Recurrent Pregnancy Loss. Aspects of Epidemiology, Immunology, Treatment and Obstetric Outcomes.

Råsmark, Emma

2019

Document Version:
Publisher's PDF, also known as Version of record

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EMMA RÅSMARK RØPKE | FACULTY OF MEDICINE | LUND UNIVERSITY

Printed by Media-Tryck, Lund 2019 NORDIC SWAN ECOLABEL 3041 0903

FACULTY OF MEDICINE

Department of Obstetrics and Gynaecology

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2019:118
ISBN 978-91-7619-847-6
ISSN 1652-8220

Recurrent Pregnancy Loss

Aspects of Epidemiology, Immunology, Treatment and Obstetrical Outcomes

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LUND
UNIVERSITY

DOCTORAL DISSERTATION
with due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended in the Auditorium of the Dept. of Obstetrics and Gynaecology, Skåne University Hospital, Malmö, on November 30th 2019, at 10.00 a.m.

Faculty opponent
Professor Christina Berg, Gothenburg University

Organisation LUND UNIVERSITY	Document name DOCTORAL DISSERTATION
	Date of issue 2019-10-25
Author(s) Emma Råsmark Røpke	Sponsoring organisation

Title and subtitle Recurrent Pregnancy Loss. Aspects of Epidemiology, Immunology, Treatment and Obstetric Outcomes

Abstract

Aims: Recurrent pregnancy loss (RPL), defined as three consecutive miscarriages, is a multifaceted problem whose resolution needs a broad approach. In this thesis, epidemiology, immunology, treatment and obstetrical outcomes are explored. The aims are as follows: To establish the incidence of the diagnosis of RPL in the National Patient Register (NPR) in Sweden from 2003 to 2012 (Paper I). To evaluate the validity of the ICD-10 diagnosis codes of RPL registered in the NPR (Paper II). To systematically review the evidence for different treatments' efficiency at improving live birth rate (LBR) in women with idiopathic RPL (Paper III). To determine whether treatment with low-molecular-weight heparin (LMWH) exerts immunological effects during pregnancy in women with a history of idiopathic RPL (Paper IV). To study if women with a subsequent pregnancy after the diagnoses of RPL are associated with adverse obstetrical outcomes (Paper V).

Methods: Papers I, II and V were register-based studies and used nation-wide data on women with RPL diagnosis registered in the NPR, as well as data from the Medical Birth Register (MBR) in Paper V. In Paper I an incidence of women diagnosed with RPL in pregnant women and in women 18-42 years of age was calculated for a 10-year period. In Paper II medical records were reviewed to validate the diagnosis codes of RPL in the NPR and a positive predictive value (PPV) was estimated. Paper III was a systematic review of 21 RCTs regarding acetylsalicylic acid (ASA), LMWH, progesterone, intravenous immunoglobulin (IvIg) or leukocyte immune therapy (LIT). Meta-analyses on treatment efficiency regarding live birth rate (LBR) in women with idiopathic RPL were conducted. Paper IV was an open randomised trial, including women with idiopathic RPL in which the immunological effect of LMWH was measured by assessing cytokine/chemokine levels during pregnancy. Paper V was a retrospective cohort study in which placenta-associated complications such as preeclampsia, small for gestation infant, preterm birth, stillbirth and placental abruption, were evaluated in women with a first subsequent pregnancy after the diagnosis of RPL compared with outcomes of pregnancies of women without RPL.

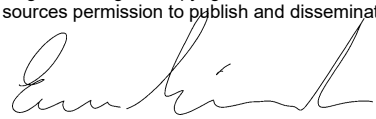
Results: In Paper I, the incidence of RPL diagnosis was increased from 478 to 875/100 000 pregnant women and from 39 to 73/100 000 women aged 18-42 years in the study period, 2003-2012. In Paper II, 202 out of 238 medical records had correct diagnoses of RPL, resulting in a PPV of 85% (95%CI 78–89%). In Paper III, meta-analyses showed no increase in LBR when treated with LMWH (RR 1.47, 95% CI 0.83–2.61) or IvIg (RR 1.07, 95% CI 0.91–1.26) but an increase in LBR with LIT (RR 1.8, 95% CI 1.34–2.41). No meta-analyses were possible for ASA and progesterone, although two large RCTs on the latter have shown higher LBR in treated women compared with placebo. In Paper IV, the Th1-associated chemokines CXCL10, CXCL11 were significantly higher (p=0.01 and <0.001) in LMWH treated women compared to untreated women. In Paper V women with RPL showed a higher risk of placenta-associated disorders: preeclampsia (OR 1.45 95%CI:1.24-1.69), stillbirth <37 gestational weeks (GWs) (OR 1.92 95%CI:1.22-3.02), SGA birth (OR 1.97 95%CI:1.42-2.74), preterm birth (OR 1.46 95%CI:1.20-1.77), and placental abruption <37 GWS. (OR 2.47 95%CI:1.62-3.76).

Conclusion: The incidence of RPL diagnoses increased over the study period, although further studies are needed to obtain causative explanations. The diagnosis of RPL in the NPR is acceptably accurate and can be used for studies in this field. Because of low study quality, large diversity in the study groups and treatment start no recommendation can be given of one particular treatment to improve LBR in women with idiopathic RPL. Treatment with LMWH does not modulate the immune response in a favourable direction during pregnancy and is not recommended for women with idiopathic RPL. Women with RPL are at higher risk for developing placenta-associated disorders; therefore antenatal surveillance ought to be intensified for this group.

Key words recurrent pregnancy loss, reproductive immunology, epidemiology

Classification system and/or index terms (if any)

Supplementary bibliographical information		**Language** English
ISSN and key title 1652-8220		**ISBN** 978-917619-847-6
Recipient's notes	**Number of pages** 88	Price
	Security classification	

Signature  Date 2019-10-24

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Printed in Sweden by Media-Tryck, Lund University
Lund 2019

To my family

Life brings tears, smiles and memories.
The tears dry, the smiles fade,
but the memories last forever.

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Abbreviations

ACA	Anticardiolipin antibodies
AOR	Adjusted odds ratio
ASRM	American Society of Reproduction Medicine
aPL	Antiphospholipid antibodies
APS	Antiphospholipid syndrome
ASA	Acetylsalicylic acid
BMI	Body mass index
CGH	Comparative genomic hybridisation
CI	Confidence interval
CONSORT	Consolidated standards of reporting trials
ELISA	Enzyme-linked immune sorbent assay
ESHRE	European Society of Human Reproduction and Endocrinology
EVT	Extravillous trophoblast
GM-CSF	Granulocyte macrophage- colony stimulating factor
GRADE	Grading of Recommendations Assessment Development and Evaluation
GWs	Gestational weeks
HB-EGF	Heparin-binding epidermal growth factor
hCG	Human choriongonodotropin
HLA	Human leukocyte antigen
ICD	International classification of diseases
IUFD	Intrauterine foetal death
IFN	Interferon
IL	Interleukin
IUGR	Intrauterine growth restriction
IVF	In vitro fertilisation
IvIg	Intra-venous immunoglobulin
KIR	Killer immunologlobulin receptors
LAC	Lupus anticoagulant
LBR	Live birth rate

LIF	Leukaemia inhibitory factor
LIT	Leukocyte immunisation treatment
LMWH	Low-molecular-weight heparin
LPD	Luteal phase defect
MBR	Medical birth register
M-CSF	Macrophage-colony stimulating factor
MHC	Major histocompatibility complex
NBHW	National Board of Health and Welfare
NK	Natural killer (cells)
NPR	National patient register
OR	Odds ratio
PCOS	Polycystic ovarian syndrome
PICO	Population intervention comparison outcome
PIN	Personal identification number
RCT	Randomised controlled trial
PPV	Positive predictive value
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-analyses
RPL	Recurrent pregnancy loss
SGA	Small for gestational age
STROBE	Strengthening the Reporting of Observational Studies in Epidemiology
Tc	T-cytotoxic cells
Th cells	T-helper cells
TGF	Transforming growth factor
TNF	Tumour necrosis factor
Treg cells	Regulatory T-cells
VEGF	Vascular endothelial growth factor

Original Papers

This thesis is based on the following papers, which will be referred to by their Roman numerals. The five papers are appended at the end of this thesis.

I. Is the incidence of recurrent pregnancy loss increasing?
A retrospective register-based study in Sweden.
Rasmark Roepke E, Matthiesen L, Rylance R, Christiansen OB.
Acta Obstet Gynecol Scand. 2017 Nov 96(11):1365-1372.

II. Reliability of recurrent pregnancy loss diagnosis coding in the Swedish National Patient Register: a validation study.
Rasmark Roepke E, Christiansen OB, Hansson SR.
Clinical Epidemiology. 2019 May 8;11:375-381.

III. Treatment efficacy for idiopathic recurrent pregnancy loss - a systematic review and meta-analyses.
Rasmark Roepke E, Hellgren M, Hjertberg R, Blomqvist L, Matthiesen L, Henic E, Lalitkumar S, Strandell A.
Acta Obstet Gynecol Scand. 2018 March 8;97:921–941

IV. Low-molecular-weight-heparin increases Th1- and Th17-associated chemokine levels during pregnancy in women with unexplained recurrent pregnancy loss: a randomised controlled trial
Rasmark Roepke E*, Bruno V*, Nedstrand E, Boij R, Petersson Strid C, Piccione E, Berg G, Svensson-Arvelund J, Jenmalm M.C, Rubér M, Ernerudh J *first authors
Scientific Reports 2019 Aug 23;9(1):12314.

V. Women with a history of recurrent pregnancy loss are a high-risk population for adverse obstetrical outcome. A retrospective cohort study.
Rasmark Roepke E, Christiansen O B, Källén K, Hansson S R
Manuscript.

Introduction

Aspects of miscarriage and Recurrent Pregnancy Loss

Miscarriage is one of the most common complications in pregnancy and leads to great distress for many couples. When miscarriage is repeated it is debated if this happens only due to repeated bad luck or whether there is a disease entity. However, several arguments support the view that recurrent pregnancy loss (RPL) is a clinical disorder with an underlying cause, and it does not happen just by chance (to be further discussed later).
Despite recent advances in reproductive medicine there are still many unanswered questions regarding RPL. It is not known for certain how many suffer from RPL or if it is an increasing problem. Frequently, a cause of RPL is not found even though several risk factors seem to be associated with it. When investigation fails to determine what caused the recurrent miscarriages, it is believed that immunology defaults in early pregnancy can be an explanation. Although the research in reproductive immunology is extensive, the mechanisms are far from fully explored. Thus, we still do not know why some pregnancies are successful and why some end in miscarriage.

Medical treatment might be able to modulate the immune response during pregnancy in a more favourable direction, but few studies have been conducted on humans and *in vivo*. An open question is whether any medical treatment is effective on improving live birth rate. Another remaining issue is whether women with RPL, despite a "successful" pregnancy, might be at a higher risk of adverse pregnancy outcome caused by an immunological impairment.

The present thesis attempts to elucidate some of the above aspects of RPL concerning epidemiology, immunology, treatment and obstetrical outcomes.

Epidemiology of Recurrent Pregnancy Loss

Definition of Recurrent Pregnancy Loss

RPL has been defined in a variety of ways in regard to number of miscarriages and gestational age. How RPL is defined has clinical, economical and scientific consequences.

It is debated whether two or three consecutive miscarriages should constitute the definition.[1,2,3,4] The terminology of miscarriage and stillbirth depends on the defined limit of gestational age for viability and differs between countries as legal aspects also come into consideration. The spectrum of gestational age used in the definition of miscarriage extends from 20 gestational weeks (GWs)[1,5,6] to 24 GWs[3,7] in the literature. The viability limit has changed from 28 GWs to 22 GWs in Sweden, as advances in neonatal care have improved. Pregnancy loss after GWs 20 is rare so if the definition of 20 or 24 GWs is used it will make little difference to the population of RPL.[8] The European Society of Human Reproduction and Embryology's (ESHRE) updated guidelines[7] define RPL as two or more consecutive pregnancy losses before GWs 24, including pregnancy loss after spontaneous conception and assisted treatment, but exclude ectopic and molar pregnancy as well as implantation failure. This definition has been changed since the last guidelines in 2006,[9] and some guideline development group members have stressed that the definition should be kept to three miscarriages.[7]

The lack of a consensus on the definition of RPL creates difficulties in research and clinical work assessing investigations and treatment outcome in trials and patient care. For couples that experience miscarriages, an investigation will first take place after the definition of RPL is met: therefore, it is a matter of great concern to the patients whether the definition is two or three miscarriages.

Economically, it will make a difference if investigation is initiated after two or three miscarriages, as more women will be included for investigation after only two. Speculatively, it might be beneficial for private actors to start investigation after only two miscarriages if the patient or an insurance company pays it. Public health care would possibly prefer to restrict investigations to women with three miscarriages based on a cost-benefit calculation. The fewer the number of previous miscarriages the better the prognosis for live birth rate (LBR) in a subsequent pregnancy.[10–12] The chance of a subsequent live birth in RPL women with three, four or five miscarriages has been found to be 42-86%, 44-73% and 25-52%, respectively.[8,11] Live birth for women with previous two and three miscarriages has been also been shown in an additional study to be 81% and 73%,

respectively.[13] With this prognosis it is acceptable to start investigation after three consecutive miscarriages rather than two.

Women with RPL are divided into primary and secondary RPL groups. Primary RPL is defined as three consecutive pregnancy losses without previous live births, or pregnancy beyond GWs 22. Secondary RPL is defined as three consecutive pregnancy losses following one or more live birth(s), or pregnancy/pregnancies beyond GWs 22.[8] This grouping is done because there might be different pathophysiological backgrounds in primary and secondary RPL.[8]

In this work the definition of three consecutive miscarriages before 22 GWs is used.

Incidence and prevalence of Recurrent Pregnancy Loss

The incidence of RPL is the number of new women each year suffering their third consecutive pregnancy loss in a risk population. Even though the incidence is mentioned in several studies, most of them are actually referring to prevalence, as these two terms are often used incorrectly. The prevalence of RPL is the number of all women in a population who, at a specific time point, have had three or more consecutive miscarriages. However, the risk population is not clearly defined in the literature (discussed further below).

Several studies have reported a frequency of RPL to be between 0.5% and 2.3%.[5,8,14–16] The above studies actually report the prevalence rather than the incidence of RPL. Incidence is important when comparing the risk rate of RPL and to identify trends over time. Incidence can be used when planning research designs or investigations and treatment protocols in health care clinics.

The prevalence of clinical miscarriages is estimated at approximately 15% of all pregnancies, in several studies.[17–20] If miscarriage is always a random event and the incidence of clinical miscarriages is 15%, the theoretical calculated risk for three consecutive events would be 0.34%. RPL is thought to affect 1% of couples,[5,21] which is higher than the theoretical estimated risk above. This suggests that RPL is not just repetitive events of bad luck. Moreover, the risk of miscarriage increases according to the previous number of miscarriages, which supports the view that RPL is a clinical entity.[1,8,10]

Some obstacles are met when estimating the incidence of RPL. The first is the lack of consensus on the definition of RPL. The second is the description of the population at risk of experiencing RPL, which is the denominator in the calculation of the incidence. There is a huge diversity in the risk population in the literature.[1,3,9,10,22–25] A risk population can be all women in the population, all women of fertile age, women trying to conceive, women who have been pregnant

twice (to complete the family with two children) or women who have been pregnant at least three times (a group that is enriched by women with repeated miscarriages).

The third obstacle is the inclusion or exclusion of biochemical pregnancies, defined as a rise of urine or serum human choriogonadotropin (hCG), in the estimation of the incidence of RPL. It has been suggested that the risk for miscarriages is up to 60% of pregnancies defined biochemically, while the risk is 15% in clinically defined pregnancies.[10,26,27] However, these figures will differ in different subgroups and age groups.[28] ESHRE[7] suggests including both biochemical and clinical pregnancy loss in the definition of miscarriage but excluding unsolved pregnancies of unknown location, as well as molar and extra uterine pregnancies.

A fourth concern in estimating the incidence of RPL is how to study the occurrence of miscarriages. Hospital-based studies tend to underestimate the frequency of miscarriages, as they tend to only include miscarriages where medical intervention was needed or include only those women who appear in medical birth registers having achieved a live birth after repeated miscarriages. When retrospective questionnaires are used there will be a recall bias and even more uncertainty about the definition of miscarriage.

It is still uncertain how many women suffer RPL and if it is an increasing problem.

Normal implantation and placentation in pregnancy

Since miscarriages often occur early in pregnancy, during the first trimester, it is believed that all stages from pre-implantation, implantation and placentation are essential for a normal pregnancy. Therefore, a brief review of the normal early pregnancy phases is presented.

Endometrial receptivity and implantation

Decidualisation is the remodelling of the endometrium to prepare and make it receptive for a potential implantation of a blastocyst. During decidualisation the endometrium undergoes a radical change, influenced by oestrogen and progesterone, with increased vascularisation, differentiation of stromal cells to decidual stromal cells, and infiltration of leukocytes. The decidualisation creates "the window of implantation", which lasts for approximately five days and the decidua continues only if fertilisation and implantation succeed.[29] Secretory products like cytokines and growth factors, secreted from leukocytes and

endometrial glands, prepare the endometrium for an implantation. Oestrogen-regulated cytokines like macrophage-colony stimulating factor (M-CSF), granulocyte macrophage-CSF (GM-CSF) and tumour necrosis factor (TNF) increase during the proliferative phase. Additionally, the pro-inflammatory interferon-ɣ (IFN-ɣ) contributes to implantation. During the luteal phase, increasing progesterone weakens the above pro-inflammatory cytokines. Cytokine expression shifts towards anti-inflammatory cytokines, like M-CSF, leukaemia inhibitory factor (LIF) and transforming growth factor-β (TGF-β) once implantation begins. The implantation is a controlled inflammatory stage. Epithelial cells also secret chemokines and vascular endothelial growth factors (VEGF) that regulate leukocyte infiltration and vascular remodelling.[30]

Implantation is a reciprocal signalling of hormones, cytokines, chemokines and growth factors with a complex interaction between the blastocyst and the maternal decidua. The cytokines synthesised by epithelial cells and local leukocytes support blastocyst development, and protect it from cell stress and apoptosis as wells as promoting implantation.[30] The implantation starts with adhesion of the blastocyst to the decidua, where the outer layer of the blastocyst, the trophoectoderm, penetrates the endometrial epithelium and becomes completely embedded into the endometrium (Figure 1).[31,32] The blastocyst also expresses signals, for instance hCG, that maintain high levels of progesterone and stimulate LIF expression.[30]

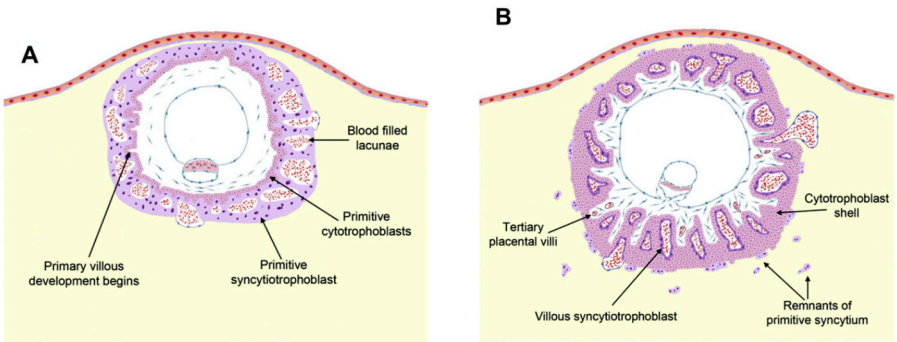

Figure 1. Implantation and development of the placenta. **A** Human blastocyst implanting into decidua, embedded by the endometrium. Development of the first placental structures and the embryonic disc. Formation of primary villi with maternal blood lacunes in between the syncytiotrophoblast cells. **B**. Development of secondary and tertiary villi. Reprinted from "Human placentation from nidation to 5 weeks of gestation. Part I: What do we know about formative placental development following implantation?" James J. *et al*. Placenta 2012 with permission from Elsevier.

Trophoblast differentiation and artery remodelling

Trophoblast cells proliferate and differentiate into cytotrophoblasts. The cytotrophoblast cells take two pathways, differentiation into syncytiotrophoblast or extravillous trohpoblast (EVT). In the first pathway they lose their cell membrane and fuse into the syncytiotrophoblast. The syncytiotrophoblasts constitute a thick surrounding layer around the embryo, and maternal blood vessels form lacunas between the syncytiotrophoblasts (Figure 1).[31,32] Syncytiotrophoblasts form an outer layer of the villi of the placenta. The villi divide into primary, secondary, and tertiary villi and, in addition, anchoring villi that attach to the decidua basalis. Beneath the outer layer of the syncytiotrophoblast is an inner layer of cytotrophoblast cells and the villous mesenchyme, which together form the placental membrane. The membrane function is a barrier between the foetal blood in the villi vessels and the maternal blood in the intervillous space. The barrier regulates the passage of oxygen and nutrition.[32]

The second pathway of the cytotrophoblast cells is differentiation into EVT cells, termed interstitial and endovascular cells. The EVT cells invade and migrate through the anchoring villi into the decidua (Figure 2). The interstitial EVTs secure the anchoring of the placenta to the maternal decidua. The endovascular EVTs invade the uterine spiral arteries, lymph vessels and glands for remodelling.[33] At first, so-called trophoblast plugs prevent maternal blood flow from entering the interstitial villi. This ensures a hypoxic environment, which is important for trophoblast differentiation and artery remodelling. Endovascular trophoblast cells replace vascular smooth muscle cells and endothelial cells so the spiral arteries dilate and become non-vasoactive vessels. After the trophoblast plug dissolves at the end of first trimester,[34] the artery remodelling allows maternal blood flow in the intervillous space without resistance.[35,36] This is crucial for adequate nutrition and development of the growing foetus.[31] Defects in trophoblast invasion and artery remodelling can result in placenta dysfunction and may lead to miscarriages, preeclampsia and intrauterine growth restriction (IUGR).[37]

Further significant cells affecting placentation are discussed further in the next section.

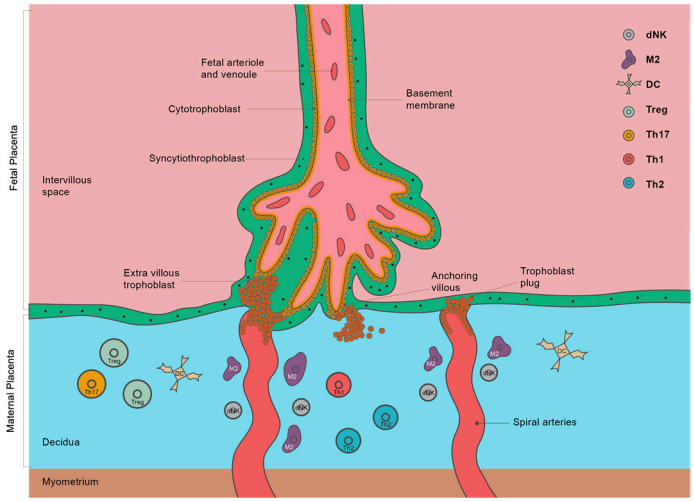

Figure 2. Placental villi with extra villous trophoblasts invading the spiral arteries, developing throphoblast plugs and remodelling the maternal arteries. Immune cells are present at the maternal–foetal interface. Early pregnancy is characterised by the presence of mainly innate but also adaptive immune cells, including decidual NK (dNK) cells, macrophages (mainly of M2-like type), dendritic cells (DCs), T helper cells (mainly Th2) and regulatory T (Treg) cells. The phenotypes, distributions and functions of these cells are determined by signals that originate from the placenta and the decidua. Immune cells not only have a central role in the immunological aspects associated with tolerance and immune protection, but also support biological processes that are crucial for the development of the foetus, such as trophoblast invasion, spiral artery transformation and decidual differentiation. Illustrated by Elinora Frolova.

Immunology in early pregnancy

Immunology has an important role in early pregnancy, and impairment of the immune system might play a part in RPL. A brief overview of the immune system is presented. Furthermore, the immune modulation in early pregnancy is described.

Brief overview of the immune system

The immune system is traditionally divided into the innate and adaptive immune system. The innate system acts as a first line of defence towards foreign pathogens. The main cells of the innate system that have been most studied in relation to pregnancy are dendritic cells, macrophages and natural killer (NK) cells (explained in more detail later). These cells are activated when they meet pathogens, which leads to killing of the pathogens by phagocytosis (macrophages) or cytotoxic effects (NK cells). Dendritic cells and macrophages are able to activate the adaptive immune system (see below). The cells also secrete inflammatory mediators, such as cytokines and chemokines, which activate other

cells and recruit specific immune cells for specific and different immune responses.[38]

The adaptive immune system takes longer to activate but it is more specific and long-lasting as it can develop memory to recognise the antigens. Cells of the adaptive immune system that are especially important for pregnancy are cytotoxic T-lymphocytes (Tc), helper T-lymphocytes (Th) and regulatory T-lymphocytes (Treg). T-cells are activated by antigen-presenting cells, such as dendritic cells and macrophages that present the antigen on the major histocompatibility complex (MHC) on the cell surface that T-cell receptors bind to. During the activation of naive T-cells, the presence of specific cytokines regulates the differentiation of the naive T-cells into different subsets of effector cells. The most established Th subsets are Th1, Th2, Th17 and Treg (Figure 3). There is a positive feed-back loop promoting T cell proliferation and differentiation but also inhibitory signals that suppress and counter-balance the other types of subsets.[38] Figure 3 shows cytokines that induce different Th subsets and their general function in pregnancy.

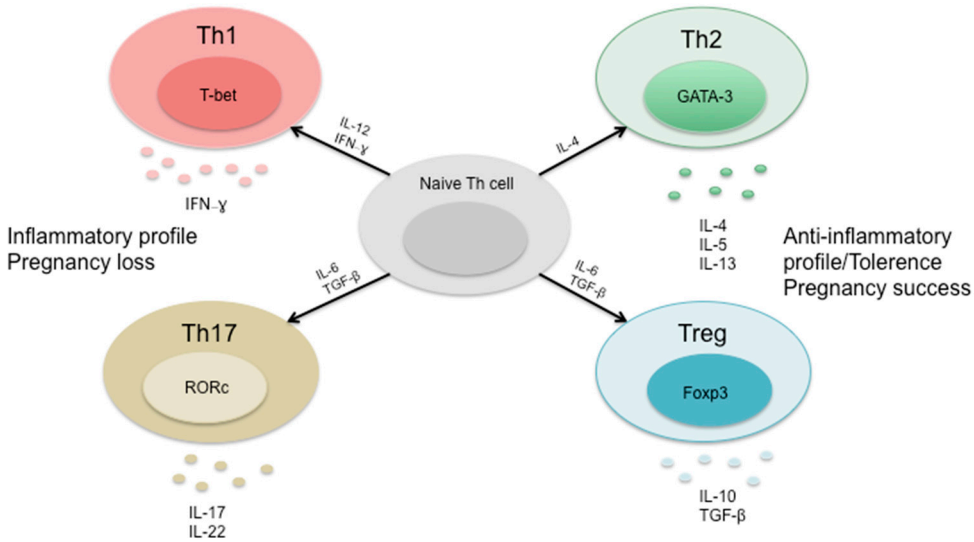

Figure 3 Schematic representation of T-helper cells. Cytokines, mainly produced from innate immune cells like dentritic cells, macrophages, and NK cells, induce differentiation from naïve Th cell to specific Th subsets. Transcription factors (GATA-3, Foxp3, T-bet, RORc) are thereby activated and induce expression of specific Th subset cytokines. Th2 and Treg are regulatory subsets that promote pregnancy success. Th1 and Th17 are pro-inflammatory subsets that can induce pregnancy loss. Image illustrated by Emma Råsmark Røpke.

Chemokines, unlike cytokines, are often measurable in plasma and can be used as markers for different forms of immune polarisation. The different Th subsets

produce specific cytokines and chemokines, which bind to specific receptors, expressed by that each particular Th subset. Table 1 shows Th subsets, their effector cytokines and chemokines and receptor expression.

Table 1. T-helper subsets with their main cytokines, chemokines and receptors

Th subset	Induced by	Cytokines expressed by Th cells	Chemokines expressed by macrophages, epithelial cells	Chemokine receptor	Effect in infections/ in pregnancy
Th1	IFN-γ, IL-12	IFN-γ	CXCL9, CXCL10, CXCL11	CXCR3	intra-cellular virus and bacteria/ pro-inflammatory
Th2	IL-4	IL-4, IL-5, IL-13	CCL17, CCL22	CCR4	extra-cellular parasites/ pregnancy promoting
Th17	IL-6,TGF-β	IL-17, IL-22	CCL20	CCR6	extra-cellular bacteria and fungi/ pro-inflammatory
Treg	TGF-β	TGF-β, IL-10	-	-	regulating inflammation /tolerance anti-inflammatory

Maternal immune adaptation during pregnancy

It is not completely understood how a mother, who has a potent immune system, can accept a foetus presenting foreign paternal antigens. The maternal immune system needs to adapt and modulate in order not to reject the semi-allogeneic foetus. At the same time as showing such immune tolerance the maternal immune system needs to protect the mother and foetus from pathogens. Several immunological components need to fall into place for the pregnancy not to fail.

Briefly, a successful pregnancy is believed to depend on modulation of the maternal immune system and balance in the Th1/Th2 immunity with an anti-inflammatory Th2-skewing. Cytokine, chemokines, hormones and prostaglandins all play a role in regulating the immune system at the foetal-maternal interface. Both the mother and the embryo contribute to a successful pregnancy and numerous immune mechanisms are involved.[39]

The placenta membrane keeps the foetus separated from direct contact with the maternal blood. However, foetally derived cells like the EVT cells come into direct contact with maternal leukocytes such as NK cells, macrophages and T cells (Figure 2). The reason why the mother's immune system does not attack the EVT cells is partly the fact that EVT cells express a restricted MHC profile. They express MHC I: human leukocyte antigen (HLA) class I molecules HLA-C, HLA-E and HLA-G but not HLA-A, HLA-B or HLA class II (HLA-DR and DQ).[39,25]

The trophoblasts present the HLA for NK receptors, like killer immunoglobulin receptors (KIR). Only the HLA-C/KIR system is highly polymorphic, which means that paternal and maternal HLA-C alleles can differ[40] and thereby stimulate an immune response towards the foetus.[39] This specific HLA expression is involved in the modulation of the maternal immune response.[41]

Trophoblast cells also contribute to hormone production including progesterone, estradiol and hCG that are all involved in immune modulation. Progesterone is highly anti-inflammatory and immune-suppressive as it stimulates anti-inflammatory cytokines (IL-10, TGF-β, LIF, CSF) and promotes Th2 differentiation.[42,43] Oestrogen is pro-inflammatory at lower concentrations, enhancing the Th1 response, although a high concentration is thought to stimulate IL-10 secretion and Th2 immunity.[44] hCG has been proven to suppress Tc cells, and the production of IFN-γ and TNF, and to increase the anti-inflammatory cytokines IL-10 and TGF-β. Additionally, hCG increases the number of Treg cells and their suppressive function at the foetal-maternal interface.[45]

Innate immune system in pregnancy

The dNK cell is the most prominent leukocyte in the decidua and has a $CD16^{-}$ $CD56^{bright}$ cell surface phenotype that differs from peripheral NK cells ($CD16^{+}$ $CD25^{dim}$). Compared with peripheral blood NK cells, dNK cells express higher levels of chemokines, cytokines, and angiogenic factors[46] but display low NK cytotoxicity[47]. These cells are specialised NK cells in pregnancy and play a major role in decidua spiral arteriole remodelling and in promoting trophoblast invasion.[47] Inhibitory and activating KIR on uNK cells bind to HLA-C on foetal trophoblast cells. The reduced cytotoxic potential towards the trophoblast is believed to be controlled by inhibitory receptors (discussed above). However, activation receptors are important for promoting angiogenic factors and for proper invasion of trophoblasts in remodelling the spiral arteries.[48,49] dNK cells can produce both Th1-associated cytokines, like IFN-γ, and anti-inflammatory cytokines such as IL-10, and this diversity in cytokine production seems important in the regulation of the trophoblast invasion.[50]

Decidual macrophages are multifunctional and can change their phenotype according to the environment. The decidual macrophages are the most frequently occurring antigen-presenting cells at the foetal-maternal interface. They are important in balancing the tolerance and pro-inflammatory response. Differentiation is required to develop the M2-like phenotype, a process in which M-CSF and IL-10 are important.[51] This anti-inflammatory M2-phenotype contributes to both remodelling of the endometrium and to a "foetus friendly" environment.[51] These M2 macrophages primarily produce anti-inflammatory cytokines like IL-10. Decidual macrophages are at a high concentration around

invading trophoblasts and have a phagocytic ability, where they phagocytose degraded extra-cellular matrix and apoptotic cells.

Like decidual macrophages, dendritic cells in the decidua are antigen-presenting cells and act as immuno-suppressor cells. The decidual dendritic cells control the activation and recruitment of Treg cells and are therefore important for immune tolerance.[39]

Th subsets in pregnancy

Traditionally, it is believed that a foetal tolerance is associated with a shift from a pathogenic Th1 to a benign Th2 immunity and that the maternal immune system needs to be suppressed for the pregnancy to continue.[52] Recent data rather suggest an active immune response at the foetal-maternal interface and a successful pregnancy requires a robust, dynamic and responsive immunity. Immune modulation and tolerance at implantation is crucial. It has been suggested that an early pregnancy with invasion of trophoblasts into the decidua is more like a tumour metastasis with its immunological response rather than a graft-host rejection.[53] The Th1/Th2 theory is believed to be a too simplified theory.[54] It has been suggested that the pre-implantation stage is dependent on a pro-inflammatory stage, induced by a low concentration of oestrogen. In early pregnancy, decidual Th1 cells have been seen elevated, indicating a mild inflammation.[43,55] However, a too strong inflammatory response can lead to pregnancy complications such as miscarriage, intrauterine growth retardation and preeclampsia.[47,56]

The stage of the pre-implantation window is later followed by an anti-inflammatory response that is essential for post-implantation embryo survival and development, and for a balanced trophoblastic invasion.[57] At the implantation phase the trophoblast and decidua cells secrete cytokines and chemokines that attract maternal immune cells, such as macrophages, decidual NK cells and Treg cells, which contribute to an anti-inflammatory environment. There is a shift towards anti-inflammation and Th2 immunity.[53]

Treg cells are believed to have a central role in maintaining an anti-inflammatory environment. They have a potent anti-inflammatory, immune-regulatory and vaso-regulatory function that is significant in pregnancy. Already in the luteal phase Treg cells are increased in the decidua to prepare for a controlled inflammation and also for an anti-inflammatory environment.[43,44] It has been suggested that peripheral circulating Treg cells are recruited to the decidua, where they proliferate and act locally for a tolerance response.[58] Treg cells continue to be increased in the decidua throughout pregnancy until the inflammatory phase of delivery is triggered.[43] Treg cells are suggested to be in a reciprocal relationship with Th17. Fewer pro-inflammatory Th17 cells are seen in the decidua than in blood during pregnancy.[55] Imbalance in pro-inflammation and anti-inflammation,

with disparity in the ratio of Th2/Th1 and Treg/Th17 subsets, can be associated with pregnancy loss (Figure 4).

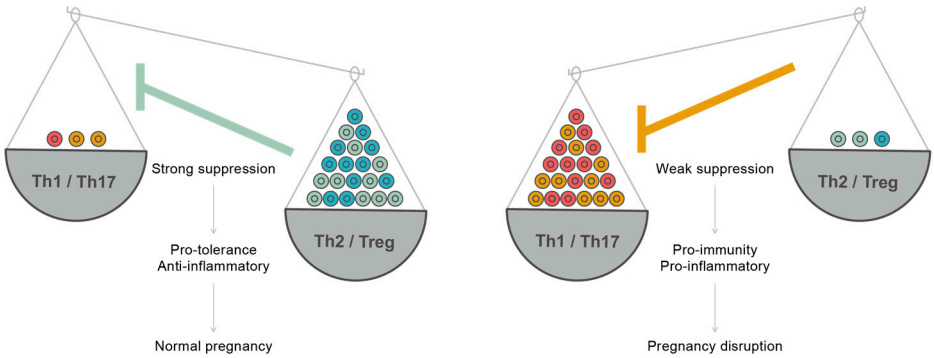

Figure 4. Accurate frequency and function of Treg cells suppress/modulate inflammation and Th1/Th17 subsets. Deficiency in Treg cell numbers and/or suppressive/modulating function leads to pro-inflammation and is associated with for instance pregnancy loss. Reprinted (with minimal atlering) from "Immunology of Pregnancy" Robertson *et al*. in Knobil and Neill's Physiology of Reproduction, 2015 with permission from Elsevier.

Maternal systemic immune response in pregnancy

The interaction between the foetus and the mother occurs locally at the foetal-maternal interface. However, there is evidence that systemic immune changes do occur, probably as a result of placenta-originated cytokines, chemokines, growth factors and hormones together with the release of micro-vesicles derived from the trophoblasts.[59,60] At the local level, immunity seems to be skewed towards a tolerant activity, while at the systemic level more diverse directions of immune responses are recorded. In complications during pregnancy it seems that both the local and systemic immune response skews towards a more pro-inflammatory Th1 immunity.[58] Treg cells are found to be increased in the local decidua although this increased size of the Treg population in peripheral circulation is debated. Some[61] argue that Treg cells increase in the first and second trimesters, peaking in the second trimester, although other evidence indicates there is no increase of Treg cells in the circulation during pregnancy.[58]

The fact that inflammatory and autoimmune diseases such as multiple sclerosis and rheumatoid arthritis are improved during pregnancy supports a systemic influence of pregnancy.[62,63] However, the systemic immune response cannot directly represent the local immune response in pregnancy.[64]

It is important to keep in mind that the immune cells and responses differ depending on whether peripheral blood, endometria or decidua are investigated. The last two are difficult to study in human pregnancy and many results are from murine pregnancies or *in vitro* studies. This should also be taken into consideration when results are interpreted. As the most important place, the uterus, is a "black box" during pregnancy, there are still many knowledge gaps in the area of immunology in human pregnancy.

Pathology of Recurrent Pregnancy Loss

Risk factors for miscarriage and Recurrent Pregnancy Loss

Women with RPL have a higher prevalence of parental chromosomal abnormalities, uterine anomalies, endocrine defects, thrombophilia and autoimmune diseases. It is important to keep in mind that many women with RPL do not only have one risk factor but several, hence RPL should rather be seen as a multifactorial disease regarding its pathogenesis.[65] There is diversity in how extensive investigation for possible risk factors is conducted, which will lead to variations in study results. Investigation and treatment will however not be discussed in detail here.

Uterine anomalies

Anatomic abnormalities may account for 10-20%[66–69] of RPL cases. Several anatomical changes within the uterus have been reported to be associated with RPL but not always with sufficient proof that they are causative.[70] Abnormalities thought to cause RPL are uterine septa, intrauterine adhesion (Asherman's syndrome), fibroids and polyps. There is data to suggest that these structural changes are associated with an increased risk of miscarriage due to impairment of implantation.[65] Diagnostic evaluation of anatomic anomalies includes hysterosalpingography and hysteroscopy.

Endocrinology

Several endocrinological factors might be associated with RPL including thyroid disease, diabetes mellitus, polycystic ovarian syndrome (PCOS), luteal phase defect (LPD) and hyperprolactinemi.

Untreated hypothyroidism has been proven to be associated with RPL,[69] while well-regulated thyroid dysfunction is not.[65,71] Whether anti-thyroid antibodies are associated with RPL in euthyroid women is a matter of debate.[72,73]

Well-controlled diabetes mellitus is not found to be associated with RPL, although insulin resistance has been discovered more frequently in women with RPL compared with controls.[74,75]

The prevalence of PCOS among women with RPL ranges from 8-41% in different studies.[76,77] The association between PCOS and early pregnancy loss may not be direct rather the presence of PCOS-associated hyperinsulinemia, leading to hyperandrogenemia, has been implicated in the pathophysiology of early pregnancy loss. Higher androgen levels might affect the endometrium negatively for implantation.[1] Further studies are needed to understand the relationship between hyperandrogenism and RPL or other possible association between PCOS and RPL.[65]

LPD is thought to originate from inadequate production of progesterone by the corpus luteum and inadequate endometrial maturation for implantation. Decreased progesterone production by the corpus luteum is hypothesised to occur because of poor follicular development, stress, exercise, weight loss or hyperprolactinaemia, or combinations of these. Low progesterone levels may indicate inability to maintain pregnancy. Alternatively, the low progesterone might be the result of a failing pregnancy with low production of hCG leading to low progesterone levels. However, the actual presence of LPD and its relation to miscarriage and RPL is controversial.[65,69,78]

Hyperprolactinemia has been suggested to be associated with RPL although data show no evidence of such an association.[65,79] Normal prolactin levels may play an important role in the growth and maintenance of early pregnancy as high levels inhibits progesterone production. Further studies are required to clarify the role of prolactin abnormalities in RPL.[78]

Chromosomal aberrations

Approximately 2-4%[66,68,69,71] of RPL women are associated with parental chromosomal abnormalities. The most common aberration is balanced reciprocal or Robertsonian translocation. Several studies[80,81] have found a higher risk for a subsequent miscarriage and a lower chance for LBR in couples carrying an abnormal karyotype. It is recommended that couples with RPL are screened for parental chromosomal abberations.[7,69]

Thrombophilia defects

Hereditary thrombophilia involves the factor V Leiden, prothrombin and methylenetetrahydrofolate reductase (MTHFR) mutations as well as deficiency in protein S, protein C or antithrombin. Two meta-analyses concluded that there was an increased prevalence of factor V Leiden and prothrombin polymorphisms in women with RPL compared with controls.[82,83] Protein S deficiency was also

associated with RPL, while no association was found between RPL and protein C and antithrombin deficiency.[83]

The ESHRE RPL guideline group[7] summarises that there is no, or a weak association at best, between hereditary thrombophilia and RPL, and screening for hereditary thrombophilia is not recommended. If additional risk factors are present such as a previous deep vein thrombosis or a family history of thrombophilia screening for hereditary thrombophilia can be considered. This conclusion was drawn since a positive test result does not improve outcomes for the couples.

Acquired thrombophilia refers to the antiphospholipid syndrome (APLS). It has been established that antiphospholipid antibodies (aPL), such as lupus anticoagulant (LAC) and anticardiolipin antibodies (ACA), are associated with RPL.[1,65,69] APLS is defined as repeatedly positive tests for aPL and association with thrombosis and/or pregnancy complications. Morbidity in pregnancy includes unexplained stillbirths at ≥10 GWs, preterm delivery due to eclampsia, preeclampsia, or placental insufficiency, and three or more consecutive miscarriages.[84,85] APLS can be primary or caused by other underlying disorders (secondary), such as systemic lupus erythematosus (SLE). There is consensus that the RPL patients should be screened for aPL because these antibodies can be found with significantly increased prevalence in RPL patients[86] and possible treatment with acetylsalicylic acid (ASA) and/or low molecular weight heparin (LMWH) exists.[87] It is unclear how the antibodies exert their pathological effects. The following suggestions are given: the antibodies act on coagulation factors or placental endothelial cells and thereby promote thrombosis of placental vessels; the antibodies act directly on villous or extravillous trophoblast cells, inhibiting implantation processes or cell fusion; or the antibodies are only markers of other immunological processes harmful to the pregnancy.[70]

Life style and environmental factors

Epidemiological studies have provided evidence that certain life style factors increase the risk of spontaneous miscarriage. Such factors are obesity, alcohol consumption and daily caffeine intake >300 mg. These life style factors are linked to sporadic miscarriages, but the relation to cause RPL is debated.[65,88] There are methodological difficulties in studies of life factors as many studies rely on questionnaires with a risk of recall bias.

Obesity, defined as BMI over 30kg/m^2, is associated with RPL, as shown in several studies. In women with RPL there was a higher miscarriage rate in obese than in non-obese women.[88–91] Lashen et al.[92] showed that obese women had a three times higher risk for RPL than normal-weight women.

It is still uncertain whether *caffeine* is a risk factor for RPL. Some studies[93] suggest caffeine it is while others[94] have not found such an association. The

ESHRE guidelines[7] have concluded that it is unclear whether caffeine is a risk factor for RPL.

Stress is suggested to influence the risk of miscarriage and RPL but it is difficult to study, and the impact of stress is today unclear at present. There is a low quality of evidence to determine whether stress is a result of RPL or whether it is a causal factor.[7] Prospective studies are warranted.

Increasing *maternal age* is a predictive factor for miscarriage rate.[28] The prevalence of aneuploidy miscarriage increases with higher age.[80,95] Women over 40 years of age have a higher risk for RPL[7] and worse prognosis for a live birth.[11]

Environmental factors like air pollution and chemicals and their association with RPL are poorly studied. A review[96] reported an association between air pollution, chemicals bisphenol A, some phthalates, pesticides, fracking chemicals and miscarriage. The possible mechanism through which these act and how they affect the risk of RPL needs to be determined.

It is important to keep in mind that there can be more than one factor causing RPL and it might be possible that several factors are required for RPL to occur. There are still a large group of women where no cause or risk factor for RPL is found, and further studies are needed.

Idiopathic Recurrent Pregnancy Loss

About half of the women with RPL who are investigated for risk factors are left without a possible explanation for the miscarriages.[68,71,97] This group has been widely discussed and there has been debate about whether idiopathic RPL may be just a chance occurrence.[98] Saravelos et al.[99] argue that there are two subgroups of idiopathic RPL. Group I comprises women with RPL that has occurred predominantly by chance, and the women have no specific underlying pathology. This type has a relatively good prognosis for live birth in the future. Group II refers to women with unexplained RPL that occurs due to an underlying pathology, not currently identified by routine clinical investigations or due to significant environmental and lifestyle risk factors. This type has a poorer prognosis compared with women of a similar age.

Euploid vs. aneuploid pregnancy loss

If no maternal cause for RPL is found it is classified as idiopathic RPL, although some of these pregnancy losses might have a foetal cause with chromosomal aneuploidy. The rate of aneuploidy embryos causes about 50-70% of sporadic miscarriages.[100–102] Ogasawara et al.[100] have shown that abnormal chromosomal rate increases as the maternal age increases and the number of miscarriages decreases. They also found that the embryonal aneuploidy rate was significantly lower in women with RPL compared with controls. Still, it is debated whether women with RPL have pregnancy loss less frequently with abnormal chromosomal karyotype.

Several studies[101,103–105] have investigated women with idiopathic RPL and their subsequent miscarriages in regard to chromosomal abnormality. The prevalence of abnormal embryonic karyotypes in these women is reported to be 25-51%, which is lower than the prevalence of 70% expected in sporadic miscarriages.[101] This indicates that a different mechanism other than the aneuploidy karyotype may be responsible for the majority of RPL. However, other studies have reported the prevalence of truly unexplained RPL to be only 10-24% of all RPL women after excluding women with abnormal embryonal karyotypes in the idiopathic group.[68,100]

In general, for all studies only one particular pregnancy loss was karyotyped, which does not necessarily rule out other underlying conditions responsible for RPL. Further studies in this area are warranted. Whether the truly idiopathic RPL group is smaller than previously suggested is yet to be proven.

Immunological mechanism in Recurrent Pregnancy Loss

It has been suggested that pregnancy complications, such as RPL, might have an immunological aetiology.[106] Christiansen et al.[107] reported an increased prevalence of autoimmune diseases, such as SLE, inflammatory bowel disease and hyper/hypothyreosis, in women with RPL compared with historical control groups. It has been suggested that an autoimmune disease can be a marker for increased sensitivity to impaired immunological tolerance to trophoblastic antigens.[47] It has also been suggested that women with RPL are already predisposed to a generally higher inflammatory response before pregnancy.[108,109] Supporting this, the TNF/IL-10 and IFN-ɣ/IL-10 T cell ratio in peripheral blood was found to be increased in non-pregnant women with RPL compared with controls.[110] TNF was also suggested to be a risk factor for a new miscarriage.[111] Aberrations in NK cells, both in the blood and in the endometrium, have also been associated with RPL. It has been suggested that there is increased NK cell activity and cytotoxicity in peripheral blood in women with RPL compared with controls.[112] Compared with

controls women with RPL have been reported to have a higher proportion of the more cytolytic active dNK cell phenotype, $CD16^{+}CD65^{dim}$, and a lower percentage of the $CD16^{-}CD65^{bright}$, which are normally dominant in the decidua. An increased number of the cytolytic NK cells in women with RPL has been suggested to be causative of pregnancy loss.[112]

In addition to dysfunction in the innate immune system, dysfunction in the adaptive immune system is also believed to be required for pregnancy to fail.[47] Lissauer et al.[113] have found an increased Th1 and Th17 response in non-pregnant women with RPL. This is in line with other studies showing an enhanced inflammatory response with elevated pro-inflammatory cytokines like TNF, IFN-ɣ and IL-6 in women with a history of RPL.[106,108,109] Normal pregnancy seems to have a higher ratio of Th2/Th1 than pregnancy in women with unexplained RPL.[106,114] An increased production of IFN-ɣ in women with idiopathic RPL implies a Th1 bias in these women.[108] Although a Th1 response is necessary at pre-implantation and in the first stages of implantation, an excessively strong Th1 immune response is suggested to be harmful for pregnancy.[30,115]

Aberrations in both the frequency and/or function of Treg cells have been reported in women with RPL.[58,61] Several studies[61,110,116–120] have shown a significant decrease of Treg cells both in the decidua and peripheral circulation in women with RPL compared with controls. Wang et al[121] found an increase in Th17 cells in both circulation and locally in the decidua in idiopathic RPL women compared to normal pregnant women. The ratio of Th17/Treg has been reported as increased in women with RPL compared to normal pregnant and non-pregnant women.[116]

Another theory presented is that endometrial cells in women with RPL fail to express the necessary decidua phenotype for a successful pregnancy. An impaired decidualisation might be more receptive to implantation and the "window of implantation" may be prolonged. The impaired decidua may also lack natural embryo selection, which may be presented as miscarriage, regardless of the embryonic karyotype.[122]

Placenta-associated disorders

As described earlier, a well-coordinated interplay between the maternal immune cells and foetally derived cells is needed to develop a functional placenta. Accordingly, immune cells play a vital part in the invasion of the trophoblasts into the decidua. If the trophoblasts do not sufficiently invade and remodel the spiral arteries, this leads to oxidative stress and placental dysfunction. The throphoblastic plug maintains a hypoxic environment, which is favourable for cell differentiation and protects from the damaging effect of oxygen free radicals.[35,123] If the placentation is completely impaired it leads to miscarriage, but if impairment is

partial it will lead to placenta-associated disorders, such as preeclampsia, IUGR, premature birth, stillbirth and placental abruption.[29,124] It has also been suggested that an earlier dysfunction leads to pregnancy loss while dysfunction at a slightly later phase leads to preeclampsia.[35,64] Preterm labour has multiple pathological aetiologies, for example infection and cervix insufficiency. A subset of women with preterm birth present vascular lesions. Vascular lesions consist of failure of physiologic transformation of the spiral arteries during placentation, which can lead to utero-placental ischemia and thereby preterm birth.[125–127] Failure of the physiological vascular transformation is a sign of defective placentation and seen in preeclampsia, IUGR, preterm birth, placental abruption and pregnancy loss.[128]

The basic pathophysiology in placenta-association disorders is not fully understood. The possible continuum from early pregnancy loss to preeclampsia needs further investigation.

Medical treatments of idiopathic Recurrent Pregnancy Loss

Despite lack of evidence, different medical treatments are frequently used to reduce the risk of a subsequent miscarriage and to increase the live birth rate (LBR) in women with idiopathic RPL. Some treatments have a postulated immune modulating effect on the pregnancy. These treatments include intravenous immunoglobulin (IvIg), leukocyte immunisation treatment (LIT), progesterone, LMWH and ASA, while some of these drugs are also given on the basis of their antithrombotic effect, such as LMWH and ASA.

IvIg can inhibit the immune system with suppression of autoantibodies, attenuation of NK cells, inhibition of complement binding, modification of cytokine production and expansion of Treg cells.[129]

LIT has been used as a treatment in idiopathic RPL women based on the theory that a miscarriage is an act of a rejection, as in an organ transplant, because of improper HLA matching between the mother and father. The theory is that the mother lacks anti-paternal antibodies that protect the foetus from rejection. Injection with foreign, either paternal or third party leukocytes, could work on the same basis that pre-transplantation blood transfusion has improved organ graft survival.[130] Subsequent production of antibodies, protecting the foetus, should in theory be induced by LIT.[130,131]

Progesterone is a known anti-inflammatory hormone[44] that induces Th2-associated cytokines, which promote a pregnancy favourable environment.[132]

Progesterone helps to mature the endometrium and regulate local cytokines in preparation for implantation. It has been shown that progesterone can suppress

Th1 and enhance Th2 immunity and thereby could potentially modulate the Th1/Th2 balance to ensure a successful pregnancy.[132–135]
LMWHs is known for its anticoagulation effect and is widely used for prophylaxis during pregnancy in thrombotic diseases.[136] In addition, heparin has been suggested to possess anti-inflammatory effects including suppression of NK cytotoxicity, antagonising IFN-ɣ signalling and inhibiting the complement system, and hence acts as an immune modulator in pregnancy. LMWH also enhances several growth factors essential for trophoblast survival.[136–138] Today, the golden standard is to treat women with APS and RPL with LMWH in combination with ASA to improve the obstetrical outcome.[139]
ASA is used with the rationale of preventing placental thrombosis based on the hypothesis that RPL is caused by impaired placental circulation due to microthrombosis.[140] It has been suggested that women with RPL have a pro-thrombotic phenotype with increased levels of pro-coagulant factors and decreased levels of naturally occurring anticoagulants during pregnancy. The combination of circulating pro-coagulant micro particles and a defective maternal haemostatic response could lead to platelet aggregation and thrombosis in the placenta.[141] ASA is an antiplatelet agent that inhibits platelet cyclo-oxygenase and thereby decreases potent vasoconstrictors and platelet activators.[142]

Research results for treatment efficiency on live birth rate are contradictory, and there is no definite evidence that supports any of the treatments in idiopathic RPL. Methodology and definition differences, together with heterogeneous study groups of women with unexplained RPL are some of the reasons for this diversity in the results, and will be discussed in more detail in Paper III. Since there is no clear consensus yet, further studies are needed.

In summary

RPL is a multifaceted problem with several subgroups and heterogeneous aetiologies. It becomes even more complex in the absence of straightforward definitions of the concept of miscarriage and RPL. Without consensus on definitions of RPL is it is difficult to examine what causes it and to come to a conclusion on effective treatments. Like many other multifactorial disorders, RPL should not be investigated with the aim of resolving only one issue. The topic needs to be approached from different angles. In this thesis, epidemiology, immunology, treatment and obstetrical outcome are explored.

Aims of the thesis

The general aim of this thesis was to improve knowledge of RPL related to the areas epidemiology, immunology, treatment and obstetrical outcomes.

The specific aims were:

I. To study the incidence of the diagnosis of RPL in the National Patient Register in Sweden from 2003 to 2012.

II. To study the validity of the ICD-10 diagnosis codes of RPL (N96.9, O26.2) registered in the National Patient Register.

III. To systematically review the evidence for different treatments' efficiency at improving live birth rates in women with idiopathic RPL.

IV. To assess if treatment with low-molecular-weight heparin has immunological effects during pregnancy in women with a history of idiopathic RPL in a randomised controlled trial.

V. To study if women, with a subsequent pregnancy after the diagnosis of RPL, have an increased risk of adverse obstetrical outcome.

Methods

Study material

In this thesis, three of the studies (Papers I, II, V) were observational register-based studies, and therefore the sources of information will be described: the Swedish National Patient Register and Medical Birth Register, both delivered by the National Board of Health and Welfare.

The National Patient Register

The National Board of Health and Welfare (NBHW) is a government agency, which maintains health registers and official statistics. One of these registers is the National Patient Register (NPR) that started to collect information regarding inpatients in the 1960s. It is mandatory for all county councils in Sweden to contribute with information to this registry. From 1987 the NPR included all inpatient care and it has a coverage of 99%, and from 2001 it has also covered outpatient visits from both private and public caregivers.[143]

Information is delivered to the register to the NBHW once a month from each of the county councils. The information consists of admission and discharge dates, hospital ID, primary and secondary diagnoses, as well as procedures. All information is connected to the individual personal identification number (PIN). Register data is protected by strict confidentiality but individual-based data can be made available for research after a special review and the granting of ethical consent from a regional ethical board. A quality and validity control of the register is performed by the NBHW on the submitted data. If the data is incorrect above a certain threshold new data is requested.[143]

The Medical Birth Register

The Swedish Medical Birth Register (MBR) started in 1973 and includes data on deliveries and covers more than 98% of all births in Sweden.[144] It is compulsory for every health care provider to report to the register and the information available is collected from medical records from the prenatal, delivery and

neonatal care services provided. After delivery, the responsible midwife or doctor records the woman's diseases and complications during pregnancy and delivery, according to the International Classification of Diseases (ICD). A number of ongoing diseases are indicated in check boxes where information is provided based on interviews performed by midwives.

The information in the MBR includes demographic data of the mother e.g. country of birth, smoking status, BMI, information on reproductive history, gestational age, mode of delivery, diagnoses of mother and child, and complications during pregnancy and delivery and in the neonatal period.[145]

A quality report was made,[144] in which data from 500 patients in the MBR was compared to the information found in the original medical records. Data was in general found to be consistent; for example information on birth weight was lacking in the MBR for only 0.32% of infants, and the vast majority of birth weights were correct. For the diagnosis of stillbirth, 1-2% of records were missing, which is still acceptable although it may lead to an underestimation of stillbirth.

The Swedish personal identification number

Since 1947, every individual who is a permanent resident in Sweden has been assigned a unique PIN, which is recorded in the Total Population Register. Immigrants without a residence permit receive a temporary PIN.[146] The PIN is the unique identifier and the key variable when matching between different registers including the NPR and the MBR. When such a linkage is made, the PINs of all study participants are removed from the data files, and replaced by unique "serial numbers" which are delivered from the NBHW to the researcher. Hence the researcher cannot use the delivered data to discover the identity of patients or controls.[147]

Study subjects and study design

Paper I.

Paper I included women 18-42 years of age, registered in the Swedish NPR from 2003-2012, with the ICD-10 codes of RPL (N96.9 or O26.2). Women without the diagnosis codes of RPL in the register, but who had experienced three or more consecutive miscarriages, diagnosed with the following ICD codes (primary or secondary diagnosis): O02.1, O03 (including sub-diagnosis O03.0-O03.9) were also included. The last group was included since it was considered a "false

negative", as the definition of RPL was fulfilled although no registrations of RPL codes in the register were made.

This was an observational register-based study where the main outcome was the incidence of RPL diagnosis over a 10-year period. This incidence was calculated as the number of new women diagnosed with RPL per year in a risk population. As the population at risk was not well defined in the literature two different risk populations were defined; 1) women of fertile age (18-42 years) and 2) women who had been pregnant (miscarriage or childbirth) in the year.

Paper II.

A sample size of 711 women diagnosed with the ICD 10 codes of RPL (N96.9 and/or O26.2) during the period 2003-2012, in the NPR was randomly selected by the NBHW. The sample data selected by NBWH comprised a PIN and a hospital and clinic code for each woman. All relevant available hospital clinics were contacted to obtain medical records for 485 women, where 254 medical records from 38 hospitals were obtained and 238 individual samples were conclusive for assessment.

A cross-sectional observational study design was used to validate the RPL diagnosis registered in the NPR and compared it with the information obtained in the medical records. The medical records were considered as the “reference standard”. The main outcome was to estimate "true positive" with RPL of those who were registered with RPL diagnosis in the NPR.

Paper III.

This systematic review included randomised controlled trials on treatments with ASA, LMWH, progesterone, IvIg or LIT to improve LBR in women with three or more consecutive miscarriages of unknown cause. After 126 full articles were reviewed, 21 RCTs were included for assessment.

To identify relevant literature a systematic search was made in the MEDLINE PubMed, EMBASE, and Cochrane Library databases using the search terms: “recurrent miscarriage”, “recurrent spontaneous abortion”, “recurrent pregnancy loss”, “habitual abortion”, and therapy. The search-line for therapy was: “LIT”, “leukocyte immunotherapy”, “paternal lymphocyte immunisation”, “third party donor”, “pre-implantation genetic screening”, “heparin”, “low-molecular-weight heparin”, “lipid emulsion”, “progesterone”, “acetylsalicylic acid”, “psychological support”, “tender loving care”, “supportive care”, “emotional support”, “intravenous immunoglobulin”, and “corticosteroids”. The search was filtered for

studies on humans, conducted in English or Scandinavian languages. There was no time limit for first publication and the last search was conducted in September 2017. The flow chart illustrates the selection process (Figure 5).

This systematic review was conducted according to PRISMA guidelines.[148] A population, intervention, comparison and outcome (PICO) were defined (Table 2). Two authors in the research group independently identified trials fulfilling the PICO criteria for inclusion, assessed the risk of bias, and extracted data from all included trials. The main outcome was to evaluate evidence for treatment efficiency regarding live birth rate and complications. The Grading of Recommendations Assessment, Development and Evaluation (GRADE) criteria were followed to assess the certainty of evidence across the selected studies.[149]

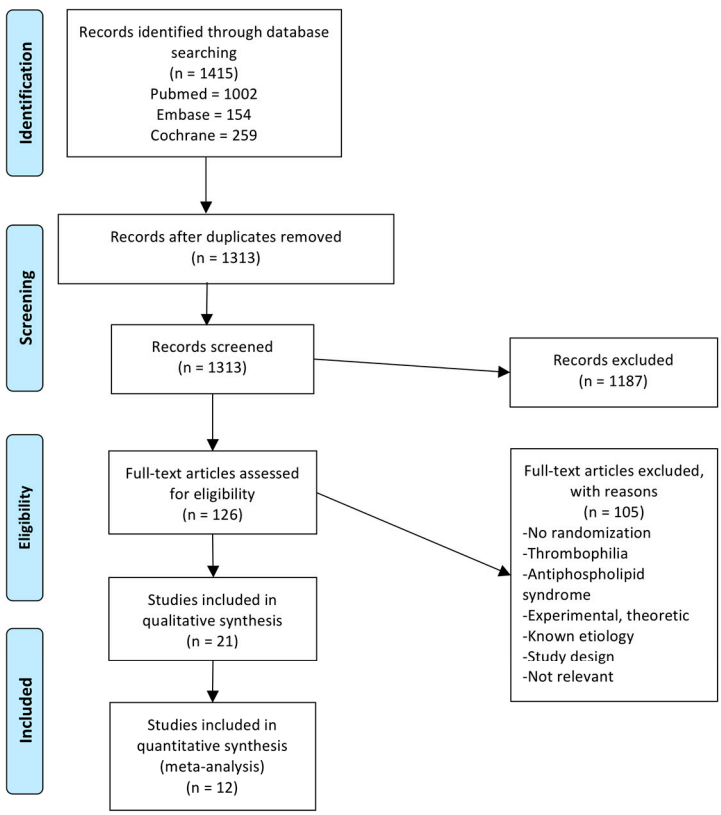

Figure 5. Flow chart of the literature search.

Table 2. PICO: Population, Intervention, Comparison, and Outcome of the systematic review on treatment efficacy in unexplained recurrent pregnancy loss.

Population (patients)	1 ≥3 miscarriages <22 gestational weeks No known risk factors
Intervention (treatment)	Acetylsalicylic acid Low molecular weight heparin Progesterone Corticosteroids Intravenous immunoglobulin Leukocyte immunotherapy Lipid emulsion Tender Loving Care Pre-implantation genetic screening
Comparison	Placebo No treatment All treatments in interventions
Outcome	Live born children Pregnancy ≥22 weeks of gestation Complications

Paper IV.

This study included 87 pregnant women with a history of unexplained RPL recruited from six centres in the southern part of Sweden; Helsingborg, Lund, Kalmar, Jönköping, Karlskrona and Linköping from January 1, 2012 to December 31, 2015. Of the included participants, 45 women were randomised to treatment with tinzaparin and 42 women to no treatment. For blood sample analyses of the included women, there were 35 participants in each group after six were excluded, due to loss to follow-up or did not fulfil the study protocol. Another seven miscarried and four samples were missing.

This open multi-centre randomised controlled trial's main outcome was to study LMWHs immune modulatory effects during pregnancy in women with a history of RPL. Samples of plasma were longitudinally collected during pregnancy at GW 6, 18, 28 and 34. The samples were analysed by multiplex bead technology for levels of 11 cytokines and chemokines, chosen to represent inflammation and T-helper subset-associated immunity.

Paper V.

This retrospective register-based cohort study consisted of a cohort of women with pregnancy continuing >22 GWs, registered in the MFR, with and without a history of RPL. Women registered with ICD-10 codes of RPL, N96.9 or O26.2 in the

NPR during the period 2003-2012, and their subsequent childbirth registered in MFR were included. A comparison group without a history of RPL who had given birth and were registered in the MFR was selected by the NBHW. Only singleton pregnancies were included.

Pregnancy complications of women with and without RPL were compared in the two groups. The main outcome measures were: preeclampsia, IUGR, placental abruption, spontaneous preterm birth and IUFD.

Guidelines of epidemiological studies and meta-analysis

Strengthening the Reporting of Observational Studies in Epidemiology (**STROBE**)[150] is a network of methodologists, researchers, and journal editors that was established to strengthen the reporting of observational research. Observational studies are common in epidemiology and have a significant role in research where they can sometimes answer study questions that are not suitable for RCTs. It is also important that epidemiological observational studies are fully transparent, so that the study plan, methods, results and conclusion are easy to follow. STROBE has assessed recommendations on transparent reporting of cohort, case-control and cross-sectional studies. Their checklist of 22 items,[151] which should be addressed in studies of analytical epidemiology, was used in studies I, II and V.

The *Preferred Reporting Items for Systematic Reviews and Meta-Analyses* (**PRISMA**)[152] statement was developed by a group of 29 review authors, methodologists, clinicians, medical editors, and consumers specifically to improve the reporting of systematic reviews and meta-analyses. A PRISMA checklist[153] has been developed and is considered essential for transparent reporting in systematic reviews and meta-analyses to allow readers to assess the strengths and weaknesses of the investigation. The PRISMA also includes a flow chart to show numbers of identified records, excluded articles, and included studies (Figure 5).[154] Paper III followed the PRISMA statement methods.

The Grading of Recommendations Assessment, Development and Evaluation (**GRADE**) group has developed a common, sensible and transparent approach to assess and grade the quality of evidence and strength of recommendations when systematic reviews or guidelines are conducted.

The approach provides a structured and transparent evaluation of the importance of outcomes. It takes into account *study design*, *study quality*, *consistency* and *directness* in assessment of the quality of evidence in the selected outcomes (live

birth rate and complications in Paper III). *Study design* refers to RCT or observational studies. *Study quality* refers to the detailed study methods and execution where assessment for different biases is evaluated in each individual study (Table 3). All risks of bias are joined in a combined evaluation.

Table 3. Risk of bias

Type of bias	Explanation
Selection bias	Handling selection of study population intervention- and control population
Performance bias	If intervention and control group are exposed to something other than the study they are intended to measure
Detection bias	Regarding measurements and analyses of the results (blinded, right statistical method, right timepoint)
Attrition	Handling of dropouts
Reporting bias	How results are reported regarding the original study protocol (post-hoc analysis, subgroup analyses of negative results)
Other considerations	Financial or other conflicts of interest

Consistency or heterogeneity refers to the similarity of estimates of effect across studies. *Directness* refers to the extent to which the people, interventions, and outcome measures are similar to those of interest.

The balance between benefits and harms, quality of evidence, applicability, and the certainty of the baseline risk are all considered in judgements about the strength of recommendations. Recommendations should be stated as follows:

- High: Further research is unlikely to change our confidence in the estimate of effect.
- Moderate: Further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate.
- Low: Further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate.
- Very low: Any estimate of effect is very uncertain.

The GRADE system was systematically used in Paper III.

The *Consolidated Standards of Reporting Trials* (**CONSORT**)[155] statement was developed by a group of scientists and editors to improve the quality of reporting of RCTs. It was first published in 1996 and updated in 2001 and 2010. The statement consists of a checklist[156] and flow diagram[157] that authors can use for

reporting an RCT. RCTs are often described as the "golden standard" because of their ability to minimise bias and rule out confounders, although inadequate reporting and design are associated with biased estimates of treatment effects. Without transparent reporting, other researchers cannot judge the reliability and validity of trial findings or extract information for systematic reviews. Therefore, it is important to follow the CONSORT statement, which is done in Paper IV.

Laboratory methods

Luminex

The Luminex method is a bead-based sandwich immunoassay that combines the principle of the traditional enzyme-linked immunosorbent assay (ELISA) with flow cytometry. It allows simultaneous detection of a large number (up to 500 analytes) of extra-cellular molecules, such as cytokines and chemokines in small sample volumes.

The technique is based on microspheres, called "beads", which are individually dyed with a fluorescent colour (red and infra-red) in different concentrations, and is specific to a particular analyte. Each bead is pre-coated with an analyte-specific capture-antibody. This antibody binds to the analyte of interest, e.g. cytokines/chemokines. Another biotinylated detection-antibody, specific to the analytes of interest, is added and forms an antibody-antigen "sandwich" (Figure 6). Thereafter, another complex, streptavidin conjugated with the fluorochrome phycoerythrin, is added. Biotin, on the detection antibody, binds to several streptavidin molecules with high affinity, which makes a "bead complex" and the fluorescence signal from phycoerythrin determines the amount of the bound analyte by flow cytometry (e.g. Luminex 200).[158]

The beads are measured by two laser signals (dual laser flow cytometry); one red laser excites the red dyes of the bead and classifies the bead according to light emission, and the other laser excites the green dyes of the reporter fluorochrome (phycoerythrin). The intensity of the emitted green light is directly proportional to the amount of analyte bound to the surface of the bead.[158]

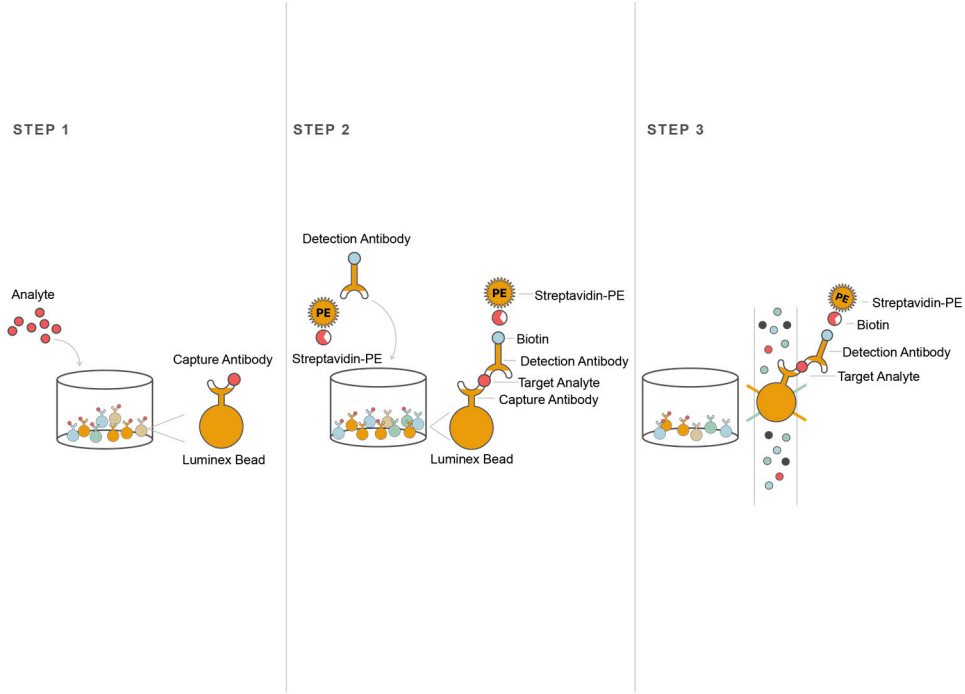

Figure 6. Luminex method.
Step 1. The sample is added to a mixture of colour-coded beads, pre-coated with analyte-specific capture antibodies. The antibodies bind to the analytes of interest (cytokines/chemokines).
Step 2. Biotinylated detection antibodies specific to the analytes of interest are added and form an antibody-antigen sandwich. Phycoerythrin (PE)-conjugated streptavidin is added, which binds to the biotinylated detection antibodies.
Step 3. Luminex beads are read on a dual laser flow-based analyser. One laser classifies the bead and determines the analyte that is being detected. The second laser determines the magnitude of the PE-derived signal, which is in direct proportion to the amount of analyte bound. Illustrated by Elinore Frolova.

Statistical methods

Statistical analyses were made with IBM SPSS STATISTICS Versions 23 and 24 for Mac (IBM Corp., Armonk, NY, USA) in Papers I, II, IV and V. REVIEW MANAGER 5.3 (RevMan) was used in Paper III.

A difference with a p-value of <0.05 was considered statistically significant in all studies.

In *Paper I*, descriptive statistics were presented and the incidence calculated as: "number of new cases of a disease per year"/"population per year". A description of incidence over the 10-year period was presented in two risk populations, women aged 18-42 years and pregnant women. Data on mean age of the women diagnosed with RPL for each year was presented.

In *Paper II*, the positive predictive value (PPV) was calculated as: [true positive register cases] divided by [true positive in reference standard] plus [false positive register cases] with 2×2 cross tables and a χ-square test with 95% confidence intervals (95% CI). Sensitivity was estimated as: [true positive register cases] divided by [true positive register cases] plus [false negative register cases] with 2×2 cross tables and a χ- square test with a 95% CI. A sample size analysis, assuming that PPV would be 85% and the accepted width of a 95% CI was 10 percentage units (e.g., 80–90%), calculated that a sample size of 228 medical records were needed to achieve a power of 90%.

In *Paper III*, data was extracted and pooled in meta-analyses when possible. This procedure was carried out using RevMan. A random-effects model was applied. This model was used, as it is a more robust model than fixed-effect model, when the effect is small or the heterogeneity of the studies is high. The random-effects method and the fixed-effect method will give identical results when there is no heterogeneity among the studies. However, when heterogeneity is present, an estimated CI in the random-effect model is wider than a CI in the fixed-effect model, and the preferable model to use. Statistical heterogeneity was examined using the χ-square test. I^2-characteristics were used to quantify inconsistency across studies, which tell more about its impact on the meta-analysis than the heterogeneity. I^2 describes the percentage of the variability in the effect estimates.[159] Clinical sources of heterogeneity were explored by stratified analyses.

The effect estimate was expressed as a risk ratio (RR) with a 95% CI. Forest plots presented the individual studies and the pooled estimates graphically.

In *Paper IV*, a priori power analysis showed that the sample size of 70 women was sufficient to detect a 40% decrease or increase in an immunological parameter (CXCL10) with a power of 80% and a α-value of 0.05.

The data on cytokines and chemokines were not normally distributed, tested with a Kolmogorov-Smirnov test. It was therefore normalised by logarithmic transformation. First, Linear Mixed Models (LMM) was used to evaluate differences between treated and untreated women of the predefined cytokines and chemokines at four time-points, including inclusion, and throughout pregnancy. This model allows for both within and between subject variations and estimates the data longitudinally. If $p<0.05$ in the LMM, a post-hoc test was done to decide at which time point(s) and in which direction there was a difference. Since only three chemokines were <0.05 in the linear mixed model test, multiple testing was not a major problem and the post-hoc test in these cases was performed with an ordinary Student´s t-test or in one case with Fisher's exact test, because of the low proportion of samples with detectable levels. With the exception of CCL20, the cytokines and chemokines were detectable in most samples. In a multivariate test

like repeated measures ANOVA, missing data at one time point, will drop those women from the entire analysis. In the mixed approach, only that time point will be dropped and the remaining data will be retained. Data was expressed as geometric means and 95% confidence intervals in the figures and tables. Graphs and figures were made using GraphPad Prism version 7.0.

In *Paper V*, basic descriptive statistics were used to describe maternal characteristics for women with and without a history of RPL at each pregnancy. Comparison in characteristics between the groups was done with χ-square tests. Logistic regression analysis was used, as our outcomes were binary (yes/no, disease/no disease). Analyses were performed to assess the association between history of RPL and the risk of placenta-associated disorders (preeclampsia, IUGR, preterm birth, placental abruption and stillbirth). The results were reported as proportions and odds ratios (OR) with a 95% CI. Since the absolute risk of the obstetrical outcomes were low OR could be used equivalent to relative risk.

First, confounders were tested for in univariable analyses. If p was <0.2 for the plausible confounder in the univariate analysis, it was included in the multiple logistic model. Adjustment for confounding factors was obtained by estimating the adjusted OR (AOR) in a stepwise unconditional multiple logistic regression analysis. The regression model was adjusted to relevant confounder variables, as following, for each different outcome: maternal age, early pregnancy BMI, smoking habits, the mother's nationality, assisted fertilisation, chronic hypertension, and pre-gestational diabetes. These factors were chosen because of their known or potential association with RPL and the placenta-associated disorders.

Missing values were handled with imputed mean for BMI. Multiple imputations would not play a significant difference to the imputed mean, since the frequency of missing data is low (about 10%). Missing data for SGA infant was assumed as normal birth weight. Missing data for preterm birth were assumed as term birth.

Methodological considerations

Papers I, II and V are register-based studies with data from NPR and MFR. Register-based studies have several strengths. Epidemiological studies are economical when they are based on already existing registers comparing to other study designs, such as RCTs. A great strength is that data already exists so data collection is more efficient and less expensive in register-based studies. Data is collected independently to the study, which reduces recall bias. Another advantage is that register-based research has a large sample size, which gives great statistical power. Registers are non-selective regarding inclusion, which limits selection bias

and registers do not have problems with non-response and loss to follow-up of participants, which are issues found in for example RCTs.[160]

Some of the strengths of register-based research can also be limitations. As data is retrospectively collected it can result in limitations due to missing necessary information and inaccurate or misclassified data. The investigator is limited to using the variables in the register and to the level of detail reported, and cannot control variations in coding by persons, departments, hospitals and regions or over time. There can, for example, be a diagnostic drift towards diagnosis codes that give more financial compensation.[160]

Register-based research with large populations can detect even small and unimportant differences between groups, with high statistical significance, even though they may not be of clinical relevance. Sometimes registers lack confounder information, which makes it difficult to adjust an outcome for possible confounding. Missing data is also a limitation that can affect the quality of the register. Missing data will affect all estimates of prevalence, but will usually have little impact on risk estimates if the lack of information is random.[145]

The validity of the register is important for knowledge about the completeness of the register, i.e. whether all individuals are included in the register. One method to check this is to make a comprehensive patient chart review to evaluate whether all patients with a given disease are correctly classified into the register. Such review procedure can provide a positive predictive value. This is done in Paper II. False and true negative individuals are difficult to identify and if they are missed, this can lead to underestimation of an incidence.[160]

A limitation in observational studies is that no causality can be proven, although associations can be found. This is one weakness in Paper I where the incidence of RPL diagnosis seems to be increasing but, assuming that the finding is correct, the study design cannot explain why.

Selection bias and missing data

Register-based research regarding miscarriages and RPL has to be interpreted carefully to avoid biased conclusions. It is well known that far from all women with miscarriage come to a health care unit but instead they miscarry at home. Even women who come to health care units are not always offered an examination by a physician. Only those women diagnosed by a physician will be registered with an ICD code in the NPR. This leads to a possible selection bias in the group of women who get examined and diagnosed with miscarriage vs. the group that does not come to a health care unit or get examined. The missing diagnosed women in the NPR, "false negatives", are of great concern when estimating an

incidence of RPL diagnoses. The RPL diagnoses are often registered after asking the woman about her medical history. So even if many miscarriages are not registered in the NPR, the diagnosis of RPL, set after asking the women about medical history, can still be adequate. Most important is to know the weaknesses of data when interpreting results, which is why Paper II, validating diagnosis codes of RPL in the NPR, is of great importance. This gives a good indication of how well we can trust the register.

Missing data can be a problem in analyses and is a potential source of bias when interpreting results. It is of great importance to be aware of the extent of missing data and how to handle it. This can be done with different statistical methods, for example imputed mean (used in Paper V). Missing data also needs to be presented properly to ensure transparency of data.

Strengths and limitations in meta-analysis

A well-designed systematic review and meta-analysis can provide valuable information for researchers and clinicians as they summarise and integrate results from several individual studies. Also, differences in the results between studies can be analysed. Furthermore, small sample sizes in individual studies can be overcome by combining the results to detect effects and analyse end points that require larger sample sizes and thereby increase precision when estimating effects. A meta-analysis can determine whether new studies are needed in a research area and can also generate new hypotheses for future studies. However, there are several potential pitfalls when performing and interpreting meta-analysis, which can cause misleading conclusions.[161]

One critical issue in meta-analysis is the identification and selection of studies. When searching relevant studies, much effort is needed to secure the right set of key words so as not to miss relevant studies. Publication bias is another problem when identifying articles, as only published studies are available in most databases.[161] Published studies tend to skew towards positive results, as compared to those that do not show any effects, they are more likely to be published, because of both investigator and editorial preferences.[162] Another critical step is the selection of studies, where differences between studies are to be reduced, duplicates eliminated, and data quality assessed. In order to eliminate selection bias, it is important to use predefined criteria that are checked for in each study (objectives, study population, study design, sample size, treatment, selection criteria, outcomes, quality of the data, analysis and reporting of results, accounting and reporting of attrition rates, length of follow-up, and when the study was conducted). This is followed in Paper III. Still, subjectivity to some extent is difficult to avoid.

Heterogeneity refers to the degree of dissimilarity in the results of individual studies and if the level of heterogeneity is high it becomes more difficult to justify a pooling of the results in a meta-analysis. A forest plot (as in Paper III) can visualise if the results are heterogenic or contradictory. Often (including Paper III) meta-analysis only includes RCTs, although some outcomes can only be measured in observational studies. It is therefore not necessarily better always to stick with RCTs. RCTs are seen as the golden standard, but meta-analyses provide valuable complementary information.

In Paper III there was a great diversity in study designs, treatment start and doses, which made it difficult to pool studies in meta-analyses. The fact there was no clear consensus on the definition of RPL made it even more complex to compare or pool study populations. A strict definition of three or more consecutive miscarriages was used in this review, and therefore several studies with the definition of only two miscarriages were excluded. This could have altered the results concerning the efficiency of a treatment. Women with two miscarriages have better prognoses on LBR, than women with three miscarriages and the effect of treatment would be difficult to interpret if studies with different definitions were included.

Confounding

A confounder is a variable or factor that influences both the exposure (independent variable) and the outcome (dependent variable) studied, independently of each other. Confounding can lead to both false negative and false positive associations. Confounders that are often found are gender, age, smoking and BMI. One example of a confounder factor from Paper V is age, which influences RPL and preeclampsia, independently of each other.

There are different ways to avoid confounding factors, either in the study design or when analysing the results. During the design phase, confounders can be avoided by randomisation (Paper IV) and by limiting the study population to exclude cases with the confounding factor (e.g. by excluding smokers). It can also be done by matching cases and controls so confounders are evenly distributed among the study population. If randomisation or matching has not been possible it is important to take that into account when analysing, so adjustments can be made. This is done in Paper V. One example is the adjustment for age and SGA when analysing the association between RPL and preeclampsia, although unknown confounders might remain.

Laboratory methods

The Luminex method can analyse several cytokines and chemokines simultaneously, which saves time, cost, and sample volume. This makes it possible to systematically examine the effects of a clinical intervention. Conventional methods are both more time-consuming and costly than the Luminex.[163]

The method has some limitations that need to be taken under consideration. The dyed microspheres are light-sensitive and must be kept at a temperature of 4ºC and can potentially aggregate. It is also important that cross-reactivity is checked for and excluded, so that the antibodies do not detect other analytes than those intended. It is an advantage that these assays generally have a broader dynamic range than classical ELISAs.[158] However, the sensitivity in the very low range of concentrations is surpassed by some other methods. When reporting exact values with multiplex bead arrays it can be difficult to draw parallels with other arrays such as ELISA, and caution is needed if a comparison is made.[163]

Measuring inflammatory responses in pregnancy

A potential limitation in Paper IV was that cytokines and chemokines in maternal peripheral blood are not equivalent to the foetal-maternal interface environment. Several cytokines are produced by the embryo itself and by the endothelial tissue of the female tract, which modifies both autocrine and paracrine regulations.[30] It is not known how the maternal immunological response in peripheral blood reflects this local immunological milieu. There is a need to understand the local milieu of the foetal-maternal interface better but for obvious reasons it is difficult and unethical to take biopsies of human early pregnancy tissue as this can cause miscarriage. We are therefore often referred to studies of tissue from other species that might not be representative for humans. Even placental tissue from human miscarriages has limitations, as it is difficult to know if the cytokines and chemokines observed are a cause or consequence of the miscarriage.

Another consideration in Paper IV was that LMWH cannot pass over the placental barrier and can therefore only affect the maternal side during placentation and pregnancy. Different LMWH preparations, such as enoxaparin, dalteparin and tinzaparin, differ in many respects, such as their molecular, structural, physiochemical, and biological properties and might therefore not be considered as clinically equivalent.[164] Tinzaparin was used in Paper IV, but using a different LMWH might have given other results.

Ethical considerations

Papers I, II and V in this thesis were approved by the Research Ethics Committee in Lund (Dnr. 2014/1) and Paper IV was approved by the Research Ethics Committee in Linköping (Dnr. 2010/295-31). Paper IV was also registered at EudraCT (protocol number: 2010-022715-19) and written informed consent was obtained from all participants. In the register-based studies (Papers I, II and V) individual consent was not obtained as it is not needed for specific research projects using registers with unidentified data, regarding Swedish law (SFS 1998:543).[165]

Results

Incidence of Recurrent Pregnancy Loss

In Paper I, 6,852 women were identified with the ICD-10 diagnosis of RPL (O26.2 or N96.9) registered in the NPR during a 10-year period, 2003-2012. Another 990 women were found in the NPR with the diagnoses of miscarriages on three or more occasions without an interspersed live birth, although they remained without a registered diagnosis of RPL in the register. Altogether 7,842 women were identified with RPL through these two approaches.

Two incidences of RPL diagnosis were estimated in the two different risk populations defined in the study. The diagnosed RPL incidence of women age 18-42 years was 39/100 000 women in 2003 and 73/100 000 women in 2012 (Figure 7a). The diagnosed RPL incidence in pregnant women was 478/100 000 women at the beginning and 875/100 000 women at the end of the study period (Figure 7b). The relative increases of the incidence of RPL in the two groups during the 10 years were 74% and 58%, respectively.

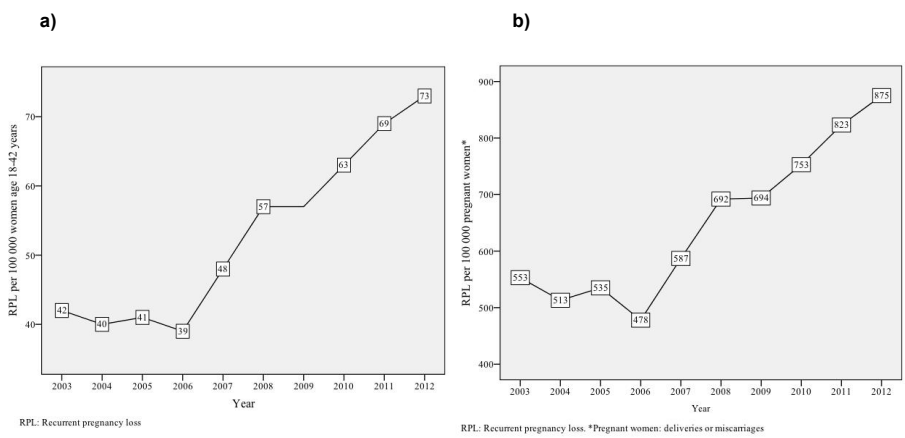

Figure 7. Estimated incidence of RPL diagnosis in the period 2003-2012. a) Incidence of RPL diagnosis in women age 18-42 years b) Incidence of RPL diagnosis in pregnant women (*miscarriage or child birth)

Validation of Recurrent Pregnancy Loss diagnoses in the National Patient Register

The NBWH randomly selected 711 women registered with RPL in the NPR during 2003-2012. Initially 485 medical records were requested from 60 different hospitals and private caregivers. Out of the 60 hospitals, 73% of the university hospitals, 60% of the regional hospitals and 50% of the private caregivers responded. In total, 254 medical records were available for review from 38 hospitals, although there was insufficient information in 16 of the medical records. A correct diagnosis was registered in 202 out of the 238 medical records, resulting in PPV (202/238)=85% (95% CI 78–89%). A positive predictive value informs how many diagnosed with a disease (e.g. RPL) in the register (or a test) actually have the disease.

Treatment efficiency at improving live birth rate in idiopathic Recurrent Pregnancy Loss

In Paper III, a systematic review on medical treatment efficiency at improve LBR in women with idiopathic RPL identified 1,415 relevant publications (102 duplicates). After the exclusion of 1,187 articles based on title and abstract, 126 full-length articles were reviewed. Another 105 were excluded according to the exclusion criteria and 21 RCTs were finally included (Figure 5).

RCTs that studied women with a history of idiopathic RPL treated with ASA, LMWH, progesterone, corticosteroids, IvIg or LIT were assessed for their effect on LBR. Meta-analyses were possible for studies on LMWH (Figure 8), IvIg (Figure 9) and LIT (Figure 10). A forest-plot of progesterone is presented for illustration (Figure 11) but no meta-analysis was possible for progesterone or ASA because of differences in the control groups or the starting point for treatment.

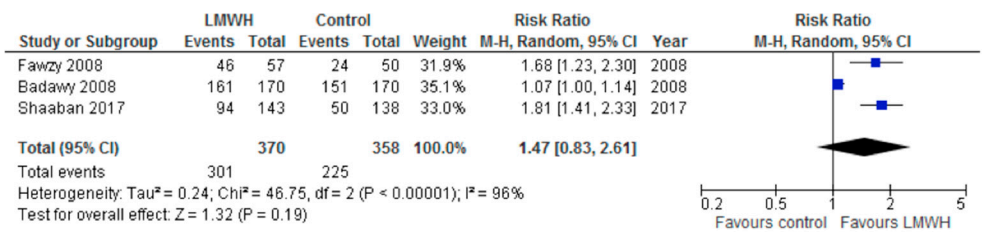

Figure 8. Forest plot and meta-analysis of LMWH treatment

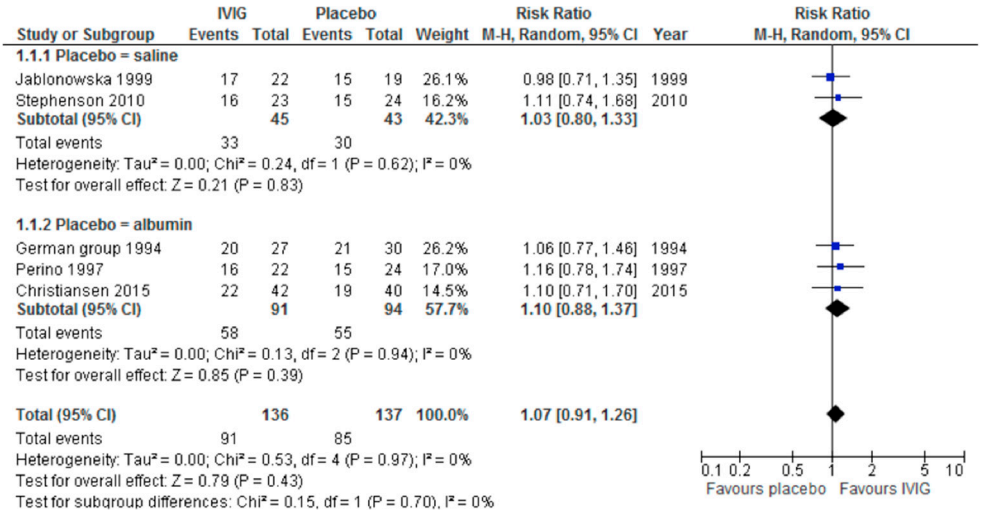

Figure 9. Forest plot and meta-analyse of Ivlg treatment

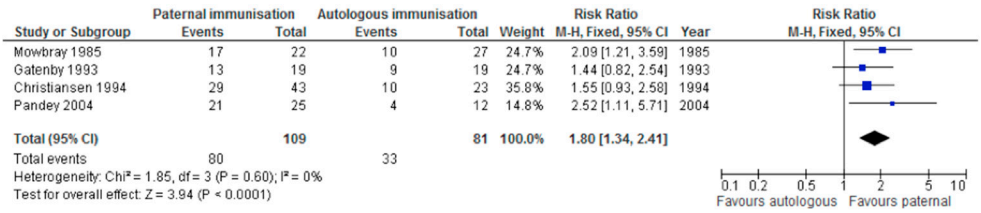

Figure 10. Forest plot and meta-analyse of LIT treatment

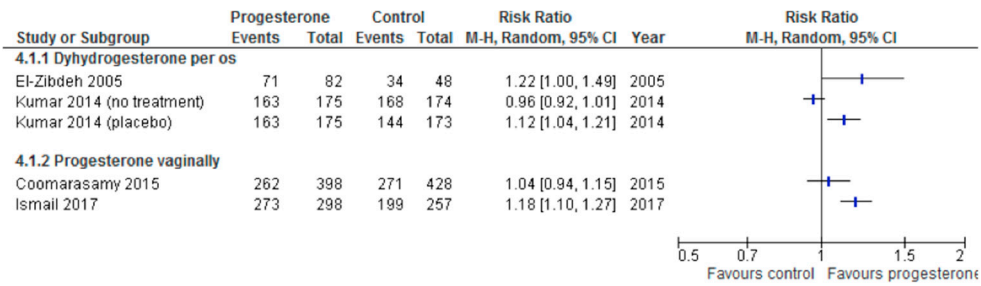

Figure 11. Forest plot of progesterone treatment

Women with RPL treated with ASA showed the same LBR as the compared group in the two studies[142,166] included in this review.

The meta-analysis on LMWH treatment did not show significant difference in LBR in RPL women compared to the controls (Figure 8). However, looking at the results in the separate LMWH studies, three[167–169] of them did show a significant effect on LBR, 81% vs. 48%, 71% vs. 42% and 66% vs. 33%, respectively.

None of the studies[170–175] on IvIg treatment could find an improved LBR, in line with the results in our meta-analysis (Figure 9).

Treatment with LIT did show a higher rate of LBR in the meta-analysis (Figure 10). The one study[176] not included in the meta-analysis, because it had a different comparison group, found a lower LBR (63% vs. 79%) in women with idiopathic RPL vs. expectant management with LIT.

Two large studies[132,177] on vaginal progesterone showed contradictory results with the starting point as the main difference. In one[177] where treatment started after a positive pregnancy test the LBR values were 66 vs. 63%, while the other study,[132] which started at ovulation, showed LBR values of 92% vs. 72% (Figure 11). Kumar et al.[133] showed an improved LBR with oral dydrogesterone treatment (93%) compared with placebo (83%).

LMWH's immune modulating effect during pregnancy in women with idiopathic Recurrent Pregnancy Loss

Compared with the control group (n=35), LMWH-treated women (n=35) had higher plasma levels of the Th1-associated chemokines CXCL10 (p=0.007) and CXCL11 (p=0.000) (Figure 12). In post-hoc tests there were significantly higher levels of both these pro-inflammatory chemokines at GWs 28 (CXCL10 p=0.048, CXCL11 p=0.023) and 34 (CXCL10 p=0.026, CXCL11 p=0.005) in treated compared with untreated women. The study also found a significant difference (p=0.037) between the two groups regarding the Th17-associated chemokine CCL20 (Figure 12). Many samples were under the detectable level for CCL20 but the proportion of women with detectable levels was higher in the treated (15% and 12% at GWs 18 and 34 respectively) compared with the untreated group (0% at both GWs 18 and 34, p=0.025 and p=0.053, respectively).

Women who miscarried (n=7) had significantly higher levels of CXCL11 compared with the women who continued their pregnancy beyond 22 GWs (n=70) (geometric mean 49 pg/ml vs. 27 pg/ml, Student t-test p=0.001).

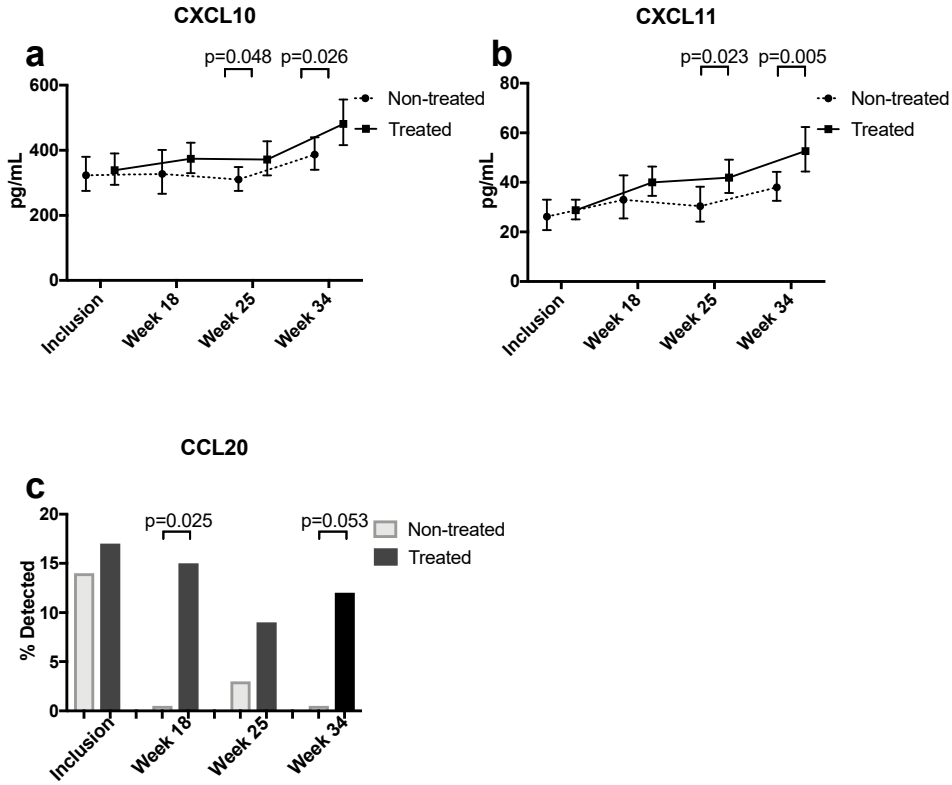

Figure 12. Chemokine levels in treated with low-molecular-weight-heparin (LMWH) (n = 35) and non-treated (n = 35) women with unexplained recurrent pregnancy loss during pregnancy.
(a, b) Longitudinal levels of CXCL10 (a) and CXCL11 (b) during pregnancy. Linear Mixed Models of log-transformed data showed differences between treated and untreated women; CXCL10, p=0.007; CXCL11, p=0.000. A Student´s t-test (log-transformed data) was used as a post-hoc test to denote differences at specific time points. Geometric mean and 95% CI are shown (c) Linear Mixed Models on log-transformed data showed a difference in CCL20 levels between treated and untreated women during pregnancy (p=0.037). Fisher's exact test was used as a post-hoc test. The proportion (%) of women with detectable levels is shown.

Obstetrical outcomes in women with previous Recurrent Pregnancy Loss

In Paper V a total of 62,381 pregnancies in 30,139 women were included for this study for analysis. There were a total of 4,971 first subsequent pregnancies in women with RPL compared to 57,410 pregnancies by women without RPL.

Of the 4,971 women with a history of RPL and their first subsequent pregnancy in MFR, 2,334 (47%) and 2,637 (53%) had primary and secondary RPL, respectively.

Compared to pregnancies of women without RPL, we found that pregnancies of women with RPL had an increased risk of preeclampsia, stillbirth, SGA birth, preterm birth and placental abruption in the first pregnancy subsequent to the diagnosis of RPL. The association with all the above placenta-associated disorders was stronger when the birth was preterm (<37 GW) compared to a term birth. The risk of an SGA infant or stillbirth was significantly higher when the child was born preterm but not when born at term (Table 4).

Table 4. Obstetrical outcome in the first subsequent pregnancy after the diagnosis of recurrent pregnancy loss (RPL) and pregnancies by women without RPL, with and without preterm birth.

Obstetrical outcome Terms and preterm	RPL n=4,971 (%)	No RPL n=57,410** (%)	Crude OR (95% CI)	Adjusted OR (95% CI)
Preeclampsia[a]				
No PE***	4,759 (95.7)	56,140 (97.8)	1.00*	1.00*
PE and preterm	65 (1.3)	248 (0.4)	3.09 (2.35-4.07)	2.26 (1.70-3.01)
PE at term	147 (3.0)	1,022 (1.8)	1.70 (1.42-2.02)	1.26 (1.05-1.51)
Stillbirth[b]				
Live birth***	4,939 (99.4)	57,178 (99.6)	1.00*	1.00*
Stillbirth and preterm	24 (0.5)	126 (0.2)	2.21 (1.42-3.42)	1.92 (1.22-3.02)
Stillbirth at term	8 (0.2)	106 (0.2)	0.87 (0.43-1.79)	0.76 (0.37-1.57)
SGA[c]				
No SGA***	4,800 (96.6)	56,001 (97.5)	1.00*	1.00*
SGA and preterm	75 (1.5)	343 (0.6)	2.55 (1-98-3.28)	2.00 (1.54-2.60)
SGA at term	96 (1.9)	1066 (1.9)	1.05 (0.85-1.30)	0.97 (0.78-1.20)
Placental abruption[d]				
No PA***	4,921 (99)	57,200 (99.6)	1.00*	1.00*
PA and preterm	29 (0.6)	123 (0.2)	2.74 (1.83-4.11)	2.47 (1.62-3.76)
PA at term	21 (0.4)	87 (0.2)	2.81 (1.74-4.52)	2.42 (1.49-1.96)

*reference **Number of pregnancies by women without RPL ***both birth at term and preterm
PE; preeclampsia, SGA: small for gestational age, PA; placental abruption
[a]adjusted for age, parity, assisted conception, body mass index, mother's country of birth, chronic hypertension, pre-gestational diabetes, smoking,
[b] adjusted for age, parity, body mass index, smoking
[c] adjusted for age, parity, body mass index, smoking, mother's country of birth, chronic hypertension,
[d] adjusted for age, parity, body mass index, smoking

Discussion

Reflections on Recurrent Pregnancy Loss as a potentially increasing problem

The incidence of RPL diagnoses was found to be increasing over a ten-year period in Sweden (Paper I). Other studies have reported a prevalence of RPL of 0.5-2.3%[5,8,14–16] but no study has attempted to estimate the incidence of RPL. To our knowledge this was the first register-based study on incidence of RPL diagnoses over time. A main question is whether this is a true increase, and if so, it is of great interest why we see this trend? The causes of RPL can be multifactorial both on the population and individual levels.[107] No causal conclusion can be drawn with the study design used. However, there are several speculative explanatory factors presented as followed.

It cannot be ruled out that the increase of the diagnosed RPL incidence identified in the NPR might be caused, at least in part, by variations in the quality of the register over time. According to NBHW, many diagnoses in general have increased over time and the quality of diagnosis coverage has improved from 2001 to 2006.[178] As the outpatient register started in 2001 a start-up phase before the register reached a reliable and fully acceptable quality was expected, even though mandatory reporting has always existed. It is noteworthy that the increase in incidence was highest after 2007.

Another potential issue, in addition to registration practice, is whether the registered diagnoses are correct. Therefore, In Paper II, validation of the diagnosis of RPL in the NPR was investigated and found to be of acceptable high quality. The data on the increasing incidence of RPL diagnoses in the register (Paper I) is therefore suggested to be reliable as regards the correctness of the diagnosis. The validation was not varied out separately for each year in Paper II. However, analyses performed after this study was published showed no major difference in accuracy over the study period (unpublished data).

Furthermore, many women who miscarry do not come into contact with the health care system and will never appear in any registers. Changes in the threshold for seeking a physician among women with miscarriage and the availability of

examinations and ultrasound examinations could influence registrations of miscarriages and RPL.

Another possibility is that an increased prevalence of risk factors for RPL really has changed the incidence of RPL. Known risk factors include chromosomal abnormalities, uterine anomalies, endocrine defects, thrombophilia, autoimmune diseases or changes in environmental and life style factors. Genetic factors cannot change so rapidly as over a period of only ten years. A risk factor that could increase substantially during a 10-year study period is maternal age at time of conception. The risk of miscarriage due to foetal aneuploidy increases with maternal age. However, the median maternal age in our study population did not change over the study period, and is therefore not an obvious explanation for the increased incidence of RPL diagnosis. Although data was lacking on risk factors such as BMI and smoking in our specific study population, the NBHW reported a lower rate of smoking during pregnancy, from 10 to 6 %[145] and only a slight increase in BMI in women aged 18-42 years during the study period,[179] thus ruling out these factors as causes of an increased incidence of RPL diagnosis.

In Paper V the RPL cohort had a higher rate of women born outside the Nordic countries and a higher frequency of women who had gone through IVF compared with a control group without RPL. These two factors may possibly have caused an increase in the incidence of RPL diagnosis as both have increased over time. However, Tamhankar et al.[180] did not find a higher rate of miscarriages (13%) in women treated with IVF than the background population, stated to be 10% to 15%.[17,18,20] Therefore, an increase in IVF treatment does not seem to be the explanation for an increasing incidence of RPL diagnosis. Differences in prevalence of RPL in distinctive countries and ethnicities are not well studied. Explaining the higher rate of RPL diagnosis in women from countries outside of Nordic countries is therefore difficult.

A large group of women have idiopathic RPL, where immunological risk factors are suspected to play a role. Women with RPL have a higher prevalence of several autoimmune diseases, which also have increased over time in the population. Immunological and inflammatory stages can change over a short time frame and are speculatively factors causing an increased incidence of RPL diagnosis.

There are many factors that can influence the maternal immune response and thereby pregnancy outcome. For instance Familari et al[181] have shown that air pollution can effect trophoblast cells in terms of reduced growth, oxidative stress and inflammation. Air pollution has been associated with a higher rate of miscarriage.[181,182] However, further studies on exposure and changes in air pollution are needed to be able to draw any conclusions on whether this may have been a contributing factor to an increase in RPL in our study.

It is necessary to investigate whether the increase in incidence of RPL, found in this study, can be verified in other cohorts. The NBHW has presented an ongoing increase of diagnosed women registered with RPL until the year 2014. Afterwards the increase halted and the trend was stable until 2017 (where data ends).[183] It is important to continue to investigate changes in the incidence of RPL in Sweden and to carry out further explanatory studies of causes to the changes. It is important to keep in mind that causes to RPL are probably multifactorial and not only one explanation should be looked for. Evidence suggests that one important factor for a successful pregnancy is a balanced inflammation at the foeto-maternal interface. With that said, an increase in immunological/inflammatory factors can be one explanation for an increased incidence of RPL. In addition, further studies are needed to clarify the association between RPL and factors influencing the maternal immune response during pregnancy.

Role of inflammation from early to late pregnancy

The immunological interactions taking place at the foetal-maternal interface are complex and far from fully understood. There is some evidence that impaired trophoblast growth and invasion may be caused by an increased local or systemic immunological reaction.[111,107] In Paper IV we saw higher levels of the Th1 associated chemokine CXCL11 in RPL women who miscarried than in those who continued their pregnancy. Several other studies have pointed out a pro-inflammatory (Th1) skewed immunity in RPL women compared with controls.[27,44,104,106,107,111] An impaired immune reaction can lead to pregnancy loss, but if trophoblast invasion is only partially impaired, it can lead to placenta-associated disorders such as preeclampsia, stillbirth, IUGR, preterm birth and placental abruption.[29,124,184] In Paper V we identified an overall increased risk for placenta-associated disorders in women with RPL compared with women without a history of RPL. This strengthens the hypothesis that RPL and placental dysfunctional disorders could partially share the same pathology related to an impaired placentation.[184] Impaired immune response early in pregnancy might cause adverse pregnancy complications in late pregnancy.

The results of Paper V provided additional scientific evidence that women with a history of RPL are an obstetrical high-risk group concerning placenta-associated disorders. Several other studies[185–189] are in line with our results that women with a history of RPL are a high-risk group for obstetrical complications. Fawzy et al. showed increased OR of SGA and perinatal death in women with RPL compared to controls.[189] Although some studies[97,186,189,190] did not find an increased risk for preeclampsia in women with RPL, several other studies[185,188,191] identified such an association, in accordance with our results. The discrepancy in the results can be

explained by underpowered study group (42 women with RPL)[190] and the lack of a strict definition of consecutive miscarriages.[186] There was great consensus that women with RPL had an increased risk of SGA[21,185,187,189,192] compared with controls, and this was also true for primary RPL compared with secondary RPL,[18,97,193] which is in accordance with our results. Also in line with our results, several studies[185–187,189,192,194] have proved a significantly higher rate of preterm birth in women with RPL than in control women.

Because of the increased risk of other pregnancy complications, antenatal surveillance needs to be improved for women with a history of RPL. One such improvement could be screening for increased vascular resistance in the uterine artery and umbilical artery, as this is correlated with adverse outcomes of pregnancy such as preeclampsia, intrauterine growth restriction and preterm delivery.[195–197] The suggested screening should be done along with feotometri ultrasound screening for intrauterine growth retardation in the third trimester. Further studies are needed to evaluate the effect of an intensified antenatal surveillance programme.

Prophylactic aspirin has been proven to be effective in reducing the risk for placenta-associated disorders in high-risk groups.[198–200] As women with a history of RPL were identified as a high-risk population in our study, such prophylactic management with aspirin might be effective for reducing the risk for placenta-associated disorders in women with RPL.

An important question that remains is: is it possible to modulate the immune system early in pregnancy in a favourable direction, which could lead to prevention of pregnancy loss and later pregnancy complications?

Immune modulation during pregnancy

In Paper IV the immunological *in vivo* effects of LMWH in pregnant women with a history of idiopathic RPL were assessed. To our knowledge, this was the first *in vivo* study investigating LMWH's potential anti-inflammatory effect during pregnancy in this specific study group.

It has been hypothesised that LMWH can potentially induce immune modulatory actions and act as an anti-inflammatory effector in different inflammatory diseases.[201,202] In contrast, our study did not find that LMWH decreased pro-inflammatory or increased anti-inflammatory/Th2-associated cytokines and chemokines. Instead, we found a significant increase in plasma levels of the pro-inflammatory/Th1-associated chemokines, CXCL10 and CXCL11 in the second and third trimesters of pregnancy, in women treated with LMWH compared to

untreated control women. A Th1-skewed immune response is known to affect pregnancy outcomes negatively in both murine models and in humans, and women with RPL have higher plasma levels of Th1-associated cytokines.[203,47,113,106,108] Thus, the observed Th1-associated and potentially pro-inflammatory effects of LMWH in this study are rather contradictory to the hypothesised anti-inflammatory and pregnancy-promoting effects assigned to LMWH treatment. Bruno et al.[204] have, in accordance with our results, showed a Th1 skewing *in vitro* where LMWH enhanced production of the Th1 associated cytokine IFN-ɣ, the cytokine that induces production of CXCL10 and CXCL11.

The effects recorded by LMWH would be supposedly unfavourable in relation to pregnancy. Based on our results, it can be questioned whether treatment given to modulate the immunology responses should start much earlier in pregnancy. Many immunological changes take place during decidualisation and at the pre-implantation stages. It can be hypothesised that medical modulation is needed at these time-points.

Medical treatment in women with idiopathic Recurrent Pregnancy Loss and the role of timing

The results from the review in Paper III identified knowledge gaps in the area of treatment for women with idiopathic RPL. We found no high quality evidence to support any medical treatment to improve the outcome of live birth in this group of women. As shown by the association between RPL and placenta-associated disorders (Paper V), the interactions in very early pregnancy (or even pre-fertilisation) affect the whole pregnancy.

When treatment is given to women with idiopathic RPL, the dose and timing of treatment administration relative to conception can be of great importance.[122] In our systematic review there were too few studies included to be able to stratify the starting point into separate analyses. In general, most studies started treatment either after a positive pregnancy test or ultrasound confirmed oval sac, foetal pole or heartbeat. A meta-analysis by Hutton et al.[202] showed that IvIg treatment of idiopathic RPL starting before conception increased LBR more compared with starting when pregnancy was established, in line with other studies.[206,207] IvIg treatment has not been found to be efficient regarding LBR in women with primary RPL although it has shown a significant effect in secondary RPL.[205] We did not find any beneficial effect of IvIg treatment in line with two other systematic reviews,[206,208] although no stratifying of starting point was possible in our study.

We concluded that pooling of the four studies[132,133,177,209] included on progesterone treatment was not adequate because of the different routes of administration and differences in control groups. Ismail et al.[132], included in our review, showed a positive effect of progesterone treatment on LBR in women with RPL when treatment started in the luteal phase. In contrast, a large RCT, the PROMISE study, failed to show any significant benefit, but in this study treatment was started after pregnancy was confirmed. Kumar et al.[133] concluded that oral dydrogesterone improved the chance of a live birth in women with unexplained RPL, even though the starting point was a positive pregnancy test.

No meta-analysis was possible on ASA-treatment in our review due to heterogeneity. Since then, a large RCT[141] has been published comparing ASA and placebo. This study concluded that ASA treatment was not efficient at improving LBR in women with idiopathic RPL, in line with the studies we have included in the review. The frequency of preterm birth, stillbirth and pre-eclampisa but not SGA was non-significantly less in the group treated with ASA than in the controls.[141] Although there was a large study population, the study might have been underpowered for showing significant differences for these secondary outcomes. Once more, none of the studies on ASA treatment started until pregnancy was detected with ultrasound or a positive pregnancy test.

The results of Wong's[208] review indicated no improvement in live births with paternal cell immunisation, third party donor cell immunisation or trophoblast membrane immunisation. The authors suggest that these therapies should no longer be offered as treatments for unexplained RPL. In our review, although we found a higher LBR in LIT-treated women, it was uncertain whether LIT affects the chance of live birth because of the low study quality, low number of patients, and lack of consistency in patients recruited.

Some studies[168,169,210] showed a significant effect on LBR regarding LMWH treatment while others[211–213] failed to do so. The last mentioned trials included RPL defined by only two miscarriages and were therefore not included in our review. A meta-analysis by Lin et al.,[214] found no beneficial effect on LBR in women with idiopathic RPL when treated with LMWH. We found similar results when studies were pooled in a meta-analysis, with no convincing effect on LBR. None of the studies previously mentioned started treatment before conception.

To be able to determine the efficiency of different treatments in women with idiopathic RPL, future RCTs with a large study population with a similar setup and definitions are important. Additionally, women with idiopathic RPL are a heterogonous group, probably with several aetiologies. This leaves the possibility that some women in this group might see an effect from treatment while others might not. If for example chromosomal abnormalities are not taken into account an effective treatment might be interpreted as ineffective.[215]

It is far from general practice to investigate the lost embryo's karyotype, which makes a difference regarding whether treatment is possible or not. Chromosomally abnormal embryos cannot and should not be treated in an attempt to continue the pregnancy, as they are incompatible with life.[215]

In conclusion, no definite recommendation on treatment to women with idiopathic RPL can be given, as the literature does not provide strong evidence on efficiency at improving LBR. However, treatment efficiency cannot be ruled out and treatment should be given in the context of RCTs for further investigation. A factor that has been mainly overlooked so far is that the timing of treatment for women with idiopathic RPL, which may be crucial. Wanting to modulate the immune response towards a favourable direction in pregnancy probably needs start of treatment in very early stages.

Summary

The incidence or RPL diagnoses were found to be increasing over a ten-year period in Sweden. It remains to be confirmed whether this is a true increase or if it is, at least in part, due to factors such as registration habits. No causal explanation for an increase can be given but several possible causes, such as an increase in risk factors and changes in life style factors, are presented. Also, immunological/inflammatory factors can be a cause of RPL as the immune system can play a major part in early pregnancy where correct modulation is needed for a successful pregnancy. In this thesis, an association between RPL and adverse late pregnancy disorders was found. Notably, some of these disorders may originate from an impaired immune response during early pregnancy and placentation. Improved antenatal surveillance is needed for women with RPL in order to improve obstetrical outcomes.

Ideally, medical treatment could modulate the immune system in a favourable direction so pregnancy loss could be prevented. We found that one of the most studied drugs, LMWH, when given during pregnancy seemed to modulate the immune system. However the modulation was towards Th1-immunity in later stages of pregnancy, which is believed not to be favourable. There is no evidence for the efficiency of any treatment given to women with idiopathic RPL in improving LBR. Possibly, the timing of these treatments might have been too late for modulation of the immune response at an early stage of pregnancy.

Conclusions

The general aim of this thesis was to improve knowledge of RPL in regard to epidemiology, immunology, treatment and obstetrical outcomes.

I. The incidence of RPL diagnoses in the National Patient Register in Sweden has increased over a ten-year period. This might be an indication of a significant and possibly increased problem with more women suffering RPL.

II. The diagnosis codes for RPL in the National Patient Register are sufficiently reliable and can be used for epidemiological studies of RPL.

III. No specific treatment can be recommended with the current evidence on the efficiency at improving live birth rate in women with idiopathic RPL. The timing of treatment onset might be of importance.

IV. Immunological modulation with LMWH during pregnancy in women with idiopathic RPL, direct the immune response into an unfavourable pro-inflammatory direction. It is not recommended to give LMWH to idiopathic RPL women, in an immunological aspect.

V. Women with RPL have a higher risk of placenta-associated disorders in a subsequent pregnancy. They should therefore be recognised as a high-risk group for late adverse pregnancy outcome and be offered improved antenatal care.

Future perspectives

It is of great interest to conduct further research on why an increased incidence of diagnosed RPL was found in this thesis, and also to establish if the noted increase of RPL diagnosis was biased by changes in registration coverage. The most unbiased way to estimate the incidence of RPL would probably be to conduct a prospective cohort study where a population of women would be studied during their fertile period. A prospective study, incorporating potential risk factors for RPL, is needed to confirm if a "true" population-based incidence of RPL is increasing and if so, to establish why. It would also be of interest to study the incidence of RPL diagnosis in other Nordic countries, which also have access to registers, to evaluate if changes in the RPL incidence is noted there as well.

Since RPL has many causes, and since unexplained RPL is a heterogeneous condition, efforts to categorise subgroups are warranted. In the case of unexplained RPL, immunological tests have been suggested, but they have not become established because of inconsistency and lack of consensus. Joint initiatives at an international level should be performed. Also, monitoring of immuno-modulating treatments should be carried out according to international guidelines. Regarding future immunological studies in RPL, it is also important to include modern technology, which has improved greatly with regard to the level of detailed resolution with which the immune system can be studied. A personalised approach to find individual immunological mechanism and choose treatment according to those is appealing, as it is increasingly used in the fields of cancer, autoimmunity and allergy.

It is still not clear if earlier treatment onset could be efficient improving LBR or decrease the risk of pregnancy complications. The timing of treatment onset should be investigated further. It is important to do so in RCTs where clear inclusion criteria and a clear definition of RPL are provided. It is also important to stratify women by age, number of previous miscarriages and karyotype analyses. As many miscarriages occur outside the hospital and there is no tradition of conducting chromosomal analyses on miscarriages, many pregnancy losses are of unknown chromosomal karyotype. More investigation of the products of conception could lead us closer to discovering how large the "truly" idiopathic RPL group really is.

This thesis identified women with RPL at a higher risk for placenta-associated disorders, and therefore improved antenatal care was recommended. Concrete surveillance programmes need to be described and implemented, and then evaluated.

Svensk sammanfattning (Swedish summary)

Bakgrund

Missfall är den vanligaste komplikationen under tidig graviditet. När detta drabbar ett par flera gånger klassificeras det som upprepade missfall. Definitionen av upprepade missfall är omdiskuterad. Det finns ingen enighet om huruvida det krävs två eller tre på varandra följande missfall för diagnos. Denna definition har betydelse när förekomst av upprepade missfall ska beräknas, och kan påverka om utredning ska utföras. Även prognosen påverkas; efter två missfall är chansen till en lyckad graviditet över 80%, medan efter tre missfall är chansen c:a 55-75% till en lyckad graviditet. Antalet som drabbas av upprepade missfall uppges ofta till ca 1-3%, men det är inte helt tydligt hur många kvinnor som drabbas årligen eller om det är ett ökande problem.

Det finns flera möjliga riskfaktorer som ökar risken för upprepade missfall. Kromosomförändringar hos paret, anatomiska förändringar i livmodern, hormonella störningar, koagulationsstörningar och autoimmuna sjukdomar är några exempel på vanliga riskfaktorer. Kvinnans livsstilsfaktorer spelar roll, högt BMI och hög ålder är kända riskfaktorer för upprepade missfall. Vidare kan vissa miljöfaktorer också ha betydelse, exempelvis föroreningar med kemikalier. Trots detta är det endast hos hälften av alla kvinnor som blir utredda för upprepade missfall som en orsak kan påvisas.

För en lyckad graviditet krävs att immunförsvaret accepterar en delvis främmande kropp (fostret) då hälften av fostrets gener kommer från mannen. För att fostret och moderkakan skall koppla samman med livmodern och därigenom mammans blodcirkulation, måste moderkakans celler växa in i livmoderslemhinnans blodkärl. Om kvinnans immunförsvar reagerar för kraftigt på dessa inväxande celler uppstår ett missfall och om de bara delvis tillåts växa in uppstår en ytlig koppling vilket kan resultera i andra graviditets-komplikationer såsom avlossning av moderkakan, tillväxthämning av fostret och havandeskapsförgiftning. Immunförsvaret kan inte undertryckas helt då det samtidigt måste skydda fostret och mamman mot infektioner under graviditeten. Det finns teorier om att en

kraftig obalans i immunresponsen leder till tidigt missfall medan en partiell obalans leder till graviditetskomplikationer senare i graviditeten. Dessa teorier är dock inte bevisade, och det finns idag ingen immunologisk utredning eller behandling med bevisad betydelse.

Ett flertal medicinska behandlingar har utvärderats hos kvinnor med upprepade missfall där orsak är okänd. Strategierna har varierat från att dämpa immunförsvaret till att minska risken för blodproppar i moderkakan. Många behandlingar har använts på empirisk grund utan fastställd evidens. Det råder en stor diskrepans mellan olika studieresultat gällande dessa olika behandlingar, fortfarande är det dock inte visat om behandlingarna förorsakar nytta eller skada.

En blodförtunnande medicin, låg-molekylär heparin (LMWH), har ofta använts till kvinnor med upprepade missfall. Förutom den kända blodförtunnande effekten har LMWH föreslagits ha en effekt på immunförsvaret. Flera *in vivo* studier på djur och *in vitro* studier på odlade celler, tyder på att LMWH kan påverka immunceller och därmed vara gynnsam i tidig graviditet. Ingen tidigare studie har undersökt om LMWH har en gynnsam effekt på immunsystemet hos kvinnor med upprepade missfall.

Syfte och metoder

Målsättningen med denna avhandling var att undersöka hur många kvinnor som årligen drabbas av upprepade missfall under en 10-års period i Sverige (Arbete I) Vidare undersöktes hur pålitliga Socialstyrelsens register är med avseende på upprepade missfall (Arbete II). Detta gjordes utifrån registerdata i det nationella patient registret där diagnoskoder, på upprepade missfall, registreras. Journalgenomgång på kvinnors journaler, som registrerats med upprepade missfall i registret, utfördes för att se om diagnosen i registret överensstämde med journalen.

Ytterligare ett syfte var att studera om medicinsk behandling, till kvinnor med okänd orsak till upprepade missfall, kan leda till att kvinnan föder barn istället för åter att drabbas av missfall. En systematisk genomgång av litteraturen utfördes för att studera evidensen av olika mediciners effekt på andelen födda barn (Arbete III).

För att ta fram ett vetenskapligt underlag på effekten av LMWH undersöktes dess påverkan på immunförsvaret under graviditeten. I en randomiserad kontrollstudie av gravida kvinnor med tidigare upprepade missfall av okänd orsak, gavs LMWH till hälften av kvinnorna och den andra hälften fick ingen medicin. Immunologiska

markörer mättes vid flera tillfällen under en graviditet och jämfördes mellan grupperna (Arbete IV).

Då moderkakans (placentans) koppling till livmodern är central för en lyckad graviditet studerades placenta-relaterade komplikationer hos gravida kvinnor som tidigare haft upprepade missfall. Med hjälp av registerdata kunde vi studera kvinnor som haft upprepade missfall och specifikt titta på utfallet i efterföljande graviditet (Arbete V).

Resultat

Avhandlingen är baserad på fem delarbeten (Arbeten I-V). Förekomsten av kvinnor med upprepade missfall ökade under en 10-års period i Sverige (Arbete I). Kvaliteten i det nationella patientregister som användes granskades och resultaten visade att det var en acceptabel hög nivå gällande diagnosen upprepade missfall (Arbete II). Litteraturgranskning av ofta använda läkemedel kunde inte entydigt visa några gynnsamma effekter (Arbete III). Trots att LMWH visades kunna påverka immunologiska processer i graviditeten kunde inte några skyddande effekter påvisas (Arbete IV). I det sista arbetet kunde en association mellan tidigare upprepade missfall och graviditetskomplikationer i efterföljande graviditet påvisas. Speciellt kunde det påvisas att kvinnor med tidigare upprepade missfall hade en ökad risk för havandeskapsförgiftning, tillväxthämmade barn, för tidig förlossning, avlossning av moderkakan och dödfödda barn (Arbete V).

Diskussion

Avhandlingen visar ett stigande antal kvinnor som drabbas av upprepade missfall, registrerade i det nationella patientregistret. Resultaten har dock inte kunnat säkerställa varför antalet ökar. Det ökade antalet registreringar av diagnosen *upprepade missfall* i det nationella patientregistret kan bero på bättre tillgänglighet i vården så att diagnosen kan ställas eller att registret förbättrat täckningsgraden. Det kan även bero på att andelen kända riskfaktorer såsom kromosomförändringar hos paret, anatomiska förändringar i livmodern, hormonella störningar, koagulationsstörningar och autoimmuna sjukdomar har ökat, vilket dock är mindre sannolikt. Livsstilsfaktorer som BMI har inte ökat nämnbart under studietiden och rökning under tidig graviditeten har snarast minskat. Däremot har IVF behandlingar ökat, dock har man inte kunnat påvisa att IVF ger ökad risk för missfall. Även andra ännu okända orsaker skulle kunna bidra.

Vår litteraturgenomgång har inte kunnat visa några belägg för att läkemedel kan förebygga upprepade missfall och öka andelen levande födda barn. Det krävs ytterligare kliniska studier för att utvärdera nyttan av medicinska behandlingar och för att kunna rekommendera någon specifik behandling.

Det vore önskvärt att med hjälp av läkemedel kunna optimera immunförsvaret i syfte att underlätta kopplingen mellan moderkakan och livmodern. Behandling med LMWH, i efterföljande graviditet hos kvinnor med tidigare upprepade missfall, visade sig modulera immunförsvaret till ett mer aktivt, så kallad pro-inflammatorisk stadium. Dessa resultat stod i motsattsförhållande till hypotesen att LMWH skulle dämpa immunförsvarets aktivitet, så kallad anti-inflammatoriskt effekt. Utifrån dessa resultat är det inte gynnsamt att ge LMWH i syfte att optimera immunförsvaret i en positiv (anti-inflammatorisk) riktning under graviditeten (däremot kan LMWH vara värdefullt vid vissa ovanliga koagulationsrubbningar som kan ge upprepade missfall).

Upprepade missfall medför en ökad risk för sena graviditetskomplikationer i en nästkommande graviditet. Detta stöder hypotesen att både missfall och graviditetskomplikationerna havandeskapsförgiftning, tillväxthämning för tidig förlossning, avlossning av moderkakan och dödfödda barn, till viss del har en gemensam sjukdomsmekanism. Det kan vara indicerat att se kvinnor med upprepade missfall som en risk grupp för sena graviditetskomplikationer och att de därför är i behov av tätare kontroller under graviditeten.

Acknowledgments/ Tack

Leif Mathiesen, jag kommer alltid att minnas dig för ditt stora engagemang till livet generellt och till kvinnor med upprepade missfall i synnerhet. Du tog dig alltid tid och gav mig mental support när jag behövde det som mest. Du ultraljudsscannade mig när jag var gravid med Stella och höll Agnes som bebis under våra forskningsmöten. Jag är ledsen att du aldrig hann se eller hålla denna bok i dina händer som ett resultat av det vi startade tillsammans. Jag önskar du fortfarande var med oss idag. Du var mer än en handledare, du var en vän. Du är saknad!

Stefan Hansson, tack för din generositet och mod att hoppa på tåget som handledare långt efter tåget redan lämnat perrongen. Jag uppskattar din breda expertis, idérikedom och forskarerfarenhet. Du har låtit mig utvecklas mot en självständig forskare och lärt mig att höja blicken och perspektivera.

Ole Bjarne Christensen, tack för allt ditt stöd genom åren. Du har väglett mig och hjälpt mig enormt mycket genom allt mitt arbete från design av studier, artikelskrivning och själva avhandlingen. Jag uppskattar din stora expertis inom området upprepade missfall och reproduktiv immunologi. Du var ett stort stöd när jag stod utan handledare och du försäkrade att jag skulle klara det med din hjälp.

Jan Ernerudh, tack för den eftertänksamhet och klokhet du sprider. Du har en enorm kunskap inom immunologi och du har lärt mig mycket inom detta område, även om det är mycket mer att lära. Det har varit en ära att arbeta med dig och jag är tacksam för att du accepterade mig som doktorand fast du då aldrig hade träffat mig.

Maria Jenmalm, tack för dina kloka "inputs" till min forskning inom reproduktions immunologi. Jag beundrar din breda och djupa kunskap inom immunologi. Du är en stor inspirationskälla!

Göran Berg, tack för att du tog mig under dina vingar efter Leifs bortgång. Du gav mig en stor trygghet och visade mig vägen till Linköping och vidare introduktion till Jan och Maria.

Valentina Bruno, thank you for a great cooperation with our fellow project. It has been great to get to know you and thank you for sharing your story with me.

Tack alla övriga medförfattare: *Rebecca Rylance, Karin Källén, Margareta Hellgren, Ragnhild Hjertberg, Lennart Blomqvist, Emir Henic, Sujata Lalitkumar, Annika Strandell, Elisabeth Nedstrand, Roland Boij, Christina Petersson Strid, Piccione, Judith Svensson-Arvelund, Marie Rubér* och tidigare nämnda som bidragit med en ovärderlig arbetsinsats till mina forskningsprojekt och värdefulla kommentarer till mina manuskript.

Tack alla *barnmorskor* och *sjuksköterskor* som hjälpt med multicenter forskningsprojektet i Helsingborg, Lund, Karlskrona, Kalmar, Jönköping, Linköping. Tack alla kvinnor som deltagit i studien.

Tack *FoU Region Skåne* för forskningsanslag så att denna forskning kunde fullföljas.

Tack *Henriette Grinder Schimmel* för den fina framsidan till denna bok.

Tack alla *kollegor* för det stöd ni gett mig och det extra arbete ni har gjort i mitt ställe när jag har forskat istället för att ha bidragit på kliniken. Tack till cheferna på kliniken, Pia Teleman, Charlotte Hellsten och Andreas Herbst som har prioriterat forskning och gett mig forskningstid så att detta arbete har kunnat genomföras. *Harpa och Lana*, tack för ert stöd och främst för alla roliga stunder på tåget, i fikarummet och nattlivet i Köpenhamn. Jag hoppas att vi kommer att arbeta tillsammans igen i framtiden.

Tack *alla vänner*: barndomsvänner, vänner från resor, vänner från studietiden och vänner från livet. Ni är alltid där för mig och peppar mig när jag behöver det. Tack för att ni ger mig perspektiv på livet och tack för alla roliga stunder tillsammans. *Teresa*, tack för ditt stöd genom hela doktorandtiden och för att du lurade mig till att doktorandanmäla mig genom att säga att det var "lätt och snabbt" ordnat att doktorera. *Mia,* tack för att du alltid är där för mig.

Min *svärfamilj*, tack för er värme och omtanke. Tack för ert stöd och för att ni alltid ställer upp för oss.

Mina föräldrar, *Ann-Greth och Göran,* tack för er obetingade kärlek, generositet och ert eviga stöd. Ni är realister men har aldrig stoppat mina drömmar. Tack för att ni alltid tror på mig och för att ni format mig till den jag är idag.

Anna, min syster, tack för att du alltid är där för mig när jag behöver det. Tack till din fina familj.

Mina barn*, Stella och Agnes,* tack för den glädje ni sprider och att ni lär mig att leva i nuet. Jag är så stolt över att vara er mamma. Min kärlek till er sinar aldrig.

Anders, min man, du är mitt allt! Jag älskar dig så högt. Du är en stabil klippa genom hårda tider och en stor källa till glädje och lycka. Tack för att du hjälper mig i vått och tort. Jag hade aldrig klarat detta utan dig.

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