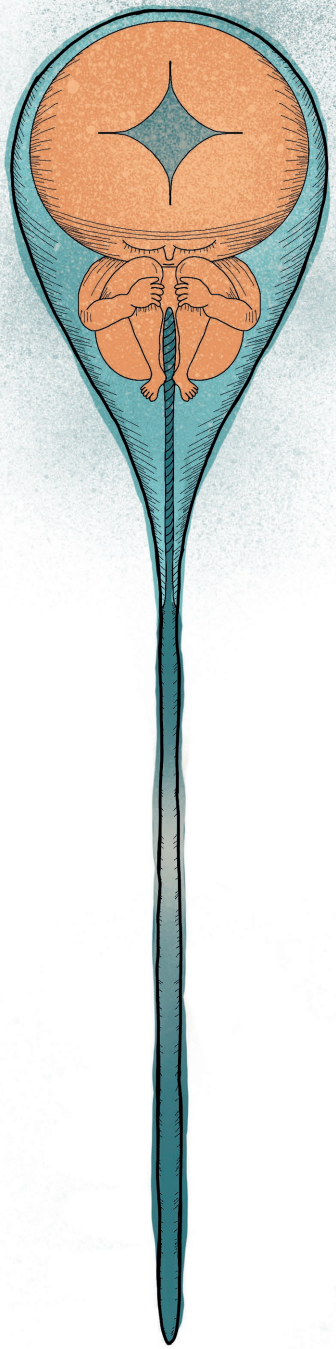
REFERENCES

1. 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).
2. Jaslow CR, Carney JL, Kutteh WH. Diagnostic factors identified in 1020 women with two versus three or more recurrent pregnancy losses. Fertil Steril. 2010;93(4):1234-43.
3. Craenmehr MH, Heidt S, Eikmans M, Claas FH. What is wrong with the regulatory T cells and foetomaternal tolerance in women with recurrent miscarriages? HLA. 2016;87(2):69-78.
4. Tsuda S, Nakashima A, Shima T, Saito S. New Paradigm in the Role of Regulatory T Cells During Pregnancy. Front Immunol. 2019;10:573-.
5. Robertson SA, Sharkey DJ. Seminal fluid and fertility in women. Fertil Steril. 2016;106(3):511-9.
6. 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.
7. 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.
8. Fraczek M, Kurpisz M. Cytokines in the male reproductive tract and their role in infertility disorders. J Reprod Immunol.. 2015;108:98-104.
9. Sharkey DJ, Tremellen KP, Briggs NE, Dekker GA, Robertson SA. Seminal plasma pro-inflammatory cytokines interferon-γ (IFNG) and C-X-C motif chemokine ligand 8 (CXCL8) fluctuate over time within men. Hum Reprod. 2017;32(7):1373-81.
10. Havrylyuk A, Chopyak V, Boyko Y, Kril I, Kurpisz M. Cytokines in the blood and semen of infertile patients. Cent Eur J Immunol. 2015;40(3):337-44.
11. Nederlof I, Meuleman T, van der Hoorn MLP, Claas FHJ, Eikmans M. The seed to success: The role of seminal plasma in pregnancy. J Reprod Immunol. 2017;123:24-8.
12. Schjenken JE, Robertson SA. The Female Response to Seminal Fluid. Physiol Rev. 2020;100(3):1077-117.
13. Robertson SA, Prins JR, Sharkey DJ, Moldenhauer LM. Seminal fluid and the generation of regulatory T cells for embryo implantation. Am J Reprod Immunol. 2013;69(4):315-30.
14. Robertson SA, Guerin LR, Bromfield JJ, Branson KM, Ahlström AC, Care AS. Seminal fluid drives expansion of the CD4+CD25+ T regulatory cell pool and induces tolerance to paternal alloantigens in mice. Biol Reprod. 2009;80(5):1036-45.
15. Meuleman T, Snaterse G, van Beelen E, Anholts JDH, Pilgram GSK, van der Westerlaken LAJ, et al. The immunomodulating effect of seminal plasma on T cells. J Reprod Immunol. 2015;110:109-16.
16. 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.
17. du Fossé NA, Lashley E, van Beelen E, Meuleman T, le Cessie S, van Lith JMM, et al. Identification of distinct seminal plasma cytokine profiles associated with male age and lifestyle characteristics in unexplained recurrent pregnancy loss. J Reprod Immunol. 2021;147:103349.
18. Wierenga APA, Gezgin G, van Beelen E, Eikmans M, Spruyt-Gerritse M, Brouwer NJ, et al. Soluble HLA in the Aqueous Humour of Uveal Melanoma Is Associated with Unfavourable Tumour Characteristics.

Cancers. 2019;11(8):1202.

19. Ward JH. Hierarchical Grouping to Optimize an Objective Function. J Am Stat Assoc. 1963;58(301):236-44.
20. Wahl SM, Swisher J, McCartney-Francis N, Chen W. TGF-β: the perpetrator of immune suppression by regulatory T cells and suicidal T cells. J Leukoc Biol. 2004;76(1):15-24.
21. 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.
22. Jin LP, Chen QY, Zhang T, Guo PF, Li DJ. The CD4+CD25 bright regulatory T cells and CTLA-4 expression in peripheral and decidual lymphocytes are down-regulated in human miscarriage. Clin Immunol. 2009;133(3):402-10.
23. 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.
24. 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.
25. Mahmoud FF, Abul HT, Haines DD, Omu AE, Diejomaoh M, Wise JA, et al. Butyrylcholinesterase activity and lymphocyte subpopulations in peripheral blood of Kuwaiti women experiencing recurrent spontaneous abortion. J Reprod Immunol. 2008;77(2):186-94.
26. 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.
27. Tang MX, Zhang YH, Hu L, Kwak-Kim J, Liao AH. CD14++ CD16+ HLA-DR+ Monocytes in Peripheral Blood are Quantitatively Correlated with the Severity of Pre-eclampsia. Am J Reprod Immunol. 2015;74(2):116-22.
28. Radysh TV, Chernyshov VP. [Immunopathophysiologic characteristics of early pregnancy in women with recurrent miscarriage]. Fiziol Zh. 2005;51(2):65-72.
29. 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.
30. Neefjes J, Jongsma MLM, Paul P, Bakke O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. Nat Rev Immunol. 2011;11(12):823-36.
31. Mansournia MA, Hernán MA, Greenland S. Matched designs and causal diagrams. Int J Epidemiol. 2013;42(3):860-9.
32. Lee SK, Kim CJ, Kim DJ, Kang JH. Immune cells in the female reproductive tract. Immune Netw. 2015;15(1):16-26.
33. Monin L, Whettlock EM, Male V. Immune responses in the human female reproductive tract. Immunology. 2020;160(2):106-15.
34. Sharkey DJ, Tremellen KP, Jasper MJ, Gemzell-Danielsson K, Robertson SA. Seminal fluid induces leukocyte recruitment and cytokine and chemokine mRNA expression in the human cervix after coitus. J Immunol. 2012;188(5):2445-54.

35. Pudney J, Quayle AJ, Anderson DJ. Immunological Microenvironments in the Human Vagina and Cervix: Mediators of Cellular Immunity Are Concentrated in the Cervical Transformation Zone1. Biol Reprod. 2005;73(6):1253-63.



CHAPTER 8

Exploring gender differences in supportive care preferences of couples with recurrent pregnancy loss

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ABSTRACT

Background

International guidelines recommend to offer supportive care during a next pregnancy to couples affected by recurrent pregnancy loss (RPL). In previous research, several options for supportive care have been identified and women's preferences have been quantified. Although it is known that RPL impacts the mental health of both partners, male preferences for supportive care have hardly been explored.

Methods

A cross-sectional study was conducted in couples who visited a specialized RPL clinic in the Netherlands between November 2018 and December 2019. Both members of the couples received a questionnaire that quantified their preferences for supportive care in a next pregnancy and they were asked to complete this independently from each other. Preferences for each supportive care option were analysed on a group level (by gender) and on a couple level, by comparing preferences of both partners.

Results

Ninety-two questionnaires (completed by 46 couples) were analysed. The overall need for supportive care indicated on a scale from 1-10 was 6.8 for men and 7.9 for women (P = 0.002). Both genders preferred to regularly see the same doctor with knowledge of their obstetric history, to make a plan for the first trimester and to have frequent ultrasound examinations. A lower proportion of men preferred a doctor that shows understanding (80% of men vs. 100% of women, P = 0.004) and a doctor that informs on wellbeing (72% vs. 100%, P = ≤0.000). Fewer men preferred support from friends (48% vs. 74%, P = 0.017). Thirty-seven percent of men requested more involvement of the male partner at the outpatient clinic, compared to 70% of women (P = 0.007). In 28% of couples, partners had opposing preferences regarding peer support.

Conclusions

While both women and men affected by RPL are in need of supportive care, their preferences may differ. Current supportive care services may not entirely address the needs of men. Health care professionals should focus on both partners and development of novel supportive care programs with specific attention for men should be considered.

BACKGROUND

Recurrent pregnancy loss (RPL) is a frustrating condition for both patients and care providers. This condition, defined as the loss of two or more pregnancies before the fetus reaches viability, is estimated to affect 1-3% of all couples of reproductive age.(1-3) Multiple risk factors have been identified, but despite extensive diagnostic investigations, RPL remains unexplained in the 60-70% of cases.(4) For these couples, there is currently no evidence-based medical treatment option. As pregnancy losses are generally experienced as significant negative life events, RPL may have serious psychological impact. A recent study reported that both women and men affected by RPL show high risks for developing depression and anxiety, while they often use different coping strategies.(5)

It is recommended by current international guidelines to offer supportive care programs for couples with RPL.(6) Some studies even suggested that supportive care during early pregnancy may have a beneficial effect on pregnancy outcome, although this evidence is limited.(7-10) Moreover, professional support and compassionate care are highly valued by couples with RPL.(11) Musters et al. elucidated what is actually perceived as supportive care for RPL and evaluated women's preferences for twenty supportive care options during a next pregnancy.(12, 13) They showed that women with RPL preferred to see the same doctor during their consultations who is specialized in RPL, takes them seriously, listens, shows understanding and enquires about emotional needs. The women wanted to make a plan with their doctor for the first trimester of a new pregnancy and they preferred frequent ultrasound examinations during this period. Furthermore, they indicated a need for psychological after-care in case of a new miscarriage. Notably, male partners' preferences and their need for supportive care were not addressed in this study.

As shown by a systematic review(14) that evaluated 27 studies on patient-centred early pregnancy care, male partners were not involved in most prior studies in this research field. The male perspective was examined in only three of the included studies and the authors considered involvement of the partner as an improvement target. Identifying male preferences for supportive care in RPL is relevant, not only because it has been shown that men do also suffer from RPL, but also because tailored supportive care programs may assist the male partner during a new pregnancy. The significance of this has been underscored by several studies showing that the male role in pregnancy is of great impact on maternal health behaviour and pregnancy outcome.(15-17)

The aim of the current study was to quantify preferences for supportive care of both men and women affected by RPL. Previously identified supportive care options for

RPL(12, 13) were used as a framework for this study and both members of participating couples were independently questioned, allowing us to compare preferences between genders but also to analyse potential discrepant preferences within couples.

METHODS

Participants

The study was conducted in couples that visited the specialized RPL outpatient clinic of the Leiden University Medical Center in the Netherlands between November 2018 and December 2019. Participating couples had at least two pregnancy losses (following the definition of the ESHRE guideline for RPL(1)) and had to be fluent in Dutch or English. The study protocol was approved by the Medical Research Ethics Committee of the Leiden University Medical Center (reference number N19.101). All participants provided written consent to take part in the study.

Procedures at the RPL outpatient clinic

When couples visit the RPL clinic for the first time, they have an intake consultation with a gynaecologist or fertility doctor. The team comprises four physicians, all specialized in RPL. All physicians adhere to the same protocol and provide similar care. New patients are discussed in the team after their first consultation. Besides obtainment of detailed obstetric history and extensive history of both partners, couples receive information about known risk factors for RPL, advices on lifestyle changes, options for diagnostic testing, potential therapeutic options, chances for future pregnancy outcome and ongoing studies.

Besides the medical approach, attention is paid to the psychological impact of RPL and consultation with a medical social worker is offered. A referral can be made immediately, or the couple can make an appointment at a later time if desired (it is estimated that 10% of all couples opt for a consultation with the medical social worker). In case of a next pregnancy, couples are offered monitoring at the RPL outpatient clinic in the first 12 weeks of the pregnancy. Ultrasound examination in the first trimester is offered, the frequency depending on the couple's preference. In addition, it is emphasized that the affiliated obstetric clinic of the Leiden University Medical Center is available 'twenty-four seven' and can be reached in case of any symptoms or distress. In case of an ongoing pregnancy beyond 12 weeks, the couple will be referred for further regular monitoring of the pregnancy to either an obstetrical outpatient clinic or a midwifery practice (depending on medical indication and individual situation). In case of another pregnancy loss, the doctor will re-evaluate their individual plan at the follow-up consult at the RPL outpatient clinic.

Data collection

After the couples had attended the intake consultation, they received the questionnaires, which were completed at home. The questionnaires were returned by post or during a next consultation. The questionnaire consisted of two parts: general demographic

questions and preferences for supportive care. The second part of the questionnaire was based on supportive care options in three domains as identified by Musters et al.(12, 13): 1: Medical supportive care (for example: ultrasound examination during early pregnancy, medical information and advices); 2: Soft-skills (for example: communication skills of the doctor) and 3: Other types of supportive care (for example: support from friends, family and peers, relaxation exercises, alternative therapies).

Two versions of the questionnaire were used, intended for either women or men. Given the purpose of the study, the couples were asked to complete the questionnaires independently, without discussion between both partners. The questionnaires were available in Dutch and English language (the English version is included as Supplementary material). Preferences and need for supportive care were quantified using 5-point Likert scale items ranging from total disagreement to total agreement and a rating scale question (grade 1-10). The estimated completion time for the questionnaire was maximum 15 minutes. The questionnaires were developed and pilot tested by two gynaecologists (specialized in RPL), two fertility doctors (specialized in RPL), a psychologist, a PhD candidate (specialized in RPL) and two patients with RPL. No major adjustments were made after pilot testing.

Statistical analyses

Descriptive data are presented in numbers and percentages. The 5-point Likert scale items for supportive care options were recoded: 1 and 2 represent the non-preference group, 3 the neutral group, and 4 and 5 the preference group (similar to Musters et al.(13)). Scale reliability was assessed with Cronbach's alpha. To prevent multiple hypothesis testing, statistical tests were not executed for the complete panel of supportive care options but restricted to predefined selected entities: whenever a supportive care option was preferred by either ≥60% of women, ≥60% of men, or both, this option was considered as potentially relevant for clinical practice and thus examined in further detail. This was done by comparing the preference rates for these selected supportive care options between women and men. To account for the statistical dependence of data derived from two partners of a couple, McNemar tests for paired data were used. The mean overall need for supportive care expressed on a scale from 1-10 is presented with standard deviation (SD) and compared between women and men with a paired samples T-test. Two-sided P-values <0.05 were considered statistically significant. Intra-couple discrepancy was defined as one of the two partners having no need (1 or 2) for a certain supportive care option and the other partner having a preference (4 or 5) for this supportive care option. The level of intra-couple discrepancy for each supportive care option was calculated as the percentage of all couples that met this definition. Analyses were performed in R studio version 1.3.9.50 (R Foundation for Statistical Computing, Vienna, Austria).

Sample size calculation

On the basis of the null hypothesis that an equal percentage of women and men would prefer a supportive care option, a sample size of 44 couples would be required for an 80% power at a two-sided alpha of 0.05 to detect a difference in preference rate of 30% between women and men, which we considered as a clinically relevant difference. The sample size was calculated with R studio package 'SampleSizeMcNemar'.

RESULTS

Between November 2018 and December 2019, 50 women and 46 men completed the questionnaire. Four questionnaires were excluded from the analyses as only the female partner returned the questionnaire. All couples were heterosexual. The majority of women and men (85% both) were born in the Netherlands. The median number of pregnancy losses at the time the RPL outpatient clinic was visited for the first time was 2 (range 2-6). No underlying condition for RPL was found in 70% of the couples. More baseline characteristics of the couples are shown in Table 1.

Table 1. Baseline characteristics of couples with RPL

Baseline characteristics of couples with RPL *n* = 46		
Referral by n (%)		
Physician of same hospital		18 (39)
General practitioner		10 (22)
Midwife		5 (11)
Secondary hospital		13 (28)
Reproductive information		
Number of pregnancy losses (median)		2 (range 2- 6)
Couples with child together n (%)		21 (46)
Fertility treatment n (%)		
IVF		2 (4)
IUI only		4 (9)
None		40 (87)
Pregnant during intake consultation n (%)		5 (11)
RPL diagnosis n (%)		
Unexplained		32 (70)
Thyroid autoimmunity		6 (13)
Uterine anomaly		4 (9)
Unknown (no diagnostic work-up)		2 (4)
Antiphospholipid syndrome		1 (2)
Parental chromosomal translocation		1 (2)
	Women *n* = 46	**Men** *n* = 46
Age (mean, (SD))	34 (4.40)	37 (5.58)
Education level		
Low [a]	1 (2)	3 (7)
Moderate [b]	13 (28)	14 (30)
High [c]	32 (70)	29 (63)

[a] Primary school/intermediate vocational education

[b] Higher general secondary education/pre-university secondary education

[c] Higher vocational education/university

IVF = in vitro fertilization; IUI = intrauterine insemination; RPL = recurrent pregnancy loss

Preferences for supportive care in a next pregnancy

The mean need for supportive care expressed on a scale from 1-10 was 6.8 (SD 1.68) for men and 7.9 (SD 1.65) for women (*P* = 0.002). Overall, Cronbach's alpha was 0.82 (0.80 for the subgroup of women and 0.82 for the subgroup of men), indicating good reliability of the Likert scales. Seventeen options for supportive care in a next pregnancy were preferred by either the majority (≥60%) of women and/or men. Preference rates and levels of intra-couple discrepancy for these specific options are shown in Figure 1, including *P*-values for the differences in preference rates between women and men. In Supplementary Table 1, also the percentages of women and men that scored neutral for these options are shown. An overview of the other supportive care options, being preferred by <60% of women and men, is shown in Figure 2.

Domain 1: Medical supportive care

The majority of both women and men preferred making a plan for the first trimester, seeing the same doctor during different consultations who has knowledge of their obstetric history, an ultrasound examination directly after a positive test, once a week during the first trimester and during symptoms and medication for RPL that is proven safe for pregnancy. Medication that is not proven safe during pregnancy (i.e. experimental medication for RPL without fully known effects and safety) was preferred by 33% of women and 24% of men. Information derived from a doctor was preferred over information derived from the internet or information derived from peers. On group level, there were no significant differences between genders for all of the above options. The levels of intra-couple discrepancy were highest for the options information from peers (26%), information from the internet (24%) and advice regarding lifestyle (22%).

Domain 2: Soft skills

The majority of men and women preferred a doctor that takes the patient seriously, listens, informs on emotional needs, shows understanding and informs on wellbeing (i.e. asks how things are going). For the last two options the preference rates significantly differed between women and men. Showing understanding was preferred by 100% of women vs. 80% of men (*P* = 0.004). Informing on wellbeing was preferred by 100% of women vs. 72% of men (*P* = ≤0.000). Couples had most discrepant preferences towards counselling from a specialized nurse (level of intra-couple discrepancy 17%; preferred by 52% of both women and men) and counselling from a psychologist (level of intra-couple discrepancy 17%; preferred by 24% of women and 13% of men).

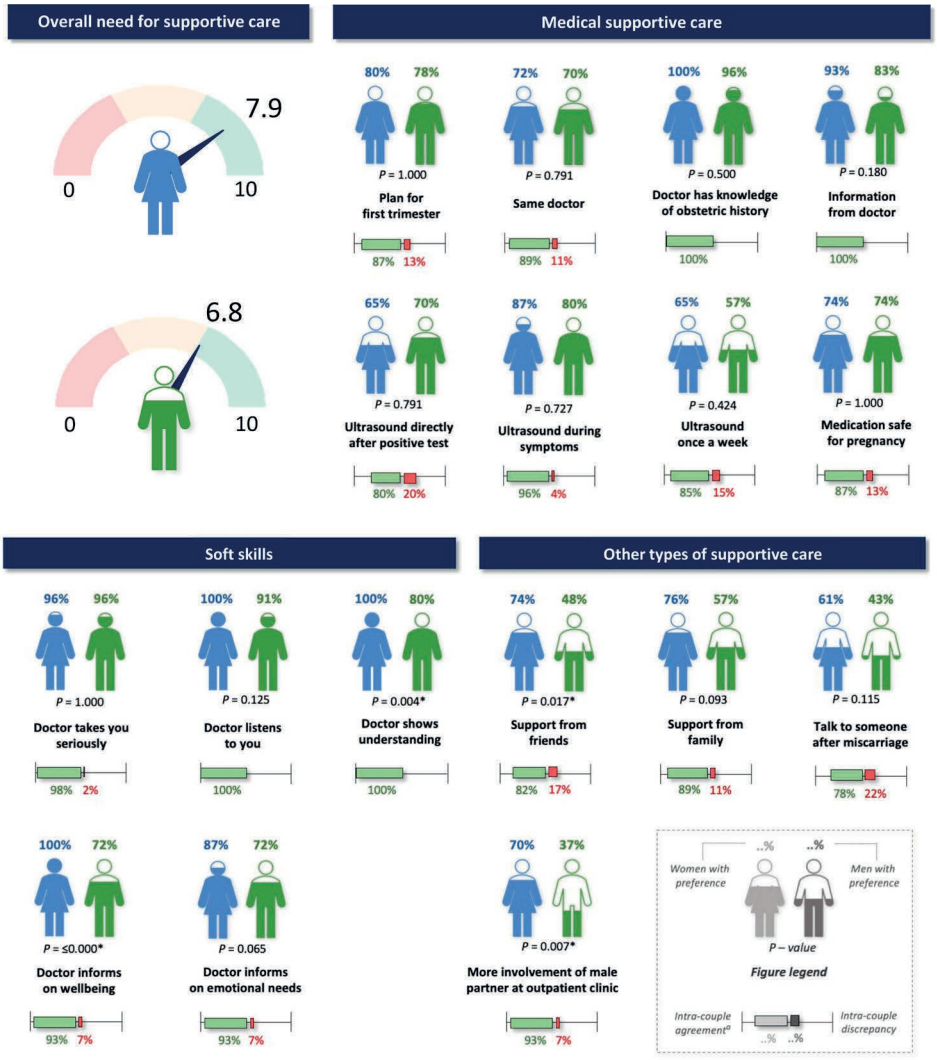


Figure 1. Overall need for supportive care of women and men affected by RPL and options for supportive care in a next pregnancy preferred by the majority (≥60%) of women and/or men

Overall need for supportive care was measured on a scale from 1-10, mean values for both genders are shown. For each supportive care option, preference rates for women and men with *P*-values and levels of intra-couple discrepancy (as defined in the Statistical analysis section) are shown. Further explanation is shown in grey text in the bottom right corner.
[a] Intra-couple agreement: both partners indicated a preference or a non-preference, or one partner responded neutral
Asterisks (*) indicate *P*-values <0.05

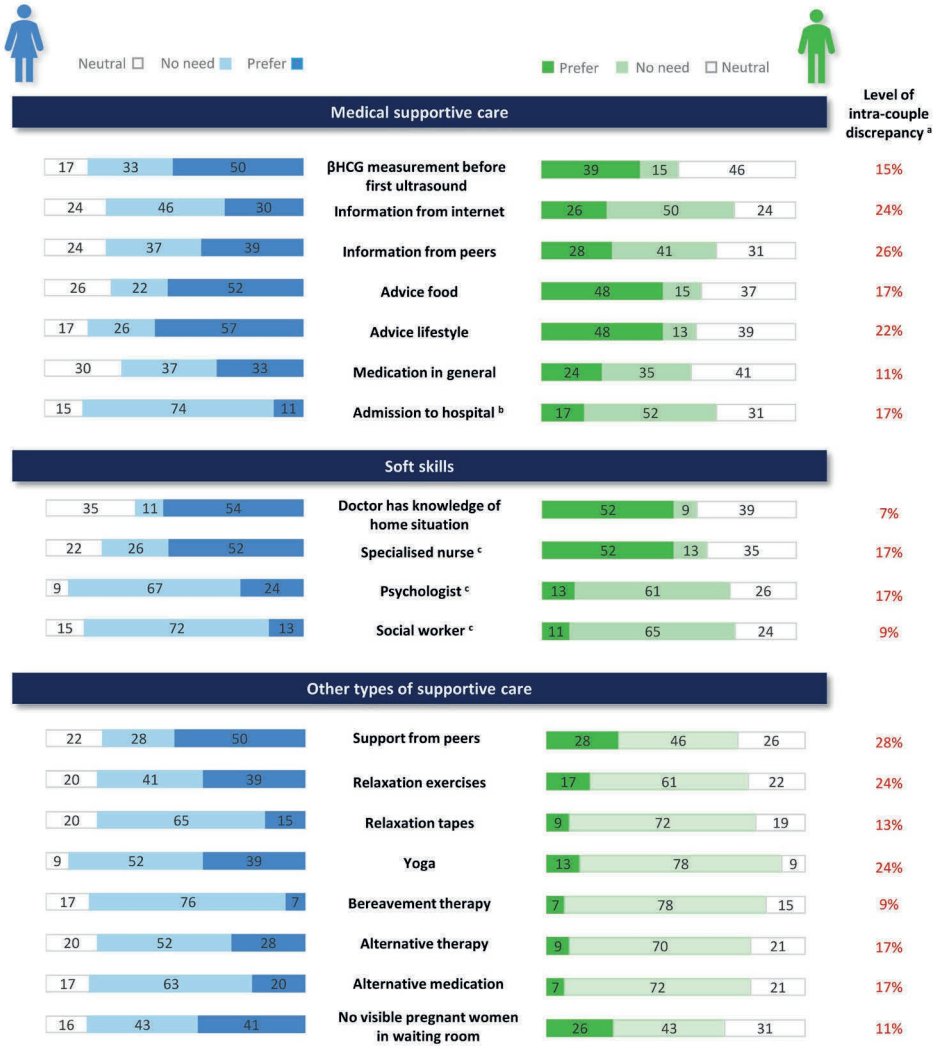


Figure 2. Options for supportive care in a next pregnancy preferred by <60% of women and men affected by RPL

[a] Level of intra-couple discrepancy: % of couples with opposing opinions (i.e. one partner indicated a preference and the other partner indicated no need), as described in the Statistical analysis

[b] Admission to hospital at same gestational age as earlier miscarriages occurred

[c] Counselling from mentioned specialist

Domain 3: Other types of supportive care

Options being preferred by the majority of women were: support from friends, support from family, more involvement of the male partner at the outpatient clinic (i.e. the doctor actively involves the male partner during consultations and in supportive care) and to talk to someone after a new miscarriage. The proportion of men that expressed a need for support from friends was significantly lower (48% vs. 74%, $P = 0.017$). None of the options in this domain were requested by ≥60% of men. More involvement of the male partner at the outpatient clinic was preferred by 70% of the women, compared to 37% of the men ($P = 0.007$). Sixty-one percent of women would like to talk to someone after experiencing another miscarriage, compared to 43% of men. The highest levels of intra-couple discrepancy were observed for need for support from peers (28%), followed by relaxation exercises (24%), yoga (24%) and talking to someone after a new miscarriage (22%).

Overall, the options for supportive care that were rejected by the majority of both women and men were bereavement therapy, listening to relaxation tapes, counselling from a social worker, counselling from a psychologist, alternative medication and hospital admission at the same gestational age as earlier miscarriages occurred. Alternative therapy (such as acupuncture or reflexology), relation exercises and yoga were not considered necessary by the majority of men. Mean levels of intra-couple discrepancy were 14% for Domain 1 (Medical supportive care), 9% for Domain 2 (Soft skills) and 17% for Domain 3 (Other types of supportive care).

DISCUSSION

This is the first study that quantified preferences for supportive care of both men and women affected by RPL and explored the existence of different needs within couples. Overall, men expressed a significantly lower need for supportive care compared to women. Regarding medical supportive care, preferences of both genders were largely similar and in line with the previous study in women by Musters et al.(13). For the other domains of supportive care, several between-gender differences were observed.

Although the majority of both men and women preferred a doctor that takes the patient seriously, listens, informs on emotional needs, informs on wellbeing and shows understanding, a significantly smaller proportion of men appreciated the last two options (differences of 28% and 22% compared to women, respectively). In addition, the majority of women expressed a need for support from family, friends and peers; men preferred this less. This is in accordance with previous research showing that men are typically more hesitant to disclose their feelings after pregnancy loss.(5, 18) Although men do experience feelings of grief, stress and vulnerability, these emotions may be less manifested.(19, 20) Men are thought to employ different coping strategies compared to women, including 'active avoidance' and distractive behaviour, related to more frequently observed risk behaviours such as excessive alcohol consumption and smoking.(5, 18) Multiple studies showed that a significant part of men affected by pregnancy loss experienced little support from their social network and a reluctance to share their loss and feelings with them; their family and friends tend to direct their acknowledgement and support largely toward the female partner.(5, 21, 22)

Also in hospital settings where support activities are profoundly targeted on or delivered by women, men have indicated that they feel excluded or marginalized from care compared to their partner.(23) In our study, remarkable gender differences were observed regarding the overall need for supportive care (mean grade 6.8 in men vs. 7.9 in women) and the need for more involvement of the male partner at the RPL outpatient clinic (desired by 37% of men and 70% of women). This seems in contrast with other studies indicating that male partners of RPL couples want to be more included.(11, 14) Multiple explanations may be underlying here. In some men's responses, a social desirability bias may be present. Various studies on experiences following pregnancy loss showed that it is not uncommon for men to view their role as primarily being a 'supporter' to their female partner, leading to a barrier to seek support for themselves. (18, 24-26) Another possibility is that the approach at the clinic and the supportive care as it is currently being offered, do not completely meet the needs of men.

Furthermore, our results suggest that it is important to offer supportive care services

to both partners individually. Although men and women may show similar preferences on group level, this does not automatically imply a high level of intra-couple agreement. For instance, while an equal percentage of the total groups of women and men (52%) preferred counselling from a specialized nurse during a next pregnancy, in almost one in five couples the partners had opposing opinions regarding this aspect (level of intra-couple discrepancy 17%). Moreover, in 28% of couples, one partner expressed a need for peer-support, while the other partner did not consider this necessary.

Previous research showed that patients with RPL want medical professionals to be aware of the psychological impact of RPL and believe they would benefit from psychological care.(Koert et al. 2018, van den Berg et al. 2018) However, in the current study, the majority of both female and male participants rejected the options of being counselled by a psychologist or a social worker. Possibly, RPL patients consider it important that there is recognition of the psychological aspect of their losses by their healthcare providers, but they are not inclined to seek specialised psychological care. This may have to do with unfamiliarity with these types of care or perceived stigma and barriers to seek care from a mental health professional. Notably, preference rates for counselling from a specialised nurse were considerably higher.

The major strength of this study is that it is the first that quantified the need for different aspects of supportive care of both men and women affected by RPL. In a recent exploratory study in 13 couples with RPL, both members of the couples were interviewed simultaneously on their need for treatment, support and follow-up.(11) This likely resulted in each partner influencing the other's perspectives, which was also recognized as a limitation by the authors themselves. In our study, the questionnaires returned by both members of each couple were carefully compared and no obvious overlap in their responses was present. This makes it credible that the questionnaires were completed independently of one another (as requested), although we cannot entirely rule out the possibility of some couples having discussed their responses. Moreover, it should be mentioned that responses of two partners will never be entirely independent, as they form a couple and they share the same experience. The study has several limitations. First, it is a single centre study and although the sample is representative for our RPL clinic, differences with RPL couples elsewhere may exist, for instance in terms of education level, being relatively high in our population. Likewise, services being offered in our RPL clinic may differ from other settings. Furthermore, the panel of supportive care options evaluated in this study was based on previous research restricted to women. It may be that some men desire other possibilities for supportive care, not being covered in this study.

It should be considered to develop supportive care programs for RPL specifically aimed at men, as supportive care in its current form may not entirely suit their needs. In a previous qualitative study, men affected by (single) pregnancy loss expressed a desire for an informal discussion with another man with the same experience. In a hospital setting, they suggested the option of a male support worker. Such possibilities may be further explored for men affected by RPL, for instance using focus group discussions, as mentioned in the study protocol of the currently ongoing study of Williams et al.(27).

Conclusions

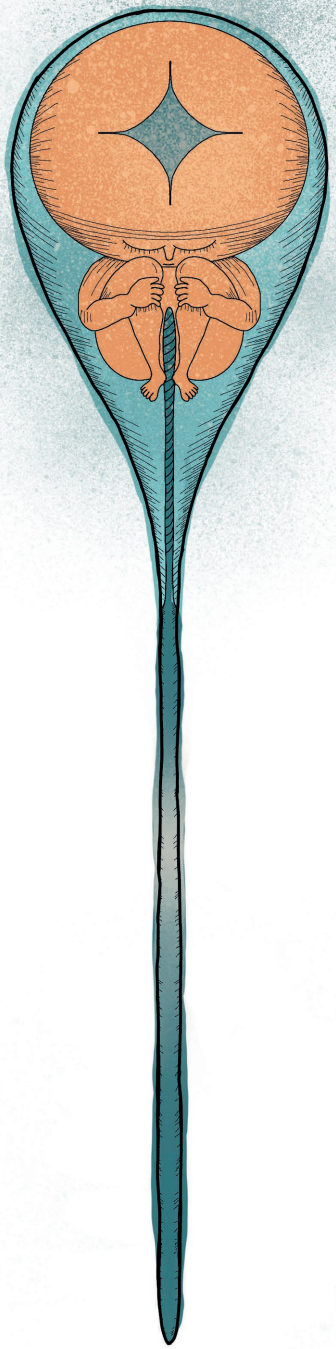
Our study shows the existence of different preferences for supportive care of men and women affected by RPL. It is important that health care providers are aware of this and take a tailored approach. We recommend to actively involve both partners, ask them about their personal preferences and discuss the most suitable approach that best fits the needs of both partners. It can be emphasized that some supportive care services may be chosen by one of the partners only. In addition, development of male-oriented supportive care programs should be explored.

REFERENCES

1. 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).
2. Rai R, Regan L. Recurrent miscarriage. Lancet. 2006;368(9535):601-11.
3. Evaluation and treatment of recurrent pregnancy loss: a committee opinion. Fertil Steril. 2012;98(5):1103-11.
4. Jaslow CR, Carney JL, Kutteh WH. Diagnostic factors identified in 1020 women with two versus three or more recurrent pregnancy losses. Fertil Steril. 2010;93(4):1234-43.
5. 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.
6. Youssef A, Vermeulen N, Lashley EELO, Goddijn M, van der Hoorn MLP. Comparison and appraisal of (inter)national recurrent pregnancy loss guidelines. Reprod Biomed Online 2019;39(3):497-503.
7. Clifford K, Rai R, Regan L. Future pregnancy outcome in unexplained recurrent first trimester miscarriage. Hum Reprod. 1997;12(2):387-9.
8. Whitley KA, Ural SH. Treatment Modalities in Recurrent Miscarriages without Diagnosis. Semin Reprod Med. 2014;32(04):319-22.
9. Habayeb OMH, Konje JC. The one-stop recurrent miscarriage clinic: an evaluation of its effectiveness and outcome. Hum Reprod. 2004;19(12):2952-8.
10. Liddell HS, Pattison NS, Zanderigo A. Recurrent miscarriage--outcome after supportive care in early pregnancy. Aust N Z J Obstet Gynaecol. 1991;31(4):320-2.
11. Koert E, Malling GMH, Sylvest R, Krog MC, Kolte AM, Schmidt L, et al. Recurrent pregnancy loss: couples' perspectives on their need for treatment, support and follow up. Hum Reprod. 2018;34(2):291-6.
12. 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.
13. 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.
14. van den Berg MMJ, Dancet EAF, Erlikh T, van der Veen F, Goddijn M, Hajenius PJ. Patient-centered early pregnancy care: a systematic review of quantitative and qualitative studies on the perspectives of women and their partners. Hum Reprod Update. 2018;24(1):106-18.
15. Alio AP, Lewis CA, Scarborough K, Harris K, Fiscella K. A community perspective on the role of fathers during pregnancy: a qualitative study. BMC Pregnancy and Childbirth. 2013;13(1):60.
16. Kashanian M, Faghankhani M, Hadizadeh H, Salehi MM, Roshan MY, Pour ME, et al. Psychosocial and biological paternal role in pregnancy outcomes. J Matern Fetal Neonatal Med. 2020;33(2):243-52.
17. Cheng ER, Rifas-Shiman SL, Perkins ME, Rich-Edwards JW, Gillman MW, Wright R, et al. The Influence of Antenatal Partner Support on Pregnancy Outcomes. J Womens Health. 2016;25(7):672-9.
18. 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-.
19. Kong GW, Chung TK, Lai BP, Lok IH. Gender comparison of psychological reaction after miscarriage-a

1-year longitudinal study. BJOG. 2010;117(10):1211-9.

20. Kagami M, Maruyama T, Koizumi T, Miyazaki K, Nishikawa-Uchida S, Oda H, et al. Psychological adjustment and psychosocial stress among Japanese couples with a history of recurrent pregnancy loss. Hum Reprod. 2012;27(3):787-94.
21. 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.
22. Obst KL, Oxlad M, Due C, Middleton P. Factors contributing to men's grief following pregnancy loss and neonatal death: further development of an emerging model in an Australian sample. BMC Pregnancy and Childbirth. 2021;21(1):29.
23. Miller EJ, Temple-Smith MJ, Bilardi JE. 'There was just no-one there to acknowledge that it happened to me as well': A qualitative study of male partner's experience of miscarriage. PLoS One. 2019;14(5):e0217395-e.
24. Obst KL, Due C. Australian men's experiences of support following pregnancy loss: A qualitative study. Midwifery. 2019;70:1-6.
25. O'Leary J, Thorwick C. Fathers' perspectives during pregnancy, postperinatal loss. J Obstet Gynecol Neonatal Nurs. 2006;35(1):78-86.
26. McCreight BS. A grief ignored: narratives of pregnancy loss from a male perspective. Sociol Health Illn. 2004;26(3):326-50.
27. Williams HM, Jones LL, Coomarasamy A, Topping AE. Men living through multiple miscarriages: protocol for a qualitative exploration of experiences and support requirements. BMJ Open. 2020;10(5):e035967.



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.

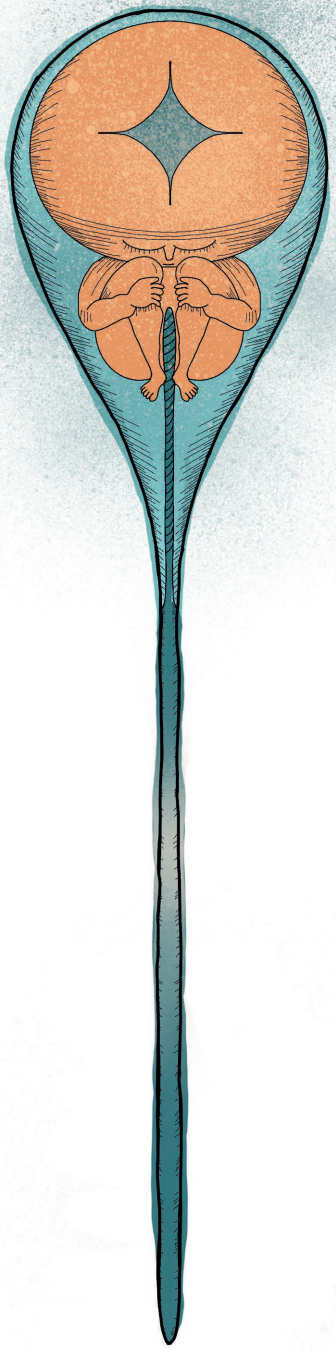
REFERENCES

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.

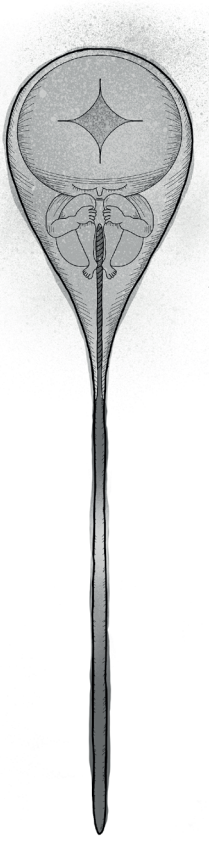
36. Perdichizzi A, Nicoletti F, La Vignera S, Barone N, D'Agata R, Vicari E, et al. Effects of Tumour Necrosis Factor-α on Human Sperm Motility and Apoptosis. J Clin Immunol. 2007;27(2):152-62.
37. Rajasekaran M, Hellstrom WJ, Naz RK, Sikka SC. Oxidative stress and interleukins in seminal plasma during 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.



APPENDICES &

Nederlandse samenvatting
List of co-authors and their affiliations
List of publications
Curriculum Vitae
Dankwoord

NEDERLANDSE SAMENVATTING



De vergeten vader bij herhaalde miskramen

Herhaalde miskramen wordt gedefinieerd als het spontane verlies van tenminste twee zwangerschappen, optredend in de periode tussen de conceptie en het moment dat de foetus de termijn van levensvatbaarheid bereikt bij 24 weken amenorroeduur. Er zijn een aantal maternale aandoeningen geassocieerd met herhaalde miskramen, waaronder het antifosfolipidensyndroom, uterusanomalieën en aanwezigheid van anti-TPO-antilichamen gericht tegen de schildklier. De internationale klinische richtlijn van de Europese Vereniging voor Humane Reproductie en Embryologie (ESHRE) adviseert om diagnostiek te verrichten naar deze aandoeningen bij vrouwen met herhaalde miskramen. Het enige onderzoek dat bij de man (en tevens bij de vrouw) wordt ingezet, is een karyogram om te screenen voor gebalanceerde chromosomale translocaties, die eveneens een risicofactor voor herhaalde miskramen vormen. Naast de reeds genoemde maternale aandoeningen bestaan er een aantal andere maternale karakteristieken die het risico op (herhaalde) miskramen verhogen, waaronder een leeftijd boven de 35 jaar, obesitas of ernstig ondergewicht, roken en alcoholgebruik.

Ondanks het verrichten van uitgebreide diagnostiek, wordt bij minder dan 50% van de koppels met herhaalde miskramen een onderliggende aandoening gevonden. Voor koppels met onverklaarde herhaalde miskramen bestaan op dit moment geen bewezen effectieve therapieën. Dit draagt bij aan het verdriet en de frustratie die gepaard gaan met onverklaarde herhaalde miskramen. Koppels dragen de last van aanhoudende onzekerheid, terwijl clinici geen wetenschappelijk onderbouwde behandeling kunnen bieden. De psychische lijdensdruk ten gevolge van herhaalde miskramen is hoog. Uit eerder onderzoek blijkt dat zowel vrouwen als mannen een hoger risico lopen op het ontwikkelen van depressie en angststoornissen na het meemaken van herhaalde miskramen.

Het wetenschappelijk onderzoek naar herhaalde miskramen is van oudsher gericht op vrouwelijke factoren. Willen we meer inzicht verkrijgen in de pathofysiologie van herhaalde miskramen en de best mogelijke zorg leveren aan getroffen koppels, dan moeten we de vader niet vergeten. Lange tijd werd gedacht dat de totstandkoming van een zwangerschap het bewijs vormde voor normaal functionerende mannelijke geslachtscellen. Eventuele complicaties, waaronder een miskraam, werden zonder meer toegeschreven aan vrouwelijke afwijkingen. Aangezien de man echter de helft van het genetisch materiaal van het embryo aanlevert, lijkt het plausibel dat zijn invloed verder reikt dan alleen de conceptie. Een grondige evaluatie van paternale factoren vormt daarom een uitstekende kans om kennis over het ontstaan van herhaalde miskramen te vergroten.

Biologische achtergrond: het semen

Om meer te weten te komen over rol van de man ten aanzien van herhaalde miskramen, is het essentieel om in te zoomen op de substantie die daadwerkelijk de mannelijke bijdrage vormt: het semen. Het semen bestaat uit de spermatozoa (de spermacellen) en het seminaal plasma (de acellulaire vloeistoffractie). Volwassen spermatozoa zijn in hoog-gedifferentieerde cellen bestaande uit een staart, middenstuk en een kop. De kop bevat het paternale genetisch materiaal, dat ligt opgeslagen in DNA-moleculen. De voornaamste functie van spermatozoa is het transporteren van het haploïde paternale genoom naar het vrouwelijke voortplantingsstelsel. Het paternale DNA is verpakt in proteïnen; het complex van DNA en proteïnen wordt chromatine genoemd. Goede bescherming van het sperma-DNA is cruciaal omdat de DNA-reparatie capaciteit van spermatozoa beperkt is in vergelijking met andere lichaamscellen.

Het seminaal plasma is een combinatie van secreties geproduceerd door de mannelijke accessoire geslachtsklieren. Naast dat het seminaal plasma dient als een beschermend en voedend medium voor de spermatozoa, bevat het ook een verscheidenheid aan bioactieve signaalmoleculen: cytokines, chemokines, prostaglandines en andere immunologische factoren. Er wordt onderscheid gemaakt tussen pro-inflammatoire, immuunregulatoire en groeifactoren. Eerder onderzoek heeft aangetoond dat deze bestanddelen van het seminaal plasma na ejaculatie kunnen interacteren met het maternale immuunsysteem.

Conventionele semenanalyse zoals uitgevoerd volgens de WHO richtlijnen is gericht op het volume van het ejaculaat en de concentratie en motiliteit van de spermatozoa. Er bestaat echter geen duidelijke associatie tussen deze semenparameters en herhaalde miskramen. Daarom hebben recente studies binnen het veld van herhaalde miskramen zich gericht op andere semenfactoren; voornamelijk genetische defecten. Er zijn aanwijzingen dat sperma DNA schade een belangrijke rol speelt bij herhaalde miskramen. Twee biologische theorieën met betrekking tot een potentiële mannelijke bijdrage aan herhaalde miskramen dienen als basis voor dit proefschrift. De eerste theorie is gefocust op sperma DNA fragmentatie en de tweede hypothese betreft verstoorde maternale immuunregulatie als gevolg van een disbalans in de samenstelling van het seminaal plasma.

Verhoogde levels van sperma DNA fragmentatie

Sperma DNA schade kan veroorzaakt worden via meerdere mechanismen, tijdens verschillende stadia van productie en transport van de spermatozoa. Een mechanisme dat vermoedelijk een grote rol speelt is het optreden van oxidatieve stress ten gevolge van een overmaat aan reactive oxygen species (ROS). Een overmaat aan ROS kan leiden tot breuken in de DNA-strengen van het sperma: DNA fragmentatie. Verschillende

factoren kunnen leiden tot (over)productie van ROS, waaronder veroudering, obesitas, roken, excessief alcoholgebruik, de aanwezigheid van varicocele en verschillende milieuverontreinigende stoffen. Spermatozoa met bovenmatige oxidatieve DNA schade zijn soms wel in staat tot bevruchting, maar kunnen mogelijk tot een miskraam leiden in het blastocyst stadium of later in het vroeg-foetale stadium. Twee systematische reviews en meta-analyses lieten een significante associatie zien tussen verhoogde waarden van sperma DNA fragmentatie en herhaalde miskramen.

Verstoring van maternale immuunregulatie ten gevolge van afwijkingen in seminaal plasma

Een voorwaarde voor een succesvolle zwangerschap is dat het maternale immuunsysteem een embryo tolereert dat voor de helft lichaamsvreemd is. Ondanks vele studies die zich hierop gericht hebben, blijft het voor een groot deel onduidelijk hoe het half-lichaamsvreemde embryo (en later de foetus) ontsnapt aan 'afstoting' door het maternale immuunsysteem. Meerdere dierstudies en humane studies hebben gesuggereerd dat seminaal plasma in staat is om het maternale immuunsysteem te moduleren en bijdraagt aan de inductie van een suppressief milieu dat gunstig is voor het tot stand komen en behouden van zwangerschap. De hypothese is dat paternale antigenen die aanwezig zijn in het seminaal plasma resulteren in activatie en expansie van suppressieve regulatoire T-cellen. Er dient een balans te zijn tussen pro-inflammatoire en immuunregulatoire signaalmoleculen in het seminaal plasma. Van pro-inflammatoire factoren wordt gedacht dat ze leiden tot initiële inflammatoire effecten zoals het rekruteren van lymfocyten en antigeen-presenterende cellen, die noodzakelijk zijn om een immuunrespons te ontwikkelen ten aanzien van paternale antigenen in het seminaal plasma. Daarentegen wordt een seminaal plasma profiel met een overmaat aan pro-inflammatoire markers juist geassocieerd met infertiliteit en zwangerschapscomplicaties. Een belangrijke regulatoire factor in het seminaal plasma lijkt TGF-β te zijn. TGF-β heeft een krachtig effect op proliferatie en differentiatie van verschillende immuuncellen en samen met andere tolerantie-inducerende moleculen in het seminaal plasma, zoals PGE2, sHLA-G en IL-10, wordt het beschouwd als essentieel voor het bewerkstelligen van een maternale immuunomgeving die bevorderlijk is voor zwangerschap. Er is nog weinig bekend over de relatie tussen seminaal plasma expressieprofielen en herhaalde miskramen. Ook moet nog onderzocht worden of de samenstelling van het seminaal plasma gerelateerd is aan leefstijl- en omgevingsfactoren.

Dit proefschrift

In **hoofdstuk 2** laten we de resultaten van een systematische review en meta-analyse zien bestaande uit epidemiologische studies die de associatie tussen paternale leeftijd en het risico op een miskraam onderzochten. Op basis van gepoolde data van tien beschikbare studies werd een significant verhoogd risico op een miskraam gevonden

vanaf een paternale leeftijd ≥40 jaar. Het risico was het hoogst in de leeftijdscategorie ≥45 jaar.

Hoofdstuk 3 bevat een overzicht van beschikbare literatuur over de relatie tussen paternale leefstijlfactoren in de preconceptieperiode en het risico op een miskraam. We richtten ons op paternaal roken, alcoholconsumptie en BMI. Een meta-analyse van acht studies liet zien dat het risico op een miskraam significant verhoogd is als een man ≥10 sigaretten per dag rookt in de preconceptieperiode. Het risico was het hoogst bij het van roken ≥20 sigaretten per dag. Het was niet mogelijk om een conclusie te trekken wat betreft het risico geassocieerd met paternaal alcoholgebruik, omdat weinig studies beschikbaar waren die tevens zeer heterogeen waren en een meta-analyse niet kon worden uitgevoerd. Er werd geen enkele studie gevonden die de relatie tussen paternaal BMI en het risico op een miskraam onderzocht.

Alle studies die geïncludeerd werden in de twee systematische reviews werden kritisch beoordeeld op het risico op bias. Voor de associatie tussen paternale factoren en het risico op een miskraam vormen maternale factoren een belangrijke bron van potentiële confounding. Als hier niet adequaat voor gecorrigeerd wordt, kan dit leiden tot incorrecte interpretatie van paternale effecten. Om deze reden werden in de meta-analyses alleen studies geïncludeerd die tenminste corrigeerden voor maternale leeftijd (in **hoofdstuk 2**) en maternaal rookgedrag (in **hoofdstuk 3**). Andere vormen van bias die een rol speelden in de geïncludeerde studies waren informatiebias en selectiebias. Onze kritische beoordeling van methodologische aspecten liet zien dat verschillende studiedesigns elk hun eigen voor- en nadelen hebben met betrekking tot het risico op bias. Al met al waren de studies van goede kwaliteit en lieten ze een duidelijke associatie zien tussen het risico op een miskraam en respectievelijk paternale leeftijd en paternaal roken. Op basis van deze observationele studies is het niet mogelijk om causale effecten te bewijzen. Dat de gevonden associaties toch causale relaties betreffen, wordt echter wel waarschijnlijker door de eerder besproken biologische theorieën.

In **hoofdstuk 4** wordt het studieprotocol gepresenteerd van het REMI III project. Dit project beslaat zowel een case-control studie als een cohortstudie, gericht op de rol van paternale factoren in etiologie en prognose van herhaalde miskramen. Een deel van de doelen van het REMI III project zijn reeds behaald en de resultaten zijn weergegeven in dit proefschrift. Andere doelen zijn het onderwerp van lopend onderzoek.

Een brandende vraag van veel koppels met herhaalde miskramen heeft betrekking op hun prognose: wat is de kans op een succesvolle zwangerschap in de toekomst? Om koppels van goed onderbouwde informatie te voorzien over hun vooruitzichten kan een predictiemodel uitkomst bieden. Van de huidige, gedateerde predictiemodellen zijn

zowel de accuraatheid van de voorspellingen als validatieprocedures niet beschreven dan wel niet uitgevoerd. Bovendien zijn deze predictiemodellen gebaseerd op slechts twee predictoren: de maternale leeftijd en het aantal miskramen in de voorgeschiedenis. In **hoofdstuk 5** verkenden we of het voorspellen van de kans op een doorgaande zwangerschap voorbij 24 weken amenorroeduur bij stellen met onverklaarde herhaalde miskramen nauwkeuriger wordt als meer kandidaatpredictoren in beschouwing worden genomen, waaronder ook paternale variabelen. We laten zien dat het voorspellen van deze uitkomst accurater wordt als de volgende additionele predictoren worden toegevoegd aan het model (naast maternale leeftijd en het aantal miskramen in de voorgeschiedenis): paternale leeftijd, maternaal en paternaal BMI, maternale rookstatus en eerdere IVF/ICSI behandeling. Het discriminatief vermogen van het model uitgedrukt in de *area under the curve* (AUC) was 0.63, vergeleken met een AUC van 0.57 voor een model op basis van alleen de twee conventionele predictoren. Dat ook paternale variabelen deel uitmaken van de nieuw geïdentificeerde predictoren is een belangrijke bevinding voor zowel patiënten als clinici. Echter moet benadrukt worden dat het voorspellend vermogen van het model nog steeds beperkt is. Om een betrouwbaarder model te ontwikkelen dat in de klinische praktijk gebruikt kan worden, moet nog meer werk verricht worden. Het toevoegen van veelbelovende biomarkers zoals sperma DNA fragmentatie zou de prestaties van het model kunnen verhogen. Tegelijkertijd moeten we ons realiseren dat zwangerschapsuitkomst zeer complex is om te voorspellen, zoals tevens geïllustreerd wordt door de resultaten van andere predictiestudies binnen de reproductieve geneeskunde.

In **hoofdstuk 6 en 7** onderzochten we de rol van seminaal plasma in relatie tot herhaalde miskramen. In **hoofdstuk 6** voerden we een clusteranalyse uit op seminaal plasma samples van mannen uit koppels met herhaalde miskramen (hierna genoemd: herhaalde miskramen-mannen). We identificeerden twee verschillende expressieprofielen in het seminaal plasma. Eén subgroep van herhaalde miskramen-mannen had relatief hoge waarden van pro-inflammatoire cytokines in het seminaal plasma. Mannen in deze subgroep waren significant ouder en hadden relatief ongunstigere leefstijlkenmerken wat betreft rookgedrag, alcoholconsumptie en overgewicht. Herhaalde miskramen-mannen in de andere subgroep hadden een seminaal plasma profiel dat meer overeenkwam met een controlegroep van mannen wiens partners gezonde zwangerschappen hadden. In **hoofdstuk 7** gebruikten we een *in vitro* model om het effect van seminaal plasma op genexpressie van T-cellen en monocyten te onderzoeken. Immuuncellen van een anonieme vrouwelijke bloeddonor werden geïncubeerd met seminaal plasma van herhaalde miskramen-mannen en mannen uit een controlegroep wiens partners gezonde zwangerschappen hadden. We toonden aan dat seminaal plasma een direct effect heeft op T-cellen en monocyten, passend bij differentiatie richting een meer immuunregulatoir fenotype. Daarnaast vonden we in deze studie een aantal opmerkelijke verschillen in

stimulatoire capaciteit van seminaal plasma van herhaalde miskramen-mannen en seminaal plasma van mannen uit de controlegroep. De resultaten suggereren dat de immuunregulatoire capaciteit van seminaal plasma afwijkend kan zijn in het geval van herhaalde miskramen. Toekomstig onderzoek moet de interacties tussen seminaal plasma en de maternale immuunomgeving in fysiologische omstandigheden en bij herhaalde miskramen in meer detail in kaart gaan brengen.

Er is toenemend bewijs dat seminaal plasma een rol speelt in het bewerkstelligen van aanpassingen in het maternale immuunsysteem die van belang zijn voor een succesvolle zwangerschap. Als hier verstoringen in optreden, bijvoorbeeld door een afwijkende samenstelling van het seminaal plasma, draagt dit mogelijk bij aan het ontstaan van een miskraam. Uiteindelijk zouden we hier op in kunnen spelen bij het ontwikkelen van nieuwe therapieën voor koppels met herhaalde miskramen, bijvoorbeeld door het toedienen van specifieke factoren uit het seminaal plasma of substanties met vergelijkbare effecten die een maternale suppressieve immuunrespons bevorderen. Echter moeten we ons niet uitsluitend richten op het seminaal plasma. Een andere stap is het ontrafelen van de interacties tussen de samenstelling van het seminaal plasma en de mate van sperma DNA fragmentatie. Sperma DNA fragmentatie lijkt een belangrijke factor te zijn in relatie tot herhaalde miskramen. Een hindernis die echter overwonnen dient te worden is het betrouwbaar en gestandaardiseerd kunnen meten van sperma DNA fragmentatie. Op dit moment zijn er vele testen en protocollen in omloop en is het nog niet geheel duidelijk wat de beste methode is. Onze en andere studies wijzen erop dat zowel de samenstelling van het seminaal plasma als de mate van sperma DNA fragmentatie geassocieerd zijn met leefstijlfactoren. Het effect van mannelijke leefstijlinterventies op deze semenfactoren en ook op zwangerschapsuitkomst moet dan ook zeker een onderwerp van toekomstig onderzoek zijn. Daarnaast is suppletie van antioxidanten een potentieel effectieve methode om sperma DNA schade veroorzaakt door oxidatieve stress te behandelen.

Naast meer inzicht te verkrijgen in de pathogenese van herhaalde miskramen en het vinden van nieuwe therapieën, moeten we ons ook inzetten om passende ondersteunende zorg te bieden aan patiënten. In **hoofdstuk 8** brachten we voorkeuren voor ondersteunende zorg in kaart van zowel mannen als vrouwen getroffen door herhaalde miskramen. Binnen het medisch domein waren de voorkeuren grotendeels gelijk. Opvallend was dat vrouwen meer belang hechtten aan ‘soft skills’ van de zorgverlener. Mannen wensten minder steun van familie en vrienden. Hoewel de exacte redenen voor de verschillen in voorkeuren tussen mannen en vrouwen onzeker blijven, zijn er een aantal mogelijke verklaringen op basis van eerdere onderzoeken. Vergeleken met vrouwen zijn mannen minder geneigd hun gevoelens te uiten en support voor zichzelf te zoeken na herhaalde miskramen. Veel mannen geven aan sterk en positief te

willen blijven ter ondersteuning van hun partner. Als zorgverleners is het van belang dat we ook aandacht besteden aan de behoeften van de mannelijke partners, zeker omdat onderzoek heeft uitgewezen dat zij eveneens een hoge psychische lijdensdruk ervaren als gevolg van herhaalde miskramen. Toekomstige studies, bijvoorbeeld interviews en focusgroeponderzoek, moeten de behoeften van deze mannen verder exploreren en richting geven aan het ontwikkelen van nieuwe interventies specifiek gericht op het ondersteunen van mannen.

Conclusie en toekomstperspectieven

Op basis van de studies gepresenteerd in dit proefschrift, kan geconcludeerd worden dat een uitsluitend op vrouwen gerichte aanpak bij herhaalde miskramen onterecht is: de mannelijke partner verdient dringend onze aandacht. We hebben de rol van de man in herhaalde miskramen vanuit verschillende perspectieven onderzocht. Zowel epidemiologische als biologische bevindingen onderstrepen een significante bijdrage van de man aan etiologie en prognose van herhaalde miskramen.

In het geval van een frustrerende, gecompliceerde en slecht begrepen aandoening als herhaalde miskramen kan er een neiging bestaan om therapieën aan te bieden die niet bewezen effectief zijn. De druk hiertoe kan extra toenemen doordat het beleid ten aanzien van het voorschrijven van (experimentele) behandelingen substantieel verschilt tussen verschillende landen en zelfs varieert op lokaal niveau. Echter, in plaats van (buiten klinische trials) experimentele behandelingen voor te schrijven met onbekende voor- en nadelen, moeten we ons focussen op het verder ontrafelen van onderliggende mechanismen, het tot stand brengen van hoogkwalitatief bewijs voor gerichte behandelingen en het bieden van uitstekende patiëntcounseling en ondersteunende zorg.

Het meer betrekken van de man, zowel in het wetenschappelijk onderzoek als in de kliniek, kan een sleutel vormen tot een lang gewenste doorbraak in herhaalde miskramen. Het is aannemelijk dat we met relatief simpele interventies gericht op de mannelijke partner, de uitkomsten voor koppels met herhaalde miskramen aanzienlijk kunnen verbeteren. Er is voldoende wetenschappelijk bewijs om te gaan starten met leefstijlinterventiestudies voor mannen (bijvoorbeeld stoppen met roken, afvallen). Voor alle toekomstige studies binnen dit veld pleiten we voor een gecombineerde aanpak van epidemiologisch en basaal-wetenschappelijk onderzoek. De link moet altijd gelegd worden tussen de interventie, de samenstelling van het semen en klinische uitkomsten. Daarnaast moeten we vol inzetten op het uitbreiden van kennis over interacties tussen het seminaal plasma en de maternale lokale immuunomgeving. Voordat we kunnen starten met specifieke immuuntherapieën, zijn er eerst meer in vitro en in vivo studies vereist, om erachter te komen welke semenfactoren nu echt het verschil kunnen maken in een succesvolle

zwangerschap en hoe we daar nieuwe therapieën op kunnen baseren. Nieuwe inzichten binnen dit onderzoeksveld zijn ook van belang voor andere onderzoeksgebieden; een beter begrip van de maternale immuunadapatie tijdens zwangerschap kan ook bijdragen aan vooruitgang binnen de transplantatie-immunologie.

Behalve samenwerking tussen verschillende disciplines, verdient het ook de aanbeveling om internationaal een uniforme definitie te hanteren van herhaalde miskramen en moeten we nationaal en internationaal meer de krachten bundelen om stappen te kunnen maken binnen het onderzoek naar herhaalde miskramen. Het zou waardevol zijn als grootschalige prospectieve studies worden opgezet die zowel klinische data als biologisch materiaal van beide partners met herhaalde miskramen verzamelen en analyseren.

Miskramen zijn lange tijd een taboeonderwerp geweest. De laatste jaren hebben een aantal bekende vrouwen publiekelijk gesproken over hun miskramen en de media-aandacht die daarop volgde heeft bijgedragen aan groeiende erkenning en het meer bespreekbaar maken van dit onderwerp. Naast het doorbreken van het taboe rondom miskramen is het ook de hoogste tijd om af te rekenen met de misvatting dat miskramen zonder meer een maternale origine hebben. Dit proefschrift benadrukt dat (herhaalde) miskramen ook het resultaat kunnen zijn van paternale factoren. Dit moet aan koppels met herhaalde miskramen worden vermeld in de klinische setting en ook worden uitgedragen aan het algemene publiek. Het is de hoogste tijd om over te schakelen van een vrouw-gerichte naar een koppel-gerichte aanpak bij herhaalde miskramen.

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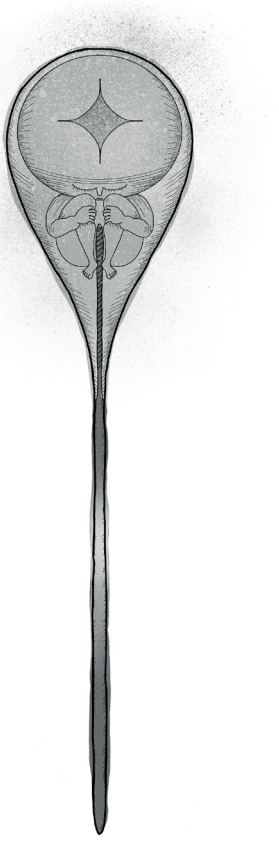

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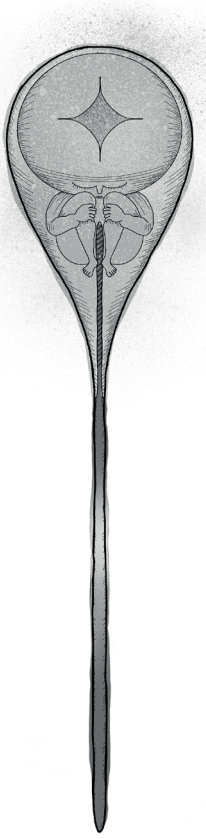
LIST OF PUBLICATIONS



Articles

1. **du Fossé NA,** Lashley EELO, Anholts JDH, van Beelen E, le Cessie S, van Lith JMM, Eikmans M, van der Hoorn MLP. Impaired immunomodulatory effects of seminal plasma may play a role in unexplained recurrent pregnancy loss.
   J Reprod Immunol 2022;151:103500.

2. **du Fossé NA**, Lashley EELO, Treurniet TT, van Lith JMM, le Cessie S, Boosman H, van der Hoorn MLP. Exploring gender differences in supportive care preferences of couples with recurrent pregnancy loss.
   BMC Pregnancy Childbirth 2021;21(1):796.

3. **du Fossé NA**, van der Hoorn MLP, de Koning R, Mulders AGMGJ, van Lith JMM, le Cessie S, Lashley EELO. Toward more accurate prediction of future pregnancy outcome in couples with unexplained recurrent pregnancy loss: taking both partners into account.
   Fertil Steril 2021;117(1):144-152.

4. **du Fossé NA**, van der Hoorn MLP, Buisman NH, van Lith JMM, le Cessie S, Lashley EELO. Paternal smoking is associated with an increased risk of pregnancy loss in a dose-dependent manner: a systematic review and meta-analysis.
   Fertil Steril Rev 2021;2(3):227-238.

5. **du Fossé NA**, Lashley EELO, van Beelen E, Meuleman T, le Cessie S, van Lith JMM, Eikmans M, van der Hoorn MLP. Identification of distinct seminal plasma cytokine profiles associated with male age and lifestyle factors in unexplained recurrent pregnancy loss.
   J Reprod Immunol 2021;147:103349.

6. **du Fossé NA**, Lutke Holzik EM, de Kroon CD. Three atypical presentations of choriocarcinoma, occurding during and shortly after a coexistent viable pregnancy.
   BMJ Case Reports 2021;14(6):e242381.

7. **du Fossé NA**, Bronsgeest K, Arbous MS, Zlei M, Myeni SK, Kikkert M, van Dongen JJM, Staal FJT, van der Hoorn, MLP, van den Akker, T. Detailed immune monitoring of a pregnant woman with critical Covid-19.
   J Reprod Immunol 2020;143:103243.

8. **du Fossé NA**, van der Hoorn MLP, van Lith JMM, le Cessie S, Lashley EELO. Advanced paternal age is associated with an increased risk of spontaneous miscarriage: a systematic review and meta-analysis.
   Hum Reprod Update 2020;26(5);650-669.

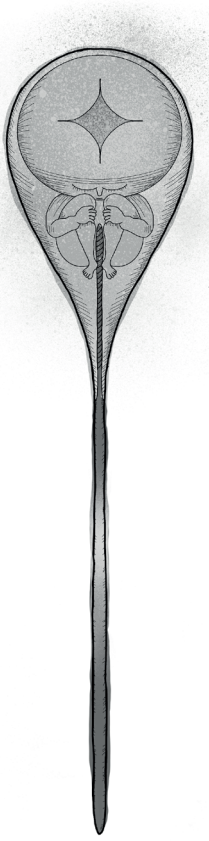
9. **du Fossé NA**, van der Hoorn MLP, Eikmans M, Heidt S, le Cessie S, Mulders AGMGJ, van Lith M, Lashley EELO. Evaluating the role of paternal factors in aetiology and prognosis of recurrent pregnancy loss: study protocol for a hospital-based multicenter case-control study and cohort study (REMI III project).
BMJ Open 2019;9(11):e033095.

10. Leuning DG, Beijer NRM, **du Fossé NA**, Lievers E, van Kooten C, Rabelink TJ, de Boer J. The cytokine secretion profile of mesenchymal stromal cells is determined by surface structure of the microenvironment.
Sci Rep 2018;8(1):7716.

Chapter in edited book

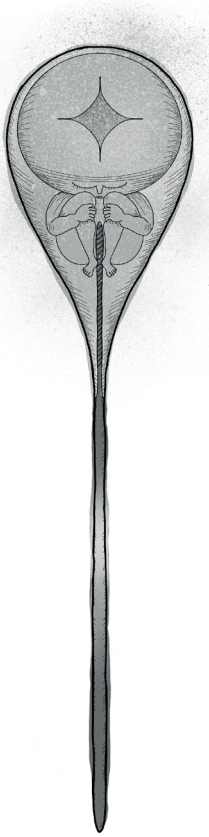
1. Verhagen I, **du Fossé NA**, van den Berg J, Heesterbeek E. Rol van patiënt en zorgprofessional. In: Stolp J, Schouten I, Boer F den, Zegers M, editors. Evidence Based Practice: Handboek voor verpleegkundigen. Amsterdam: Pearson Benelux 2017.

CURRICULUM VITAE



Nadia du Fossé werd op 23 januari 1994 geboren in het Kennemer Gasthuis in Haarlem. In haar jeugd deed zij fanatiek aan wedstrijdzwemmen en in de vakanties doorkruiste zij met haar ouders Europa per fiets. In 2012 behaalde Nadia haar gymnasiumdiploma aan Lyceum Sancta Maria in Haarlem en vervolgens startte zij met de studie Geneeskunde aan de Universiteit Leiden. Nadat zij in 2015 haar bachelordiploma behaalde, besteedde zij haar wachttijd tot de coschappen aan een klinische stage in een Ugandees ziekenhuis en een wetenschapsstage op de afdeling Nierziekten in het Leids Universitair Medisch Centrum (LUMC) onder begeleiding van prof. dr. A.J. Rabelink. Tijdens deze wetenschapsstage ontstond haar enthousiasme voor onderzoek. Gedurende haar studie had Nadia diverse bijbanen binnen de zorg, het onderzoek en het onderwijs. Aan het einde van haar master raakte zij in het LUMC geïnteresseerd in de Gynaecologie en Verloskunde. Na het cum laude behalen van het artsexamen in 2018 mocht zij op deze afdeling starten met een promotietraject, waaruit dit proefschrift is voortgekomen. Zij volgde gelijktijdig de opleiding tot Klinisch Epidemioloog B in het LUMC (opleider prof. dr. F.R. Rosendaal). Tijdens haar promotietraject maakte Nadia tevens deel uit van het bestuur van JongLUMC, met als doel jonge medewerkers werkzaam op de diverse afdelingen in het ziekenhuis met elkaar in verbinding te brengen. Vanaf december 2021 werkt Nadia als arts-assistent Gynaecologie en Verloskunde in het HagaZiekenhuis in Den Haag. In juli 2022 zal zij starten als arts-assistent op de Intensive Care in het LUMC. In de toekomst hoopt zij een klinische en wetenschappelijke carrière te combineren. Haar vrije tijd brengt Nadia graag door op de racefiets, hardlopend in de duinen of zwemmend in het buitenwater.

DANKWOORD



Promotieonderzoek doen is als fietsen in de bergen. De ene keer in vliegende vaart op je doel af koersen, dan weer zware kilometers doorstaan waarin je de pedalen nauwelijks rond krijgt. Eén ding is zeker: het is allesbepalend wie er tijdens zo'n tocht met je meefietsen. Vele mensen hebben mij geïnspireerd, uitgedaagd, aangemoedigd en uit de wind gehouden tijdens de diverse etappes die mijn promotietraject vormden.

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