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immunological risk factors and immunomodulatory treatments

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IMMUNOMODULATORY TREATMENTS

BY
CAROLINE NØRGAARD-PEDERSEN

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AALBORG UNIVERSITY
DENMARK

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by

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AALBORG UNIVERSITY
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CV

Caroline Nørgaard-Pedersen is a medical doctor who, after obtaining her master's degree in medicine in 2020, started as a PhD student because of her curiosity and compassion to make a difference in women's health. She takes pride in conducting clinical research that shapes how she and her colleagues care for patients with infertility and recurrent pregnancy loss. She finds motivation in the clinic, where she helps couples achieve their dreams of a child. Alongside her PhD, she has strived to de-taboo and disseminate knowledge about recurrent pregnancy loss to the general Danish population through interviews in podcasts and magazines and voluntary work as a medical writer for an online magazine for women of childbearing age.

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July 2022: Poster presentation at The ESHRE Congress, Milan, entitled “Is the frequency of low plasma mannose binding lectin (p-MBL) levels increased in recurrent pregnancy loss (RPL) and recurrent implantation failure (RIF) after IVF/ICSI?”

April 2022: Oral presentation at The European Society for Reproductive Immunology Congress, Paris, entitled:” A firstborn boy and maternal HLA class II polymorphism negatively affect pregnancy prognosis in recurrent pregnancy loss."

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ENGLISH SUMMARY

Recurrent pregnancy loss (RPL) is defined as two to three spontaneous pregnancy losses. The condition affects 1-3% of women of fertile age, and the incidence of RPL is increasing. Knowledge of causal factors is lacking. In approximately 60% of RPL patients, none of the evidence-based risk factors can be found, which complicates the search for effective treatments and poses a significant challenge to clinicians.

The lack of effective treatment for the large group of patients with unexplained RPL frustrates clinicians and patients. It has led to the introduction of empirical adjuvant interventions that are costly, potentially inefficient or even harmful, and often without biological rationale and unequivocal evidence. Much effort is put into research in immunological causes of RPL and immunomodulatory treatments because several studies on immune cells and signaling molecules have found the elevated frequency of specific aberrancies among RPL patients that suggests immunological rejection of the embryo/fetus as a cause of RPL. It has been hypothesized that a more comprehensive understanding of how immunological mechanisms are involved in the RPL pathogenesis will enable us to prospectively identify appropriate patients who are likely to benefit from the specific immunomodulatory treatment. So far, the evidence for the immune biomarkers suggested to predict the efficacy of immunomodulatory therapeutics is insufficient, and further confirmation is therefore needed before the inclusion of investigations for immunological aberrations in clinical settings can be recommended.

This thesis aimed to further explore the role of the immune system in RPL pathogenesis and the effect of immunomodulatory treatments. The thesis contains four original research articles investigating the association between specific immune biomarkers and RPL and one randomized controlled trial (RCT) protocol for an ongoing placebo-controlled trial that aims to examine the clinical and immunomodulatory effects of intravenous immunoglobulin combined with low-dose oral glucocorticoid treatment as well as the presence of immune biomarkers among women with RPL undergoing artificial reproductive technology treatment.

Study I is a combined cross-sectional and cohort study that found a significantly elevated prevalence of low plasma mannose-binding lectin (p-MBL) levels and a significantly reduced prevalence of high p-MBL levels in RPL patients compared to a reference group of female blood donors. The p-MBL level had no influence on the subsequent pregnancy outcome nor the prior or subsequent perinatal outcomes. However, we did find an increased frequency of birth of a boy before RPL in patients with low p-MBL levels. Since MBL is a plasma protein that aids clearance of apoptotic cells and these cells can be highly immunogenic, these study findings suggest that MBL deficiency may lead to elevated levels of apoptotic cells from the

fetus and placenta, which may trigger an inappropriate immune reaction and cause fetal rejection [1].

Study II is a cross-sectional study that found an elevated male-to-female sex ratio of older siblings and firstborn children compared with the general Danish population. In addition, the frequency of patients with at least one older brother and/or birth to a boy before RPL differed significantly from the expected frequency in the Danish population. Since cells from a genetically differing individual, known as microchimerism, can pass the placenta, it has been suggested that male microchimerism can be acquired from both the woman's prior pregnancies and her mother. These results suggest that exposure to male antigens may be an RPL risk factor [2].

Study III is a combined cross-sectional and cohort study that finds an increased male-to-female sex ratio of firstborn children in secondary RPL patients in comparison with the sex ratio in the general Danish population and that a firstborn boy exerts a negative impact on the chance of a live birth in the subsequent pregnancy in comparison to a firstborn girl. However, when the analysis was stratified according to the maternal carriage of human leukocyte antigen (HLA) class II alleles able to present male-specific minor histocompatibility antigens, the negative prognostic impact of a firstborn boy was only found among sRPL patients who carried at least one of these alleles in comparison to sRPL patients who did not carry one of these alleles and to patients with a firstborn girl irrespective of the HLA allele carriage. Since the presentation of antigens on HLA class II molecules by antigen-presenting cells is essential for triggering a type IV hypersensitivity reaction known to cause, e.g., chronic graft rejection, the significant interaction effect between HLA phenotype and sex of the child born before the disease on the pregnancy prognosis in RPL patients may suggest that a type IV hypersensitivity reaction may be involved in sRPL pathogenesis [3].

Study IV is a cross-sectional study that examined the association between autoantibody positivity and immunogenetic RPL susceptibility markers, including low p-MBL level, HLA-DRB1*03, and HLA-DRB1*07 in RPL patients. The only significant association was found between positivity for anti-nuclear antibody positivity and the presence of both a low p-MBL level and carriage of one or more HLA-DRB1*03 alleles. However, the general observation was that women with low p-MBL levels and/or carriage of one or more HLA-DRB1*03 alleles had a higher risk for autoantibody positivity than those RPL patients who had normal p-MBL levels or carried other HLA-DRB1 alleles, but the differences were not significant. In addition, HLA-DRB1*03 phenotype frequency was significantly lower in the RPL patients than in the reference group of bone marrow donors. In contrast, no difference between groups was found for the prevalence of the HLA-DRB1*07 phenotype, which suggests that these are not immunogenetic susceptibly markers for RPL. Thus, no immunogenetic susceptibility to RPL and only a weak immunogenetic susceptibility

to autoimmunity in RPL patients could be found, and this may indicate that non-genetic factors play a more dominant role in RPL pathogenesis [4].

The RCT protocol (Study V) contains a study protocol and statistical analysis plan for a double-blinded, placebo-controlled RCT comparing intravenous immunoglobulin combined with low-dose oral prednisolone with intravenous human albumin and placebo tablets in patients with a history of RPL after artificial reproductive technology treatment. The primary outcome is the ongoing pregnancy rate after embryo transfer, while the secondary outcome includes treatment safety for the woman and her child, and the tertiary explorative outcome includes treatment effects on immune biomarkers, including leukocyte cell levels and stimuli-specific cytokine production. Thus, this study will aid our understanding of immune mechanisms that occur during the window of implantation, the action of the immunomodulatory treatment on the immune system concerning the pregnancy outcome, and which immunological biomarker(s) may select patients eligible for the treatment. The full statistical analysis plan was published with the protocol to increase transparency and validity of the results that will be published when the study inclusion is complete [5].

The articles were all peer-reviewed and published with open access in journals within the field of human reproduction or immunology. An oral presentation of preliminary results from study I was given at the European Society for Human Reproduction and Embryology Congress in 2020, while an oral presentation of preliminary results from study III was given at the European Society for Reproductive Immunology Congress in 2022.

DANSK RESUME

Gentagne graviditetstab er defineret som mindst to eller tre spontane graviditetstab. Tilstanden findes hos 1-3% af kvinder i den fødedygtige alder, og antallet af kvinder med gentagne graviditetstab er stigende. Vores viden om underliggende årsager til gentagne graviditetstab er mangelfuld. Hos ca. 60% af patienterne finder vi ingen af de faktorer, som er forbundet med øget risiko for gentagne graviditetstab.

Den manglende viden om årsager til gentagne graviditetstab komplicerer vores søgen efter effektive behandlingsmidler, og aktuelt findes der ingen behandlingstilbud til denne gruppe af patienter, som er bevist effektive. Dette kan være frustrerende for både lægen og patienten. Imidlertid har flere undersøgelser af immunceller og deres signalmolekyler vist øget hyppighed af specifikke afvigelser hos denne gruppe patienter, og det tyder således på, at en årsag til gentagne graviditetstab kan være, at immunforsvaret afstøder fosteret. Derfor forskes der meget i immunologiske årsager til gentagne graviditetstab og i behandlinger med medicin, som virker på immunforsvaret.

Med en dybere forståelse af hvordan immunforsvaret er involveret i sygdomsmekanismerne ved gentagne graviditetstab, vil vi muligvis være i stand til at identificere patienter, som kan forventes at have en gavnlig effekt af immunmodulerende behandling. Vi mangler dog videnskabelige evidens for om der findes en biomarkør, der kan forudsige effektiviteten af en immunmodulerende behandling, og det er derfor nødvendigt med flere videnskabelige undersøgelser.

Formålet med denne ph.d.-afhandling var at udforske immunsystemets rolle ved gentagne graviditetstab og effekten af immunmodulerende behandling. Denne ph.d.-afhandling indeholder fire originale forskningsartikler, der undersøger immunologiske risikofaktorer for gentagne graviditetstab og en protokol for et igangværende placebokontrolleret lodtrækningsforsøg, som har til formål at undersøge den kliniske og immunmodulerende effekt af behandling med intravenøst immunglobulin (antistoffer fra bloddonorer) kombineret med lavdosis prednisolon (binyrebarkhormon-tabletter) på kvinder med gentagne graviditetstab, som gennemgår kunstig befrugtning.

Studie I er et kombineret tværsnits- og kohortestudie, som fandt en signifikant øget prævalens (hyppighed) af lav koncentration af mannose-bindende lektin i plasma (p-MBL) og en signifikant lavere prævalens af høj koncentration af p-MBL hos patienter med gentagne graviditetstab sammenlignet med en referencegruppe af kvindelige bloddonorer. Studiet fandt derudover, at koncentrationen af p-MBL ikke havde indflydelse på udfaldet i den førstkommende graviditet efter henvisning til vores center for kvinder med gentagne graviditetstab. Vi fandt heller ikke nogen sammenhæng mellem lav p-MBL-koncentration og henholdsvis lav fødselsvægt og

for tidlig fødsel ved en eventuel fødsel forud for og efter gentagne graviditetstab. Vi fandt dog en signifikant øget hyppighed af fødsel af en dreng forud for patientens gentagne graviditetstab hos patienter med lav p-MBL-koncentration. Da MBL er et plasmaprotein, der hjælper organismen med at eliminere apoptotiske (døende) celler, og da disse celler kan virke stærkt aktiverende på immunforsvarets celler, kan studiets fund tyde på, at MBL-mangel kan medføre forhøjede niveauer af apoptotiske celler fra fosteret og moderkagen, som kan udløse et uhensigtsmæssigt immunrespons og forårsage afstødning af fosteret [1].

Studie II er et tværsnitsstudie, som fandt en øget hyppighed af tidligere fødsel af en dreng og en øget hyppighed af storebrødre hos patienter med gentagne graviditetstab sammenlignet med hyppigheden i den danske befolkning. Derudover var hyppigheden af mindst én storebror og/eller en fødsel af en dreng før gentagne graviditetstab hos patienterne højere end den forventede hyppighed i den danske befolkning. Celler fra et genetisk forskelligt individ kan passere moderkagen, og disse celler antages at kunne overleve i kvindens organisme i årtier (såkaldt mikrokimærisme) og at kunne stamme fra både kvindens tidligere graviditeter og fra ældre søskende via hendes mor. Studiets fund tyder på, at eksponering for mandlige antigener der stammer fra fødsel af en dreng eller en storebror kan være en risikofaktor for gentagne graviditetstab [2].

Studie III er et kombineret tværsnits- og kohortestudie, der fandt en øget hyppighed af tidligere fødsel af en dreng hos kvinder med gentagne graviditetstab sammenlignet med hyppigheden i den danske befolkning, og at en førstefødt dreng i forhold til en førstefødt pige har en negativ indflydelse på chancen for en fødsel af et levende, rask barn i den første graviditet efter henvisning til vores center. Patienterne blev adskilt i grupper efter køn af deres førstefødte barn i forhold til om patienten bar minimum en HLA-klasse II allel (et vævstypegen), som er i stand til at præsentere antigener (stykker af molekyler) kodet af gener på Y-kromosomet. Vi fandt en negativ prognostisk indflydelse af en førstefødt dreng blandt patienter, som bar mindst én af disse alleler, sammenlignet med patienter som havde en førstefødt dreng og ikke bar en af disse alleler, og sammenlignet med patienter med en førstefødt pige uanset HLA allel-gruppe. Antigenpræsenterende cellers præsentation af antigener bundet til HLA klasse II-molekyler er afgørende for at udløse en type IV-overfølsomhedsreaktion, der vides at kunne forårsage bl.a. kronisk afstødning af et organtransplantat. Derfor kan den signifikante interaktionseffekt mellem moderens HLA-fænotype og køn af barnet født forud for de gentagne graviditetstab på graviditetsprognosen tyde på, at en type IV-overfølsomhedsreaktion kan være involveret i sygdomsmekanismerne ved gentagne graviditetstab hos disse patienter [3].

Studie IV er et tværsnitsstudie, der undersøgte sammenhængen mellem forekomsten af forskellige autoantistoffer og genetiske risikomarkører for gentagne graviditetstab, som inkluderede lav koncentration af p-MBL og HLA-DRB1*03 og HLA-DRB1*07 vævstype-allelerne hos patienter med gentagne graviditetstab. Den eneste signifikante association, som studiet fandt, var mellem forekomsten af anti-nukleære antistoffer

og tilstedeværelse af både en lav p-MBL-koncentration og en HLA-genotype med minimum et HLA-DRB1*03 allel. Den generelle observation var imidlertid, at kvinder med lavt p-MBL-niveau og/eller bærertilstand af en eller flere HLA-DRB1*03-alleller havde højere risiko for tilstedeværelse af autoantistoffer end hos patienter, der havde højere koncentration af p-MBL eller bar andre HLA-DRB1 alleler, men disse forskelle var ikke signifikante. Derudover var prævalensen af HLA-DRB1*03 signifikant lavere hos patienter med gentagne graviditetstab end hos referencegruppen bestående af knoglemarvsdonorer, mens der ikke var nogen forskel mellem de to grupper for prævalensen af HLA-DRB1*07. Det tyder således på, at disse ikke er genetiske risikomarkører for gentagne graviditetstab som rapporteret i tidligere studier. Der kunne derfor ikke findes en immuno-genetisk disponering for gentagne graviditetstab og kun en svag immuno-genetisk disposition for autoimmunitet hos patienter med gentagne graviditetstab. Dette kan indikere, at ikke-genetiske faktorer spiller en mere dominerende rolle på underliggende sygdomsmekanismer for gentagne graviditetstab [4].

Denne afhandling indeholder desuden en publiceret, fagfællebedømt protokol og en tilhørende statistisk analyseplan for et dobbelt-blindet, lodtrækningsforsøg. Lodtrækningsforsøget har til formål at sammenligne effekten af intravenøst immunglobulin i kombination med lavdosis oral prednisolon med intravenøst humant albumin og placebotabletter hos patienter med gentagne graviditetstab efter kunstig befrugtning. Det primære endepunkt er forskellen i antal patienter, som er gravide med et rask barn ved nakkefoldsscanningen i gestationsuge 12. De sekundære endepunkter inkluderer bivirkninger af behandlingen for patienterne og deres nyfødte børn, mens de tertiære, eksplorative endepunkter inkluderer behandlingens påvirkning af immunologiske biomarkører, herunder koncentrationen af specifikke hvide blodlegemer og stimuli-specifik produktion af signalmolekyler. Således vil dette studie kunne bidrage til vores forståelse af immunologiske mekanismer, der opstår på tidspunktet for embryonets implantation i livmoderen, virkningen af den immunmodulerende behandling på immunsystemet i forhold til resultatet af behandlingen med kunstig befrugtning, og hvilke immunologiske biomarkører der kan identificere patienter, som kan forventes at have gavn af behandlingen. Den fulde statistiske analyseplan blev publiceret sammen med protokollen for at øge gennemsigtigheden og validiteten af de resultater, der vil blive offentliggjort, når undersøgelsen er gennemført på alle projektdeltagere [5].

Artiklerne er alle blevet fagfællebedømt og publiceret som ”open access” i tidsskrifter inden for human reproduktion eller immunologi. Foreløbige resultater fra Studie I blev præsenteret mundtligt til *European Society for Human Reproductive and Embryology* kongressen i 2020, mens foreløbige resultater fra Studie III blev præsenteret mundtligt på *European Society for Reproductive Immunology* kongressen i 2022.

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My interest in recurrent pregnancy loss (RPL) and infertility started in my final year at medical school at Aalborg University when Professor Ole Bjarne Christiansen passionately introduced me to reproductive medicine and his research on immune system aberrations in patients who suffer from RPL and infertility. The importance of searching for answers on both causes and treatments to these medical conditions is underlined by the large size of this patient group, the great impact of RPL and infertility on the patients' physiologic and psychological health, and society's call for solutions to counteract the falling fertility rate and fecundity.

I will remember my PhD journey as an epoch in my life that has been truly exciting, fulfilling, and at times strenuous. I believe that we will be able to offer better care for these patients in the near future, but only if we, the scientific research society, take responsibility for conducting high-quality trials. That motivates me and reminds me that every minute of hard work is (and has been) worth it.

I gratefully acknowledge the great support from my principal supervisor Professor Ole B. Christiansen, who has always stood by my side to back me up professionally and personally. I would also like to thank my assistant supervisor Professor Ulrik Schiøler Kesmodel, for his great support of epidemiology and statistical analyses, and the secretary Lena Lykke Iversen Nielsen, for her indispensable help in the randomized controlled trial. Furthermore, I would like to thank the Department of Gynaecology and Obstetrics and the Fertility Clinic at Aalborg University Hospital for all of their personal support of my research. I also am grateful to the Department of Clinical Immunology for its profound expertise, support, and hard work on immunological analyses. I would also like to thank all of the patients who have contributed to my research, and I hope our findings will support improvements in the management of RPL and infertility.

Finally, I would like to thank my friends and family for their encouragement and infinite support when times were good – and when times were tough. I have never questioned their endorsement, and I am deeply thankful for that.

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Caroline Nørgaard-Pedersen

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Published in Human Reproduction Open 2022; 2022(3);hoac024

DOI: 10.1093/hropen/hoac024

Study II

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Published in Journal of Clinical Medicine 2021;10(12):2613

DOI: 10.3390/jcm10122613

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Published in Journal of Reproductive Immunology 2023;156:103817

DOI: 10.1016/j.jri.2023.103817

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C Nørgaard-Pedersen; R Steffensen; U S Kesmodel; O B Christiansen

Published in Frontiers Immunology 2023;14:1069974

DOI: 10.3389/fimmu.2023.1069974

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ABBREVIATIONS

β2-GPI	β-2-glycoprotein-1
ANA	Anti-nuclear antibody
APC	Antigen-presenting cell
aPL	Anti-phospholipid antibody
APS	Anti-phospholipid syndrome
ART	Assisted reproductive technology
ASRM	American Society for Reproductive Medicine
BMI	Body mass index
CI	Confidence interval
CRPLWD	Center for Recurrent Pregnancy Loss Western Denmark
DAG	Directed acyclic graph
DNA	Deoxyribonucleic acid
ESHRE	European Society of Human Reproduction and Embryology
EVT	Extra-villous trophoblast
G-CSF	Granulocyte colony-stimulating factor
GDPR	General Data Protection Regulation
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GVHD	Graft versus host disease
GW	Gestational week
H-Y	Male-specific minor histocompatibility antigens

hCG	Human Chorionic Gonadotrophin
HLA	Human leukocyte antigen
HYr-cII	H-Y restricted HLA class II
ICSI	Intracytoplasmatic sperm injection
InDel	Insertion/deletion
IVF	In-vitro fertilization
IVIG	Intravenous immunoglobulin
KIR	Killer immunoglobulin receptor
LBR	Live birth rate
LDA	Low dose aspirin
LH	Luteinizing hormone
LIT	Lymphocyte immunization therapy
LMWH	Low-molecular-weight heparin
MASP	MBL-associated serine proteases
MBL	Mannose-binding lectin
mHA	Minor histocompatibility antigen
MIC A/B	Major histocompatibility complex class I chain-related gene A/B
OR	Odds ratio
p-MBL	Plasma mannose-binding lectin
PCOS	Polycyclic ovarian syndrome
PGT	Preimplantation genetic testing
PGT-A	Preimplantation genetic testing for aneuploidy

pNK	Peripheral natural killer
PPR	Prevalence proportion ratio
pRPL	Primary recurrent pregnancy loss
RCOG	Royal College of Obstetricians and Gynaecologists
RCT	Randomized controlled trial
RPL	Recurrent pregnancy loss
RR	Relative risk
SCH	Subclinical hypothyroidism
SLE	Systemic lupus erythematosus
sRPL	Secondary recurrent pregnancy loss
SUSAR	Suspected unexpected serious adverse reaction
Tc	cytotoxic T
TCR	T cell receptor
Th	T helper
TNF-α	Tumor necrosis factor-α
Treg	T regulatory
TSH	Thyroid-stimulating hormone
UFH	Unfractionated heparin
uNK	Uterine natural killer
uRPL	Unexplained recurrent pregnancy loss
vs	Versus
VT	Villous trophoblast

WOC	Window of implantation

CHAPTER 1. INTRODUCTION

PREGNANCY LOSS

Pregnancy is confirmed when human chorionic gonadotrophin (hCG) is detected in urine or plasma from a female with no hCG-producing neoplastic conditions. The hCG mRNA is transcribed as early as at the 8-cell stage of the embryo corresponding to Day 3 after fertilization, the so-called “cleavage stage” occurring before implantation. Implantation occurs during the “window of implantation" (WOC) on cycle day 20-24 in a 28-day cycle, and a detectable hCG level in blood is reached by day 8-10 after fertilization peaking in gestational week (GW) 10-11 [6]. It is primarily produced by syncytiotrophoblast cells in patients with no hCG-producing neoplastic conditions and, therefore, only detectable during pregnancy.

A spontaneous pregnancy loss is an involuntary termination of a pregnancy before viability which will cause a reduction in plasma hCG (p-hCG) production and concentration with time [7]. By definition, early pregnancy loss happens before GW 12+0, while a late pregnancy loss occurs after GW 12 and before GW 20 to 24, depending on the differing national definitions [8]. After GW 20-24, a pregnancy loss is often defined as a stillbirth, although the WHO uses GW 28 as the cut-off.

Early pregnancy losses can be divided into non-visualized pregnancy losses and miscarriages. The non-visualized pregnancy losses are solely confirmed by a urine or p-hCG test and include biochemical pregnancies and pregnancies of unknown locations. Miscarriages are intrauterine pregnancy demises confirmed by ultrasound or histology [7].

In addition, two other types of pregnancies producing hCG exist. However, they will be terminated as soon as they are detected since they cannot develop into a viable fetus and can have fatal consequences to the women's health. They include ectopic pregnancies, which are pregnancies ultrasonically or surgically detected outside of the endometrial cavity, and hydatidiform moles/molar pregnancies, which are benign tumors of trophoblast cells that can develop after, e.g., two sperm cells have fertilized an oocyte with or without exclusion of maternal chromosomes [9,10].

RECURRENT PREGNANCY LOSS

Scientific papers on recurrent pregnancy loss (RPL) go back over a century. At the beginning of the 20th century, the term and the definition of the condition were debatable, just as it is today. At the time, the two terms "habitual abortion" and "recurrent abortion" were up for discussion as well as the disease criteria. Now, 100 years later, the terminology is still inconsistent. The disease criteria differ between

specialist societies on reproduction in terms of the number of losses, the need for losses occurring consecutively or not, and the inclusion of non-visualized pregnancies or not [7,11–17].

In this thesis, the term RPL will be used. During the last decade, the two largest international societies, the European Society of Human Reproduction and Embryology (ESHRE) and the American Society of Reproductive Medicine (ASRM), have changed their definition of RPL from three to two not necessarily consecutive pregnancy losses before GW 20 [13–16,18]. While the ESHRE includes all pregnancies, the ASRM does not include non-visualized pregnancies. In the Danish Society, British Society, and the German, Austrian, and Swiss Societies of Gynaecology and Obstetrics, RPL is still defined as three consecutive pregnancy losses before GW 22. Molar and ectopic pregnancies are not included in the number of pregnancy losses required to fulfill the diagnostic criteria in any RPL definition.

RPL can be divided into two subgroups: primary RPL (pRPL) and secondary RPL (sRPL). pRPL have never had a viable pregnancy beyond GW 20-24 in contrast to sRPL.

EPIDEMIOLOGY

The prevalence of RPL has been variously reported due to differing definitions of the condition (numerator), the population at risk (denominator), and different data sources and collection methods. This important background information is rarely described in detail in studies reporting the prevalence of RPL. In 2021, the Lancet published a series of three papers on miscarriages, and in the first paper, the important implications of such variations on the reported prevalence were highlighted. Nevertheless, the paper reported a population prevalence of RPL which was based on the authors' calculation of the average prevalence from four studies with highly different definitions of both the fertile age, the population at risk (i.e., parous women or all women in fertile age in the general background population within the same period) and RPL (i.e., the inclusion of biochemical pregnancy losses or not) [19]. They reported that when RPL was defined as two pregnancy losses before GW 20, the average population prevalence was reported to be approximately 2.6% of women of reproductive age, and if defined as women with three pregnancy losses, the prevalence was approximately 0.7% of women [13,19–21]. However, as described, the validity of the prevalence is questionable as the determination of the exact prevalence of RPL is complicated to measure, especially when biochemical pregnancy losses are included in the definition since these are rarely registered. Indeed, as the pregnancy loss rate has been estimated to be approximately 30% [22–24], we would expect a higher prevalence of RPL. For example, if one does not take into account the increase after each pregnancy loss [24], statistically, one would expect that approximately 3% (0.3^3) of couples would experience ≥3 consecutive pregnancy losses [22,23]. The majority of pregnancies are, however, lost before clinical confirmation is possible and

often also before the woman recognizes the pregnancy herself, which may explain the lower reported incidence [22,23]. The rate seems similar between couples conceiving naturally and through ART [22–24]. Therefore, it has been hypothesized that similar factors responsible for missing conception may also harm fetal survival in early pregnancy [25].

According to a Swedish register-based study, the RPL incidence is increasing based on data from the National Patient Register showing a steadily increasing curve with a 58-74 % higher incidence in 2012 than in 2003 [20].

At the Center for Recurrent Pregnancy Loss of Western Denmark (CRPLWD) at Aalborg University Hospital, 36% of RPL patients with ≥3 pregnancy losses undergo assisted reproductive technology (ART) treatment, including controlled ovarian hyperstimulation, in-vitro fertilization (IVF) and intracytoplasmatic sperm injection (ICSI), 4% try to conceive by intrauterine insemination. In comparison, the remaining 60% of patients try to conceive by natural conception (Data from the CRPLWD database 01.01.2023). Thus, treatment of RPL is often combined with treatments for infertility prescribed by the patients' ART clinics.

One of the most important risk factors for RPL is increasing age at conception [19]. With the steadily rising number of couples suffering from infertility and, therefore, seeking ART treatment and the increasing mean age at which women start trying to conceive in high-income countries [26,27], an increasing number of RPL patients would be expected.

ETIOLOGY

The etiology of RPL is still not known. The only factors widely accepted to have a prognostic impact on reproductive prognosis are female age and the number of previous pregnancy losses [13]. Several factors are associated with RPL, but rarely, the presence of just one risk factor is sufficient to cause a vicious circle of pregnancy losses, with each pregnancy loss increasing the risk of another loss in the subsequent pregnancy. None of the risk factors are sufficient causes that inevitably cause RPL, as each individual factor also appears in some women with no known pregnancy losses [13]. While some factors may be predisposing, others may be enabling, precipitating, or reinforcing factors. The additional problem is that approximately half of the spontaneous pregnancy losses are due to chromosome abnormalities incompatible with life that occur by chance [28]; thus, even when no known risk factors of disease appear, RPL can occur as a consequence of the naturally high rate of cytogenetic abnormalities in human embryos [29,30] (some call it “bad luck”). A study has suggested an increased rate of chromosomal abnormalities in embryos from RPL patients younger than 35 years old tested by preimplantation genetic testing for aneuploidy (PGT-A) before transfer compared to healthy women [31]. In contrast, a meta-analysis of the rate of chromosomal abnormalities from products of conception

found a lower aneuploidy rate in RPL patients compared with sporadic pregnancy losses [32]. Nevertheless, the high incidence rate of aneuploidy and mosaicism in human embryos; the rare accessibility of product of conception; and the infrequent use of genomic analysis on such tissue complicate the diagnostic workup of RPL patients concerning differentiation between patients with a consistent, underlying maternal or paternal cause requiring medical intervention in order to achieve a live born child and patients with a history of three cytogenetically abnormal fetuses, i.e., with a non-modifiable explanation.

The following describes recommendations for the diagnostic workup of RPL patients. This description is followed by a brief introduction to different important mechanisms in the immune system that, during pregnancy, mediate immune tolerance towards the fetus and to immune abnormalities that may be involved in the RPL pathogenesis. The latter will focus on mechanisms that are related to the research for this PhD. Thus, this is not a systematic review of the immune system in pregnancy and RPL but a brief elaboration of prominent factors in immune tolerance currently being investigated as RPL risk factors.

THE DIAGNOSTIC WORKUP FOR PATIENTS WITH RECURRENT PREGNANCY LOSS

Back at the beginning of the 20th century, infection with syphilis was considered the major cause of "habitual abortion," although most cases remained of unknown etiology [11,17]. Today, our knowledge about the condition has increased; nevertheless, approximately 60% of cases remain of unknown etiology and are termed unexplained RPL (uRPL) [33]. One specific pathology cannot explain all RPL cases, which rules out the "one size fits all"-theory when searching for an RPL treatment. As highlighted, RPL is a multifactorial condition with internal and external risk factors. Aberrations in almost any organ system can secondarily affect fertility. Therefore, the diagnostic workup includes a broad spectrum of investigations and examinations that may involve several medical specialties.

The factors associated with RPL include advanced maternal age; acquired and congenital uterine malformations; hereditary and acquired thrombophilia; endocrine disorders including polycystic ovarian syndrome (PCOS), poorly controlled diabetes, and overt and subclinical hypothyroidism (SCH); autoantibody positivity (anti-nuclear antibody [ANA], thyroid peroxidase [TPO] antibody, anti-cardiolipin antibody, lupus anticoagulant); and lifestyle factors including tobacco smoking, excessive alcohol consumption, and obesity [13].

Several other conditions and biological markers than those listed above have been suggested to be more prevalent in RPL patients than in healthy women. However, the evidence is more controversial, insufficient, and/or inconsistent for these factors, including chronic endometritis, hypo- and hyperprolactinemia,

hyperhomocysteinemia, luteal phase insufficiency (progesterone deficiency), and luteinizing hormone (LH) deficiency or elevation [13,34]. Therefore, examination of these factors is not recommended in a routine workup of RPL patients but only for research purposes [13,18].

Other factors related to lifestyle include excessive caffeine intake, sexual intercourse during pregnancy, drug misuse, high exposure to endocrine disrupters, and excessive exercise. However, these factors have not been investigated in well-designed studies. However, except for intercourse, RPL patients should be recommended to avoid these exposures purely based on assumptions [13].

Moderate to severe stress, anxiety, and depression are among some psychological diseases associated with RPL. While pregnancy losses can cause mental distress, the impact of maternal psychiatric disorders and stress on fertility is less evident [35,36], but a prospective study in a large cohort of females with one pregnancy loss found a higher incidence of a second pregnancy loss if the patient suffered from moderate to severe anxiety and/or depression [37]. It has been hypothesized that a vicious circle exists between pregnancy loss and psychopathology [37–41].

Although many of the risk factors for pregnancy loss in women who try to conceive naturally also apply to women undergoing ART treatment, the cause of infertility and the ART techniques may be additional risk factors in the latter patient group [42]. Thus, RPL patients may be divided according to whether ART treatment is used or not to reduce heterogeneity.

To date, the majority of research studies have focused on maternal risk factors of RPL and treatments for improving the fertility of the female patient. At the same time, the evidence for a paternal contribution is relatively limited. During the last decade, the evidence on paternal risk factors has increased concurrently with the improvement of methods for semen investigation. The evidence for an association between regular semen quality parameters and RPL is unclear [43–46]. While several studies have found a significantly higher percentage of sperm deoxyribonucleic acid (DNA) fragmentation in male partners to RPL patients than in partners to fertile women [47–50], the prognostic value of elevated sperm DNA fragmentation index is up for discussion due to the inconsistent findings on ART outcomes in infertile patients and pregnancy outcomes in RPL patients [44,51,52]. DNA damage in sperm is associated with high levels of oxidative stress, which may be exacerbated by smoking, obesity, and excessive exercise. Therefore, the most recent ESHRE guideline [13] recommends considering sperm DNA fragmentation assessment in RPL couples and to give guidance on a healthy lifestyle despite evidence of the effect of lifestyle modifications is lacking.

Based on the wide variation of RPL risk factors, it is clear that this group of patients is highly heterogenous and needs individualized treatment plans. However, even when

the conditions associated with RPL are identified and treated, the pregnancy prognosis is rarely improved. E.g., no meta-analysis has provided evidence of a positive effect on the live birth rate (LBR) of levothyroxine for SCH or low-dose aspirin (LDA) and/or low-molecular-weight heparin for hereditary thrombophilia in RPL patients [13]. Thus, there is a need for a more thorough understanding of the RPL pathogenesis – even in the subgroup of patients positive for RPL risk factors unresponsive to the associated treatment.

The psychological burden of recurrent unsuccessful pregnancies and the lack of effective treatments leave the RPL patients distressed, despaired, and often willing to do nearly anything to achieve a successful pregnancy. Many physicians are frustrated and desperate enough to offer treatments despite not being recommended by national or international guidelines due to a lack of evidence [53–55]. Consequently, this may have direct negative implications for the patient's general health and reproductive outcome and futile healthcare costs. In addition, these untenable clinical practices highlight the need for randomized controlled trials (RCTs) over observational studies as well as trial registration in public registries, (open access-) trial protocol publication, and accessibility to statistical analysis plans to increase data accessibility, credibility, and transparency; to promote responsible data management; to reduce research waste; and to limit selectively reported results.

Except for age, parental chromosomal abnormality, and uterine anatomical malformations, most of the internationally acknowledged risk factors and conditions associated with RPL involve aberrations in the immune system: e.g., SCH, anti-phospholipid syndrome (APS), autoantibody positivity, PCOS [56], endometriosis [57,58], and lifestyle factors like smoking [59] and obesity [60]. Thus, one may speculate whether RPL is a direct consequence of these specific conditions having a distinctive pathogenesis related to RPL or whether the conditions cause a common underlying aberration at the feto-maternal interface leading to an increased risk of RPL. However, for now, studies that examine the role of the immune system in RPL patients most often include only patients with uRPL.

THE ROLE OF THE IMMUNE SYSTEM IN NORMAL PREGNANCY

When none of the internationally acknowledged contributing factors for RPL is found, an abnormal immunological response to the allogeneic fetus is often suspected as the underlying cause. However, no immunological biomarker or examination has sufficient sensitivity and specificity to identify RPL patients with underlying immunological causation. Consequently, no immunological tests or treatments are commonly agreed on according to the international RPL guidelines [13–16,18]. Therefore, searching for such markers with explanatory and/or prognostic values is highly relevant and requested.

The "fetal rejection" theory has emerged since it was first proposed in the mid-1900s [61,62]. The hypothesis is based on the contradictions between the immune tolerance of the allogeneic fetus and the key principle of the immunological host defense known to fight any invading allograft parallel to an allogeneic organ transplantation. Carrying an allogeneic fetus to term requires maternal immune adaptations to acquire tolerance. Many of the mechanisms involved in pregnancy-related immunological adaptations have subsequently been investigated in RPL patients. Nevertheless, our knowledge of reproductive immunology is still limited.

When assessing the impact of the immune system on RPL pathogenesis, the acquaintance of a healthy, fertile endometrium is essential. Cells from an invading, allogeneic embryo are recognized but left unharmed by the maternal uterine leukocytes in a healthy pregnancy due to an acquired tolerogenic phenotype in the majority of leukocytes located in the feto-maternal interface.

After menstruation, multiple processes occur in the endometrium, including tissue regeneration, cell differentiation, and immune regulation. During the luteal phase, uterine natural killer (uNK) cells and dendritic cells accumulate in the endometrium, ready to support and orchestrate the vascular adaptation and trophoblast invasion if the ovulated oocyte is fertilized and arrives at the endometrium during the WOC. An initial, transient pro-inflammatory response is mounted by the decidualized stromal cells and acute senescence cells by secreting, e.g., free radicals and chemokines. This response lasts 2-4 days and leaves the endometrium receptive to embryo implantation [63–68]. If no implantation occurs, the progesterone level decreases. Subsequently, this triggers a second inflammatory response leading to an influx of leucocytes, breakdown of the endometrial tissue, and eventually menstrual bleeding.

If an embryo implants, fully differentiated, tightly adherent decidual cells and acute senescence stromal cells embed the invading fetus in an immune-privileged matrix [68,69]. This matrix forms the placenta. It consists of the differentiated trophoblast, the decidual mesenchymal cells located in the intervillous stroma, and the fetal vascular cells expanding into the maternal decidua [70]. Studies in mice models have suggested that the decidual tissue reaction inhibits residing antigen-presenting cells (APCs) surveillance of the feto-maternal interface; thus, the APCs are unable to migrate to uterine lymph nodes to initiate fetus-specific T- and B-cell response [71]. The villous trophoblast (VT) covers the villous tree. It consists of an inner cytotrophoblast layer and an outer continuum syncytiotrophoblast layer, which has close contact with the maternal systemic immune system. In contrast, the extra-villous trophoblast (EVT) consists of the endovascular trophoblast in maternal arteries, the interstitial trophoblast, and the placental bed giant cells, which have close contact with the maternal local mucosal immune cells [72]. The trophoblast possesses multiple characteristics that mediate maternal immune tolerance to the fetal alloantigens:

I. The placenta is an immuno-privileged organ because the VT does not express HLA molecules and the invading EVT only expresses the classical HLA-C and non-classical HLA-G, HLA-E, and HLA-F [72]. Thus, the trophoblast does not express the classical, greatly polymorphic HLA-A, HLA-B, and HLA Class II molecules known to be highly effective immune cell stimulators from studies on graft rejection. In addition, the expression of HLA-G and major histocompatibility complex class I chain-related proteins A and B (MIC A/B) on the trophoblast promotes tolerance by their ability to, e.g., reduce the cytotoxicity of uNK cells and T cells, elicit expansion of regulatory T cells (Tregs), inhibit differentiation, proliferation, and cytokine production of B lymphocytes, and inhibit chemotaxis of different cytotoxic lymphocyte populations [73–75].

II. The trophoblast expresses chemokines and cytokines that recruit NK cells and Tregs to the placenta and induce a tolerogenic phenotype in the decidual leukocytes, including the shift from T helper (Th) 1 dominant to Th2 dominant T lymphocyte distribution [74,76]

III. The trophoblast expresses complement inhibitory proteins on the trophoblast cell surface directed to the maternal circulation. These proteins can regulate complement activation and prevent the development of an uncontrolled local complement deposition that would otherwise attract and activate injurious neutrophils. The trophoblast also produces complement components (i.e., C1q) promoting embryo adhesion and interstitial and endovascular invasion of spiral arteries by EVT. Large tortuous vessels form during this process, characterized by a low resistance. This resistance increases the maternal blood supply to the intervillous space crucial for delivering sufficient nutrition to the fetal cells with a high proliferative capacity. Thus, during a normal pregnancy, up-regulation of both complement factors and complement inhibitors occurs [77,78].

IV. The trophoblast excretes extracellular vesicles containing immunosuppressive or apoptosis-inducing signal molecules directed to cytotoxic leukocytes (i.e., indoleamine 2,3-dioxygenase, PD-L1, TRAIL, FasL, MIC A and B, ULBP) [76,79].

Throughout the menstrual cycle, the maternal endometrium contains a high number of leucocytes, and both the concentration and the combination of different leucocyte subsets adapt to the interchanging needs at the feto-maternal interface. In early pregnancy, uNK cells make up the majority (75%) of decidual leukocytes, followed by T lymphocytes, macrophages (20-25%), and dendritic cells (1-2%). The predominant cell type at term is T lymphocytes, while the uNK cell level is significantly reduced [76,80]. Each cell type is essential for successful implantation, and the absence of just one of these cell types critically impacts the early stages of pregnancy, including trophoblast invasion, endometrial vascularity, and alloantigen tolerance [80].

NK cells are often divided into two subgroups based on their surface expression of CD56 and CD16. The $CD16^{+}CD56^{dim}$ NK cell subset has a cytotoxic phenotype, constituting approximately 90% of peripheral blood NK cells (pNK). The majority of uNK cells express $CD16^{-}CD56^{bright}$, which subset has lost its cytotoxic activity and instead acquired an immunomodulatory phenotype and is suggested to be uterus-specific due to its minor role in other tissues [81]. The uNK cell level acutely increases from 20-40% of tissue-infiltrating mononuclear cells in the proliferating phase to 60-70% in the luteal phase, and the increase continues in the decidua if embryo implantation occurs. The cells may derive from both in situ proliferation and recruitment of pNK cells that differentiate into $CD16^{-}CD56^{bright}$ cells. The number of uNK cells declines from the second trimester and returns to basal level at term [81,82]. The immunomodulatory phenotype of uNK cells is important as it assists the uterine macrophages in the clearance of senescent decidual cells, supports trophoblast invasion into the endometrium, regulates decidual spiral artery remodeling [76], and helps control local inflammation and maintain fetal tolerance [83].

A similar mechanism concerns the decidual T lymphocytes. The three main Th cell subsets are Th1, which is involved in cellular immunity and rejection processes; Th2, which is involved in humoral immunity; and Th17, which is involved in inflammation induction. These subsets act as antagonists to each other [84]. During a normal pregnancy, the cell ratio between Th cell subsets is believed to switch from a Th1-dominant profile in very early pregnancy to a Th2-predominant profile characterized by more anti-inflammatory cytokine production. The trophoblast secretes several cytokines, including granulocyte colony-stimulating factor (G-CSF), interleukin-10, and transforming growth factor-β, which induce the shift from a Th1- to a Th2 predominant environment in the decidua and inhibit Th17 lymphocytes [85,86].

Tregs are important for maintaining immunological self-tolerance and developing and maintaining tolerance to the fetus. In accordance, studies in mice models have suggested that the expansion of Tregs with gestation is alloantigen-independent but necessary to sustain pregnancy [87]. A similar expansion of Treg cells with gestation occurs in women, which immediately post-partum returns to normal or a reduced cell level compared to before pregnancy [88]. The relative increase in Tregs in pregnancies with a mismatch compared to a match between the maternal and fetal HLA-C genotypes suggests that Treg increment is a crucial response for tolerance of the increased allogeneic stimulation by the fetus [75,89].

Less is known about how uterine dendritic cells and macrophages contribute to successful and unsuccessful pregnancies. However, it is clear that they are both crucial actors in pregnancy due to their expression of HLA class II molecules presenting fetal antigens, and they act as a bridge between the innate and adaptive immune system and their cytokine production, which regulates the naïve T cell differentiation and NK cells maturation [90]. Their importance has also been reflected in studies in dendritic cell knock-out mice which showed impaired decidual cell proliferation and

disorganized vasculature in the endometrium, impaired differentiation of the uNK cells and trophoblast cells, and a lower pregnancy rate and number of implantation sites in comparison to wild-type mice [91,92].

Self and non-self discrimination relies on a successful central and peripheral immune tolerance of T cells. The central immune tolerance refers to T cell maturation in the thymus. The cortical thymic epithelial cells present self-antigens on HLA molecules to the immature CD4 and CD8 double-positive thymocytes. The thymocytes that recognize the HLA molecules differentiate into single-positive thymocytes, migrate to the thymus medulla, and interact with APCs which cells determine the fate of the T cells based on the T cell receptor (TCR) affinity for self-peptides. Self-reactive T cells undergo apoptosis or differentiate into Tregs ready to act in the periphery. Peripheral tolerance includes additional peripheral blood immune actors that act on the mature circulating T cells [93].

Cell surface presentation of peptides synthesized within the cell (endogenous) by HLA class I molecules occurs on virtually all nucleated cells, while the peptides ingested and proteolytically processed (exogenous) are presented by HLA class II molecules on primarily APCs [94]. The repertoire of peptides that can be presented relies on the genotype of the (highly polymorphic) inherited HLA molecules. The HLA class I molecules present non-self and self-peptides to mainly Tc cells and NK cells, while HLA class II molecules present primarily non-self peptides to mainly Th cells and Tregs. The cross-talk between the killer immunoglobulin receptor (KIR)/TCR, the HLA class I/II molecule, and co-receptors mediate NK cell or T cell activation or inhibition, which subsequently initiates downstream processes in the cellular and humoral adaptive immune system [75,94]. This cross-talk is important for mediating an appropriate cellular immune response to the fetus and infectious pathogens entering the placental tissue.

The primary role of the non-classical, low-polymorphic HLA class 1b molecules HLA-E, -F, and -G is not antigen-presentation as their HLA class Ia and II counterparts [95]. Only a limited set of peptides and non-protein antigens is presented to leukocytes by HLA class Ib molecules. As the VT expresses no HLA molecules on the surface, the tissue can evade T-cell binding. It is hypothesized that this evasion is greatly responsible for the maintenance of maternal tolerance to the fetus by protecting the placenta from being subject to a destructive immune attack. However, the interaction between EVT/VT and maternal leukocytes at the feto-maternal interface is essential, especially for promoting leukocyte secretion of growth factors, cytokines, and angiogenic factors stimulating modulation of maternal arteries to low resistance arteries which support the growth of the placenta and fetus [75,95].

In normal pregnancy, the woman can carry a semi-allogeneic fetus successfully to term – even with the same father twice - despite anti-HLA IgG production against paternal alloantigens. On the contrary, despite using an HLA-compatible donor, organ

transplantation often results in humoral and cellular responses similar to those seen during pregnancy. However, organ transplantation often leads to graft rejection, which contrasts the development of tolerance to the “fetal allograft” during pregnancy. The similarities between the two exposures and responses but the contrasting outcomes are called “the paradox of pregnancy” [61].

HLA compatibility is required for organ transplantation to minimize organ rejection, while in normal pregnancy, the fetus' HLA genotype seems irrelevant [96]. Nevertheless, during normal pregnancy, sensitization to the fetus' HLA antigens does occur since fetal HLA-specific maternal antibodies are often found, the prevalence of these antibodies increases over the gestational course and with multiple pregnancies, and fetal-specific cytotoxic T (Tc) cells, memory T cells, and memory B cells are developed despite restricted HLA expression on placental tissue. Moreover, these antibodies are most often against the classical, non-trophoblastic, fetal HLA antigens, which suggests that the response is not against the trophoblast. Fetal cells with classical HLA expression could derive from feto-maternal hemorrhage, retained fragments after delivery, transplacental passage of fetal erythrocytes and granulocytes, and shredding of extracellular vesicles [96–98]. The fetus-specific maternal immune cells and antibodies can persist for decades, and the same applies to the fetal (microchimeric) cells that have entered the maternal circulation [96].

While this fetal-antigen-specific sensitization may not impact a second pregnancy, a parous female recipient of an allograft has an increased risk of graft rejection, especially if the donor shares an HLA allele of the child(ren)'s father. This risk is supposedly due to the presence of anti-HLA IgG after a pregnancy [96].

During pregnancy, regulation of decidual and trophoblast cell apoptosis is important for successful trophoblast invasion and decidual modulation. Macrophages play a pivotal role in the engulfment of apoptotic cell debris. After phagocytosis of apoptotic cells, the macrophage's secretion of pro-inflammatory cytokines is suppressed, and the secretion of Th2 anti-inflammatory and immunosuppressive cytokines is promoted. Immediate clearance is important as it prevents intracellular, potentially immunogenic content release which could be fetal apoptotic or secondary necrotic cells that release fetal alloantigens if the cells are not cleared from the circulation in time, and this may trigger maternal sensitization to the fetus [99].

The activation of the complement system by the initial pro-inflammatory state of implantation is normally harmless due to the increased level of complement regulatory proteins present at the feto-maternal barrier [100]. Mannose-binding lectin (MBL) is a C-type lectin active in the innate immune system and the lectin-mediated complement pathway, which is involved in the removal of apoptotic cells and cell debris. Indeed, MBL is exceedingly present at sites of inflammation, where it enhances the opsonophagocytosis of pathogens and apoptotic cells by phagocytes and activates the complement system. In adipose tissue of MBL deficient mice compared

to wild-type mice, the macrophage infiltration and the percentage of apoptotic cells were significantly increased, which suggests a delayed clearance of apoptotic cells in case of MBL deficiency [101]. Similar findings have been described for studies in human recipients of renal grafts [102].

In sum, for a fetus to implant and develop, the maternal immune system needs to adapt to the new state, and these adaptions rely on a long list of important interplays between maternal leucocytes, complement factors, and fetal cells as well as a homeostatic external and internal environment that does not disturb their communication.

IMMUNE SYSTEM ABERRATIONS IN PATIENTS WITH RECURRENT PREGNANCY LOSS

The former section highlighted the complexity of the dynamic maternal immune response to pregnancy. As these interactive, biological processes are all important for completing a healthy pregnancy, numerous options for "system errors" causing implantation failure or fetal loss exist. However, it is hypothesized that reproduction is for every species too important to be vulnerable to a single defect in the physiological pathways involved in fertilization, implantation, and pregnancy maintenance. Therefore, it is largely unlikely that only one immunological aberration can cause RPL; rather, it is more likely that aberrations in different immune pathways, either alone or in combination, can cause RPL. Along with such a hypothesis, the approach to correct such aberrations may therefore involve using one or more immunomodulatory therapeutic drugs depending on the disturbed pathway.

Researchers in RPL immunology have different perspectives on which immunological processes to focus on when searching for RPL causations. The extensive number of cell types, functions, signal molecules, and molecular pathways can be analyzed in specimens collected from different tissues and time points by several laboratory methods. The great methodological heterogeneity complicates the assessment of the various findings on RPL immunopathology.

The following presents a (non-exhaustive) list of different findings and appertaining theories on the immune-mediated pathophysiology of RPL.

ENDOMETRIAL HYPER-RECEPTIVITY

The reiterative cyclic differentiation of endometrial stroma into the robust, tolerogenic, and receptive decidual stroma occurs in response to the postovulatory rise in progesterone and local production of cyclic adenosine monophosphate. During the WOC, a time-restricted, inflammatory decidual environment renders the endometrium receptive; thus, prepared if a fertilized oocyte enters the uterine cavity [103]. It has been hypothesized that decidualization is impaired due to the reduced number of endometrial mesenchymal stem cells and enhanced cellular senescence in RPL

patients. Lack of uNK cell-mediated clearance of acute senescent cells in the endometrium may lead to an elevated level of chronic senescence. In vitro studies have suggested that the excessive number of senescent cells and their persistence provide a prolonged inflammatory phase after ovulation due to their secretion of pro-inflammatory cytokines, chemokines, and extracellular metalloproteases [66,104], which is hypothesized to extend the WOC and impair the selectivity of the decidualized endometrium towards low-quality embryos [105] due to out-of-phase embryo implantation destined to early pregnancy loss [66,104,106–108]. Indeed, a correlation was found between the later the implantation, the higher the risk for pregnancy loss [109]. However, this hypothesis needs to be confirmed in a larger population of RPL patients and validated in clinical studies.

REGULATORY T CELLS

Developing and maintaining immune tolerance to the semi-allogeneic fetal cells are pivotal for pregnancy maintenance. Increased levels of Treg lymphocytes during early pregnancy are assumed to be crucial for the acceptance of the allograft because of the Treg anti-inflammatory cytokine profile and ability to inactivate cellular and humoral responses to self-antigens. It has therefore been hypothesized that Treg deficiency can cause an increased inflammatory response to the fetal alloantigens and subsequent fetal demise [110].

The results from studies manipulating the Treg population in mice support this hypothesis. A decreased Treg population caused implantation failure and early pregnancy loss in allogeneic pregnancy, possibly related to the increase in activation and proliferation of T cells and NK cells demonstrated in the allogeneically mated, Treg-depleted mice in contrast to syngeneically mated, Treg-depleted mice. Treg depletion in syngeneic pregnant mice did not affect the implantation rate, pregnancy rate, and number of viable fetuses per litter [87,111–113]. Furthermore, when Treg depletion was introduced after successful implantation, it did not seem to affect the pregnancy or perinatal fetal outcomes [111]. Indeed, the adoptive transfer of Tregs to abortion-prone mice prevented fetal resorption when transferred before implantation but not after this time point [113]. These findings suggest that Tregs are crucial for attaining maternal tolerance for fetal alloantigens. It is believed that the Tregs function similarly in human pregnancy since an abundance of Treg cells with anti-inflammatory and anti-cytotoxic properties can be found in peripheral blood and endometrial tissue during the first trimester of a normal pregnancy while reduced levels are found in women with a pregnancy loss [114].

Several studies in RPL patients have found a decreased level of Tregs in peripheral blood [115–117] and decidual tissue [110,116–119] as well as a reduced capacity of Tregs to suppress cytotoxic and pro-inflammatory immune responses [110,118]. Furthermore, the level of Tregs was higher in peripheral blood and decidual tissue from RPL patients whose pregnancy succeeded compared to patients with a new

pregnancy loss. The opposite was found when analyzing Th17 lymphocytes. Moreover, no differences were seen between the patients with a successful pregnancy and healthy pregnant controls [116,120,121]. A persistent inflammatory state in RPL patients may induce Treg dysfunction and consequently intolerance to fetal antigens [86,122,123].

To sum up, based on these congruent findings in connection to, a low Treg level is one of the highly suspected biomarkers of an RPL pathogenesis and an important target when searching for an effective RPL therapy.

T HELPER CELLS

Investigations on Th lymphocyte aberrations in RPL patients have mainly focused on the Th1/Th2 paradigm originating from Wegmann et al. [124]. This paradigm hypothesizes that Th1 activity is harmful during pregnancy while Th2 immunity is beneficial for reproductive fitness. The hypothesis was based on the findings in mice studies of decreased Th1/Th2 cell ratio during normal pregnancy as well as findings of fetal demise after injection of Th1 cytokines, which are generally classified as pro-inflammatory (tumor necrosis factor-α [TNF-α] and interferon-γ), in contrast to the enhanced fetal survival in abortion-prone mice after injection of Th2 cytokines classified as anti-inflammatory [124]. Moreover, a significantly enhanced Th1 inflammatory response was detected in abortion-prone mice compared to normal, pregnant mice after stimulation with paternal APCs [113] and administration of TLR-agonists activating Th1 cells induced fetal resorption. At the same time, the neutralization of Th1 cytokines prevented such events [125,126]. In general, the Th1 and Th2 cells were considered as each other's opposites and mutually inhibitory [124,127–130]. Subsequent studies found an elevated Th1 level and/or Th1/Th2 ratio in RPL patients compared to healthy controls both before and in early pregnancy [131–133]. Moreover, in pregnant women, a reduced Th1/Th2 cell ratio was found in endometrial tissue and peripheral blood collected from women with a normal pregnancy who did and did not have a history of RPL. In contrast, the cell ratio was significantly higher in RPL patients with subsequent pregnancy loss [134–136].

However, over time this dichotomous view of Th1 and Th2 cells has been criticized for being an oversimplified description that excludes the complexity and fine distinctions of the immune response occurring before, during, and after pregnancy [127,129,137]. The contradictory findings in studies testing the Th1/Th2 hypothesis may result from different timing, measurement methods, and choice of biomarkers as well as a lack of statistical power and methodological quality. Both cell types are crucial for successful implantation since both pro- and anti-inflammatory immune responses are necessary to induce endometrial receptivity, fetal tolerance, and pathogen immune defense [128,137].

Intravenous immunoglobulin (IVIG) and prednisolone are therapeutics that can reduce the Th1 cell level and increase Th2 levels [138–140]. A retrospective cohort study found that treatment with IVIG given to subfertile women with an elevated pre-conception Th1/Th2 ratio significantly increased the successful pregnancy rate compared to non-treated women with a similar immune profile [141]. However, the study did not measure if Th1/Th2 cell ratio changes occurred after treatment. Nevertheless, elevated Th1/Th2 ratio and the in vitro cytokine production upon stimulation have been suggested as markers for increased immune activity in RPL patients that could suggest a need for immune modulatory treatment [133,141].

NATURAL KILLER CELLS

Emerging theories on the role of NK cells in RPL pathogenesis are many, and validation of one theory does not necessarily exclude the relevance of the other as both may be relevant but in different patients.

In both pregnant and non-pregnant RPL patients, an elevated level of NK cells and cell cytotoxicity has been found in peripheral blood and decidual tissue in comparison to healthy pregnant and non-pregnant women [142]. Moreover, elevated NK cell levels and cell cytotoxicity have also been associated with a negative reproductive prognosis in several observational studies [133,143–149], while few other studies found no such associations [150–152]. The contradictory observations may be due to differing timing and laboratory methods as well as the treatments applied. It is hypothesized that increased NK cell level leads to an increased level of angiogenic factors causing an increased peri-implantation blood supply, and consequently oxidative stress to trophoblast [153].

NK cell parameters are widely used in clinical practice as biomarkers for specific immunomodulatory treatments for patients with reproductive failures. However, the lack of consensus regarding the most reliable laboratory methods (e.g., immunohistochemistry or FACS analysis) measures (e.g., cell concentration or proportion), and cut-off values defining abnormal NK cell parameters make it difficult to compare results between studies. Thus, the transferability to clinical practice is still controversial. Therefore, further studies are needed before measuring NK cell levels and activity in the clinic will contribute meaningful information to the counseling of RPL patients.

Another hypothesis is that depending on the combination of the inherited paternal HLA-C allele and the maternal KIR allotypes, an inadequate uNK cell activation may occur and hamper successful embryo implantation [142]. This theory is based on studies in RPL patients suggesting that an inappropriate match between the inhibitory NK receptor gene polymorphism and paternal (non-self) HLA-C allotype on the trophoblast may disturb the inhibitory NK receptor/HLA-C interaction. This interaction may impact the auto- versus (vs) allorecognition and NK cell activation,

contributing to a cytotoxic response to the paternally inherited fetal antigens and causing RPL [154–157].

HUMAN LEUKOCYTE ANTIGEN PHENOTYPE AND EXPRESSION

The majority of autoimmune diseases are associated with specific HLA alleles. The link to disease susceptibility (or protection) is highly variable, but up to as strong as 95% have been seen for Caucasians with ankylosing spondylitis being HLA-B27 positive [94,158]. As for ankylosing spondylitis [158], uRPL is a condition with a lack of strong biomarkers and increased frequency of broad autoantibody positivity and pro-inflammatory immunological changes with unclear relationship to the pathogenesis. In addition, specific HLA-DR alleles have also been associated with RPL [159–161].

HLA-DR is one of the three clinically relevant HLA class II molecules and tends to be the most highly expressed type [72]. The association with RPL is, however, controversial since one case-control study found an association with HLA-DRB1*03 [160], another study found an increased prevalence of HLA-DRB1*07 but not of HLA-DRB1*03 [161], while a meta-analysis suggested HLA-DRB1*04 and *15, as well as HLA-DR and HLA-B, sharing to be associated with RPL [162]. Thus, each study suggested distinct HLA alleles to increase susceptibility to RPL. The controversial findings may result from publication, selection, or information bias due to the relatively few studies published, limited sample sizes, and varying study criteria for inclusion. The heterogeneity between the study samples regarding whether patients with different ethnicity and patients with autoimmune diseases or autoantibody positivity were included is particularly important when searching for HLA susceptibility alleles.

It has been hypothesized that RPL patients may be genetically predisposed to miscarry since most of the factors associated with RPL are relatively frequent in the background population with no reproductive complications. Due to the similarities between autoimmune disease and RPL, HLA genetic polymorphisms have been hypothesized to act as predisposing factors. However, there is a lack of evidence to confirm any specific genetic susceptibility component in RPL [159–161,163].

Not only do the similarities between RPL and autoimmune diseases suggest HLA polymorphisms as candidates for susceptibility genes. The similarities between the immune responses found in relation to fetal demise and the response in unsuccessful organ transplantation, including graft versus host disease (GVHD) and graft loss, suggests an important role of HLA. The HLA molecules are highly immunogenic antigens, and HLA compatibility between the graft recipient and the donor is therefore important for graft survival. Furthermore, some minor histocompatibility antigens (mHA) are highly immunogenic. Especially the male-specific mHA (H-Y-antigens) are important determinants for graft survival after organ transplantation ([164,165]).

This association was also considered relevant for RPL pathophysiology. Indeed, an increased sex ratio of firstborn children was found in sRPL patients and associated with a subsequent negative prognosis [160,166].

The repertoire of peptides that an individual's APC can present depends on the HLA genotype, as the allelic variation in each HLA gene affects the structural features of the binding groove and favors binding to high-affinity peptides. For example, only specific HLA class I and II alleles can present H-Y-antigens [165,167,168]. Women who carry one or more of the H-Y-restricted HLA class II (HYr-cII) alleles can present H-Y antigens on their APCs to the T and B lymphocytes, unlike women who do not carry such alleles.

Carriage of HYr-cII alleles (to date, defined as HLA-DRB1*07, *15, and HLA-DQB1*0501/0502), but not class I alleles, in combination with the birth of a boy before the sRPL diagnosis affected the pregnancy prognosis negatively in comparison to sRPL with a firstborn girl. However, no association between the sex of the firstborn child and the subsequent prognosis was found when carrying no H-Y-restricting class II alleles [166,169]. Moreover, the negative prognostic impact seemed to increase with the increasing number of HYr-cII alleles carried by the RPL patient [170].

HLA class II antigen presentation, in contrast to class I, and activation of CD4+ T cells are necessary for the development of high-affinity antibodies by (memory) B cells which possibly explains the association found between higher frequency of HLA antibodies in sRPL after a boy than a girl and the association between HLA antibodies and a negative reproductive outcome in sRPL patients [171]. However, Nielsen et al. are the first and only group to publish such findings; therefore, further studies are needed to confirm their hypothesis.

MANNOSE-BINDING LECTIN

The amount of trophoblast and fetal cell shred to maternal circulation increases steadily during pregnancy and exerts pressure on the mechanisms responsible for clearing blood from foreign cells, apoptotic and necrotic cells, cell debris, etc. Apoptotic cells are a preferential source of autoantigens, and deficient clearance is implicated in an increased susceptibility to developing autoimmune diseases [172]. The clearance mechanisms involve phagocytosis by professional phagocytes, which rely on specific receptors and opsonins to recognize and bind to their targets. The opsonin MBL is important in especially late apoptotic and necrotic cell clearance [173]. The recognition and internalization of early apoptotic cells by the phagocytes are associated with a subsequent anti-inflammatory cellular response. However, when the apoptotic cells are not readily cleared, they reach the late apoptotic and necrotic cell stage, and the phagocytosis of such cells is associated with a pro-inflammatory cellular response. Late apoptotic and necrotic cells are membrane-damaged cells that leak immunostimulatory molecules and autoantigens to the immune system with the

potential to initiate autoimmunity [173]. Thus, efficient clearance mechanisms are fundamental for determining the subsequent adaptive immune response and are vital for maintaining immunological tolerance towards the cell-associated antigens.

Studies in abortion-prone mice demonstrated that complement activation through the lectin pathway at the feto-maternal interface occurs during implantation, and it can lead to fetal resorption in an antibody-independent manner acting upstream in a cytokine-mediated proinflammatory response. In addition, the findings revealed that selective MBL inhibition or knock-out of the MBL gene prevented fetal resorption in these abortion-prone mice [100]. These findings, in connection with the theory that deficient apoptotic cell clearance causes inflammation and intolerance to processed cell antigens, may suggest that the lectin pathway has an important role in early pregnancy.

Studies of the complement system in RPL patients have found an increased frequency of MBL deficiency based on the plasma level of the protein or the polymorphism of the *MBL2* gene, respectively [174–179]. In contrast, other studies have found no such association [180,181]. It is unclear whether a low p-MBL level has a negative impact on the reproductive outcome. Some studies have found an association between low p-MBL levels or *MBL2* gene variants and RPL, premature rupture of membranes, vaginitis, preeclampsia [182], preterm birth [178], and lower live birth weight [174]. In contrast, other studies have found no such association [183]. Moreover, one study found an association between the high MBL level genotype and premature birth [184]. However, despite few and inconclusive studies, the findings are not necessarily contradicting. A high and a low MBL level, respectively, could potentially negatively impact health when combined with other abnormal processes since the prognosis of some diseases are associated with low p-MBL levels and others with high levels. Thus, MBL may be a double-edged sword [185].

Nevertheless, it remains uncertain whether genetic or plasma MBL determination is most informative, whether both high and low p-MBL levels exhibit a risk for negative reproductive outcomes, and at what levels deficient and excessive p-MBL should be defined.

While low p-MBL levels seem to be associated with RPL, no studies have reported data on high p-MBL levels in RPL. Also, the impact of both high and low p-MBL levels on the reproductive outcome in RPL patients remains undetermined, and such information is needed to determine if and how MBL should be assessed in relation to the RPL workup and management.

This short review of the immune-mediated pathophysiology of RPL is not complete. The listed associations are some of the main ongoing hypotheses of immunological causes of RPL but are certainly not limited to them. Other immune aberrations have

been found in RPL patients but do not dominate the present research within reproductive immunology and were, therefore, not included here.

TREATMENT OF PATIENTS WITH RECURRENT PREGNANCY LOSS RISK FACTORS

An evidence-based treatment is only available for a small fraction of RPL patients, while for the majority of patients, no treatment definitively reducing miscarriage risk exists. The international societies for Obstetrics and Gynecology or reproductive medicine have differing recommendations for the treatment of RPL according to the findings in the diagnostic workup, and the few medical treatment recommendations that exist are mostly weak and/or conditional [13–16,18]. In the following, the recommended treatments for RPL patients will be presented; first, when one or more potentially contributing factors are found, and next, when no such factor is found.

PARENTAL CHROMOSOMAL ABNORMALITY

Couples with an abnormal parental karyotype should be offered genetic counseling and guidance on their options, including spontaneous conception with prenatal genetic testing in early pregnancy, ART treatment with preimplantation genetic testing (PGT), and ART treatment with a sperm or oocyte donor [13], supporting informed decision-making before trying to conceive again. PGT for aneuploidy or structural arrangements may reduce the miscarriage rate in couples with an abnormal parental karyotype [186,187]. However, it may also extend the time to live birth in couples with high fecundity rates since the risk of inheritance and new miscarriage is, on average, 50% when one in the couple carries a chromosomal translocation [188]. Also, while pregnancy losses are burdensome, so is IVF due to the financial expenses, need for (possibly many) stimulation cycles and painful oocyte collections, risk of ovarian hyperstimulation syndrome, the lack of guarantee to have an aneuploid embryo and the emotional stress and uncertainty associated with each treatment [189].

MATERNAL THROMBOPHILIA

According to a meta-analysis of randomized and quasi-randomized controlled studies in RPL patients with inherited thrombophilia, there is no benefit from antithrombotic prophylaxis, including low-molecular-weight heparin (LMWH), unfractionated heparin (UFH), or LDA or a combination of both [190].

Contrarily, RPL patients with acquired thrombophilia may benefit from treatment with LDA combined with heparin. A Cochrane review on RCTs comparing LDA combined with heparin to LDA alone found a relative risk (RR) for a live birth of 1.27 (95% CI 1.09-1.49) and a RR for a pregnancy loss of 0.48 (95% CI 0.32-0.71) in

LDA/heparin treated patients. However, due to the significant risk of bias highlighted by the authors, the certainty of the evidence was graded low [191]. The only RCT on RPL patients with APS comparing antithrombotic prophylaxis with placebo tested LDA only and found no significant difference in LBR and risk of pregnancy loss [191].

The optimal dose, initiation, and duration of LDA and LMWH/UFH for maximal benefit and minimal risk are unknown as the RCTs in the meta-analysis used highly heterogenous treatment protocols and suffered from methodological limitations [13].

Nevertheless, for RPL patients with ≥3 pregnancy losses and APS, the conditional recommendation is to initiate LDA (75-100mg/day) before conception and LMWH/UFH from positive pregnancy test and continue the treatment combination until delivery [13–16,18].

The evidence for the recommendation is weak as the available studies are of low quality, there is no RCT comparing LMWH/UFH treatment with no treatment or placebo in RPL patients with APS, and also, since the evidence on the safety of the treatments on the mother and her child is lacking.

HYPOTHYROIDISM

Overt hypothyroidism is defined as elevated thyroid stimulating hormone (TSH) and reduced free thyroxine level, and SCH is defined as moderately elevated TSH and free thyroxine within normal range. There is no consensus regarding the normal reference range since the TSH reference range depends on the iodine intake in the geographic area, ethnicity, TPO antibody positivity, and possibly body mass index (BMI) [192]. Using varying definitions is problematic for evaluating and interpreting results from different studies and making uniform guidelines for clinicians.

There is no high-quality evidence for an association between RPL and overt hypothyroidism. However, treatment with levothyroxine and close TSH level monitoring during pregnancy is effective in reducing the increased risk of adverse pregnancy complications, including preterm birth, low birth weight, miscarriage, and detrimental effects on fetal neurocognitive development associated with overt hypothyroidism [193,194]. Often adjustment of levothyroxine dose is needed as physiological changes during pregnancy cause an increased need for thyroxine [195].

Concerning SCH, the evidence for whether the treatment should follow that of overt hypothyroidism or not is contradicting, and so are the recommendations. ESHRE guideline group and European Thyroid Association Guideline suggested considering treatment of SCH with levothyroxine on a case-by-case basis where the benefits should be balanced against risks [13,196], while the ASRM and Royal College of

Obstetricians and Gynaecologists (RCOG) consider evidence insufficient for any recommendations [15,16].

Thyroid autoimmunity, the presence of TPO antibodies, is associated with RPL. Nevertheless, studies evaluating treatment with levothyroxine to euthyroid RPL patients positive for TPO antibodies do not find an improved pregnancy outcome [195,197,198]. TPO antibody positivity is associated with an increased risk of (subclinical) hypothyroidism in pregnancy [196]. TSH is therefore recommended to be monitored in early pregnancy for RPL patients with TPO antibody positivity by the ESHRE guideline group and the European Thyroid Association Guideline while no such recommendation is found in the RPL guidelines by ASRM and RCOG [13–16,18].

LUTEAL PHASE SUPPORT

Sustained progesterone production is essential for successful implantation and ongoing pregnancy. Progesterone deficiency can be caused by several conditions, including stress, PCOS, several drugs, obesity, excessive exercise, aging, thyroid dysfunction, ART treatment, and prolactin disorders [199]. However, diagnosing luteal phase insufficiency is complicated due to the unclear relation between blood level and endometrial maturation, the varying length of the luteal phase found in fertile women, and the wide variation over short periods in peripheral blood caused by the pulsatile production in response to LH pulses: i.e., up to eight-fold fluctuation in 90 minutes is seen. Therefore, none of the proposed definitions based on either biochemical, clinical, or histological tests is considered reliable, and no recommendation on progesterone supply to RPL patients exists [199].

The PROMISE trial that randomly assigned vaginal capsules of either 400mg micronized progesterone or placebo twice daily to uRPL patients with ≥3 pregnancy losses found no significant change in subsequent LBR (65.8% vs. 63.3%, p=0.45). However, a subsequent trial combining data from two RCTs [200,201] found a significantly increased LBR in pregnant women with a history of minimum ≥1 pregnancy loss and a bleeding episode in the first trimester of the present pregnancy treated with micronized progesterone compared to placebo and the beneficial effect was even greater in patients with ≥3 prior pregnancy losses [200,201]. In addition, a Cochrane meta-analysis found that RPL patients treated with progesterone compared to no treatment or placebo had a slightly lower miscarriage rate (20.1% versus 27.5%, p=0.06) [202]. Based on these findings, the recently updated ESHRE guideline recommends initiating vaginal progesterone supplementation to RPL patients with ≥3 losses and a vaginal bleeding episode from the time of bleeding and up to GW 16 [13]. In contrast, none of the other international guidelines [13–16,18] have been updated since the study by Coomarasamy et al. [203] was published. Therefore, they do not consider the evidence sufficient for recommending any luteal phase support to RPL patients.

ANOVULATORY CONDITIONS

PCOS is associated with several pregnancy complications, including miscarriage; however, no clear association with RPL has been found, although common underlying pathophysiologic factors are seen [13]. Infertile patients with PCOS have suggested treatment with metformin based on low-quality studies finding a reduced miscarriage rate compared to no treatment [204–206] and no change in risk of birth defects or pregnancy complications [207]. However, no study has focused specifically on RPL patients with PCOS, and there are concerns about a possible teratogenic effect of metformin. Therefore, the evidence for an equivalent effect of metformin in RPL patients with PCOS is considered insufficient to recommend it [13–16,18]. However, controlled ovarian stimulation for RPL patients with PCOS was considered a potentially beneficial treatment for decreasing miscarriage risk in the ESHRE guideline group [13].

RPL patients with anovulation and hyperprolactinemia may benefit from treatment with a dopamine agonist before and during pregnancy based on small, low-quality studies finding a reduced prolactin serum level and miscarriage rate [208,209]. However, the evidence was insufficient to recommend it for RPL patients with hyperprolactinemia [13–16,18].

UTERINE MALFORMATIONS

Müllerian malformations cover a wide spectrum of congenital uterine anomalies, including, among others, uterine septae and unicornuate and bicornuate uterus, and acquired uterine malformations, including, among others, endometrial polyps, fibroids, and intrauterine adhesions. The classification systems are many, and the prevalence is low, which complicates the diagnostic procedures for the individual caregivers regarding their familiarity with clinical signs and symptoms, diagnostic imaging, and treatment recommendations for each specific type. These details may complicate research on these conditions and cause diagnostic delays and inappropriate or inadequate interventions. Consequently, patients with uterine anomalies may endure persistent issues, including loss of reproductive function [210,211].

The associations between RPL and some anomalies, especially the congenital, are well documented, while the association with other anomalies, especially the acquired, are more questionable. Since surgical procedures involve a risk of both irreversible and reversible side effects, the procedure should be limited to patients for whom a beneficial outcome regarding their reproductive function is expected.

The question of whether there is an effect of surgery on fertility was raised by Venturoli et al. [212], as their study in RPL patients reported not only a reduced miscarriage rate after hysteroscopic septum resection but also a negative impact on fertility as only 52% became pregnant within the first year after surgery. The only

RCT on septum resection found no reduction in pregnancy loss rate; however, patients were only followed for 12 months post-randomization, and the study comprised a small and relatively heterogeneous group of 79 participants with a history of either subfertility, ≥1 pregnancy loss or preterm birth [213]. However, three meta-analyses of observational studies reported that hysteroscopic septum resection was associated with a lower miscarriage rate but no change in clinical pregnancy rate post-surgery compared to women having a uterine septum that was not surgically removed [214–216]. One of the meta-analyses reported results on an RPL subgroup analysis, supporting the overall findings [216]. However, the quality of the studies included in these meta-analyses based on only observational studies was considered low, and the methodological heterogeneity was large. Since the only RCT available shows no effect of septal resection on reproductive prognosis, it is recommended that treatment decision-making should have this concern in mind [13–16,18]. However, despite the weak evidence, the ASRM recommends that septate defects should be considered in RPL patients [16].

The benefits of surgical treatment of other congenital uterine malformations remain questionable and insufficiently investigated to make any evidence-based recommendations, and therefore, no well-defined best practice exists [13–16,18].

Regarding the acquired anomalies, resection of endometrial polyps larger than 1 cm can be considered when no other cause is found, while smaller polyps often regress spontaneously [13]. In contrast, there is currently no conclusive evidence for association with RPL nor an improved reproductive outcome after surgical removal of smaller endometrial polyps, intrauterine adhesions, and fibroids [13,15,16,217,218]. It is, therefore, often a clinical dilemma whether to surgically remove them or not. However, as the surgical procedure is associated with a risk of post-operative complications affecting fertility and future pregnancies [219] but has no seemingly beneficial effect on miscarriage risk, the patient should be informed about such paucity of evidence that the decision of intervention or not is made. Overall, the ASRM guideline recommends that surgical correction of significant uterine cavity defects should be considered. At the same time, the other international RPL guidelines await evidence of higher quality before making any clear recommendations [13–16,18].

PARENTAL LIFESTYLE

The evidence regarding the effect in RPL patients of lifestyle changes on LBR is either of very low quality or completely lacking, depending on the specific factor. However, informing the patient about the risks for pregnancy- and perinatal complications associated with alcohol, tobacco smoking, excessive exercise, and obesity and suggesting lifestyle modification is recommended by the ESHRE guideline group [13]. The other international guidelines describe no specific recommendation but acknowledge the potential harmful effects.

According to the male partner, evidence of any effect on RPL prognosis of lifestyle change is lacking. Nevertheless, they should be informed that smoking, obesity, specific drugs, and alcohol are associated with increased oxidative stress that can cause DNA damage in gametes and that elevated DNA damage in male gametes is associated with RPL. The information should also include suggestions for lifestyle changes if applicable and that small studies have suggested that intake of antioxidants may improve the prognosis. However, the effects on RPL patients are still insufficiently documented [13,220,221].

METABOLIC DISTURBANCES

The evidence of associations between RPL and hyper-homocysteinemia [222] or vitamin D deficiency [223] is insufficient, and determination of these factors is therefore not included in the recommended routine diagnostic workup. However, if one of these conditions is detected, general practice offering the patient folic acid 0.5 mg or vitamin D supplement up to 100 mcg/day, respectively, is recommended before pregnancy [224].

PSYCHOPATHOLOGY

Not only does the physiological aspect of RPL need attention in the diagnostic workup and therapy. The immense mental and psychological stress the patient (and her partner) suffers after RPL is often a major burden. However, since the physician rarely has the competence, time, and resources to take good care of the psychological aspect, it is often left to the patient's own responsibility.

During the last five to ten years, several women have come forward in public and shared their stories of miscarriages, including Meagan Markle and Prince Harry (Duchess and Duke of Sussex: 2020), Chrissy Teigen (model and influencer) and John Legend (musician) (2020), Beyonce and Jay-Z (musicians: 2013), Priscilla Chan and Mark Zuckerberg (Facebook founder) (2015), and Michelle and Barack Obama (former president of USA: 2018) [225]. Their purposes have often been to support other women through the stressful event, and indeed, several women report that disclosure of miscarriage by celebrities or friends assuaged their feelings [226].

Nevertheless, as the frequency of psychological stress, anxiety, and major depression is significantly higher in RPL patients compared to women with no reproductive complications [35,227], the care should be sensitive to mental health and offer support during follow-up evaluations [13]. Mental strength, healthy coping strategies, and social support aid the patient's ability to manage the presence of anxiety and grief in ensuing pregnancies as she may otherwise be at risk for post-traumatic stress disorder [228].

It has been proposed that the psychopathologic condition and RPL may exacerbate one another in a vicious circle as psychopathology affects the nervous system, hormone production, and immune pathways (the psycho-neuro-immuno-endocrine network) which subsequently can negatively influence the reproductive function [37,229]. Only a few small and non-randomized studies have evaluated the effect of medical counseling and psychological support on RPL patients after a miscarriage on the subsequent pregnancy prognosis, and they indicate a significant beneficial effect, including increased LBR [230–232]. Despite low-quality evidence, mental health is considered of great importance regardless of the effect on LBR, and it is recommended to include tender-loving care with frequent ultrasound examinations and early pregnancy support according to the patient/couple's individual psychological states and needs, as this will vary [13–16,18]. With the significantly increased prevalence of serious psychiatric diagnoses among RPL patients, including major depression and attempt of suicide [19,35], the physician may also need to consider interdisciplinary collaboration with, e.g., psychiatrists or psychologists.

IMMUNOMODULATORY TREATMENTS SUGGESTED FOR PATIENTS WITH “UNEXPLAINED” RECURRENT PREGNANCY LOSS

When no risk factor associated with RPL is identified during the diagnostic workup of the RPL patient, no evidence-based treatment for increasing LBR exists. These uRPL patients are often subject to immunological investigations as the existing evidence suggests an aberration in the immune system can be found more often than expected in these patients. However, at present, no accepted immune-based test that can identify RPL patients who will benefit from immunological treatments exists.

A major effort has been put into finding a treatment that increases LBR after uRPL. As the theory of an irregular immune response being an important factor in uRPL pathophysiology is widely acknowledged, a major scientific focus has been testing immunomodulatory therapeutic drugs for these patients. Among the immunomodulatory treatments suggested are lymphocyte immunization therapy (LIT) with paternal lymphocytes or third-party leukocytes; infusion with intravenous lipid emulsion (intralipid) or IVIG; injections with granulocyte-macrophage/granulocyte colony-stimulating factor (GM-/G-CSF), TNF-α inhibitors or heparin; or peroral glucocorticoids.

Most studies testing immunomodulatory treatments were quasi-experimental or observational and of low quality. Also, no immunological testing was performed in previous RCTs to either measure the therapeutical effect on immunological biomarkers or identify patients' characteristics among those with the beneficial effects of treatment. Thus, we lack high-quality studies that could aid in finding biomarkers

possessing sufficient discriminative value to identify patients with aberrations associated with RPL and affected by the tested therapeutic drug. As anti-inflammatory treatments may only show valid results in analyses of patients with an underlying inflammatory disorder susceptible to the drug being tested, the current lack of such inflammatory biomarkers or diagnostic tests to guide clinical decision-making may have attenuated the outcomes and consequently underestimated the true effects. In continuation hereof, it has been suggested that meta-analyses of treatment effects end up with negative findings due to no pre-selection of patients based on immune responses [133,233]. However, as the immunomodulatory therapeutic drugs often affect several mechanisms of the immune system simultaneously and several immune aberrations have been associated with RPL, it is complicated to specify a set of such patient selection criteria. If or when such criteria are found, this will aid in prescribing the immunomodulatory treatment to the selected subgroup expected to benefit while reducing harm and saving costs by not prescribing to those patients with RPL without such causal, immunological aberrations.

LYMPHOCYTE IMMUNIZATION THERAPY, INTRALIPID THERAPY, AND GRANULOCYTE COLONY-STIMULATING FACTOR

The rationale for the effect of LIT was based on the finding that RPL patients often lack anti-paternal antibodies or blocking antibodies which have been hypothesized to protect the fetus, although the impact of those antibodies is not well-known [234]. The rationale for intralipid therapy was an observation of declining NK cell cytotoxicity after infusion in females with recurrent implantation failure after ART [235], while GM-CSF and G-CSF were suggested to promote trophoblast invasion and reduce fetal resorption rate based on findings in animal studies [236,237].

Evidence for a beneficial effect of LIT, intralipid, and GM-/G-CSF on LBR in RPL patients is lacking. No positive effect from LIT on reproductive outcomes has been found; rather, a trend towards a reduced successful pregnancy rate was seen [238]. Two RCTs on G-CSF treatment found diverging effects on LBR in uRPL patients, with one RCT finding a significantly positive effect [237,239]. In contrast, a bigger multicenter RCT found a trend for a negative effect [237]. The only RCT examining the effect of intralipid on 296 uRPL patients with elevated NK cell levels undergoing ART treatment was of low quality and found no effect on the primary outcome of chemical pregnancy rate but did find a significantly increased LBR (OR 2.1, 95% CI 1.3-3.5). However, the results should be considered cautiously since the associated protocol published on ClinicalTrials.gov described no placebo group and no measurement of LBR in contrast to the published article of the study results [240].

While the beneficial effect of these treatments is controversial, the increased risk of serious adverse effects of such treatments is well-documented. These effects include sepsis, infection, embolism, and organ failure after intralipid, and fatigue, bone pain, and osteopenia after GM/G-CSF. Besides a substantial risk of transferring infections

and inducing neonatal alloimmune thrombocytopenia and anti-erythrocyte antibody production, it was recently documented that LIT increased the risk of conversion from autoantibody negative to positive, which suggests that it may increase the risk of autoimmune disorders [238]. Due to the paucity of evidence of the beneficial effect of these therapeutic drugs on uRPL and a relevant risk of serious harm, they are not recommended [13–16,18].

ANTICOAGULANTS

A widely used therapeutic drug for both RPL and recurrent implantation failure is anticoagulant treatment such as LMWH, UFH, and LDA, even for RPL patients with no positivity for anti-phospholipid antibodies (aPL) or another marker for coagulopathies. A Cochrane meta-analysis of nine randomized or quasi-randomized studies found no effect of anticoagulant treatment (i.e., heparin, LDA, or a combination versus placebo or no treatment) for uRPL patients without APS [190]. However, the studies included were considered insufficiently powered and highly heterogeneous. For example, the meta-analysis included only one study of 6 patients that compared LMWH with no treatment. Following this Cochrane review, three RCTs comparing LMWH with no treatment have been published, in which two RCTs found no significant effect on LBR [241,242]. At the same time, one RCT did report a significantly decreased pregnancy loss rate and increased LBR in uRPL patients [243]. However, evidence of the beneficial effect of anticoagulants on LBR in uRPL patients remains insufficient according to international guidelines.

GLUCOCORTICOIDS

Glucocorticoids are widely used therapeutic drugs for uRPL. The rationale for glucocorticoid use is the well-known positive effect on several autoimmune diseases due to the broad spectrum of pharmacodynamic effects on the immune system, especially the ability to suppress NK cell level and cytotoxicity (Figure 1.1) [244–247].

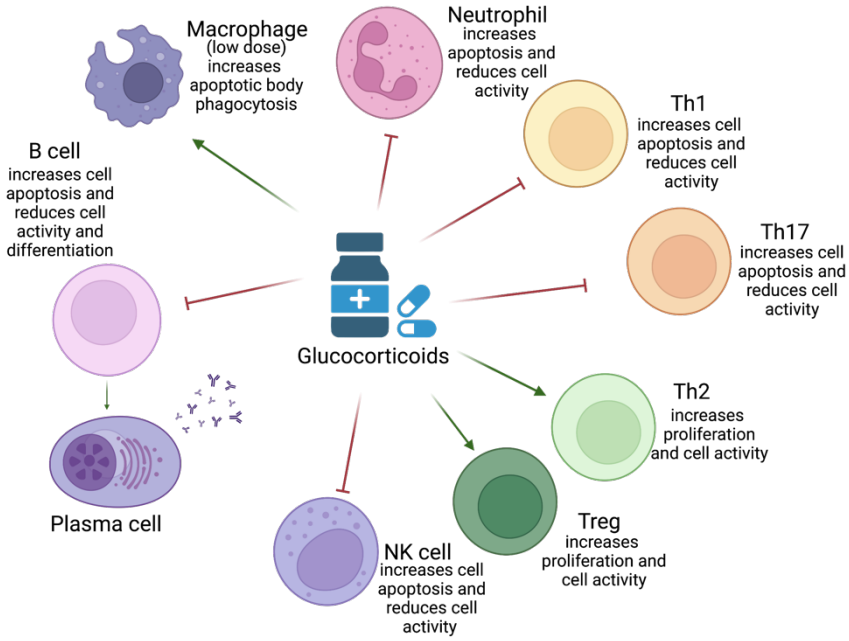

Figure 1.1: An overview of some of the immunomodulatory effects of glucocorticoids.

The three RCTs testing prednisolone in RPL patients used much different treatment protocols. One RCT compared prednisolone (20 mg/day) and LDA with LDA alone, initiated from pregnancy was confirmed and until GW 14, and found an elevated ongoing pregnancy rate (RR 1.9, 95% CI 1.4-2.5). In contrast, no adverse pregnancy or fetal outcomes were reported in either group [248]. Another placebo-controlled trial with a similar treatment protocol, besides the addition of UFH in both treatment arms and a placebo comparator, found a significantly increased ongoing pregnancy rate beyond 20 GWs compared to the placebo (RR 7.6, 95%CI 3.7-15.7) [249]. Laskin et al. [250] tested a higher dose of prednisolone (0.5-0.8 mg/kg body weight/day) from positive p-hCG measurement and until after delivery in combination with LDA in RPL patients positive for ≥1 autoantibody. Compared with placebo, they found no significant difference in LBR (65% vs. 56%). Instead, an increased incidence of preterm birth and pregnancy complications, including gestational diabetes and hypertension, was found in the group given prednisolone and LDA [250].

In a feasibility study, Tang et al. [251] randomized 40 uRPL patients who had a high uNK cell density (≥5%) during the WOC in a cycle prior to pregnancy. They initiated treatment with 20 mg/day prednisolone after intrauterine pregnancy was ultrasonically confirmed and continued for six weeks. The study found an insignificantly increased

LBR in the active treatment group (RR 1.5 95% CI 0.8-2.9) [251]. However, since funding was the limiting factor for study termination and not a preliminary estimated sample size, the study possibly ended before reaching sufficient statistical power for the comparison, and it was consequently at risk for type II error.

Several other observational studies have suggested a beneficial effect of glucocorticoids in RPL patients with elevated NK cell parameters [252,253]. Therefore, NK cell parameters are widely used as biomarkers to indicate whether glucocorticoid treatment should be prescribed. However, since there may be a substantial risk associated with glucocorticoid treatment; since the role of NK cells in RPL pathogenesis is still controversial; and since the laboratory methods and reported measures are highly variable between the studies, the validity and safety of such treatment is questionable and further studies of higher quality is needed before such clinical practice can be fully endorsed.

Overall, the sparse evidence may suggest an effect of glucocorticoids on LBR in patients with uRPL positive for certain immune biomarkers, but further studies are needed to evaluate the effect and side effects before any recommendation can be substantiated.

INTRAVENOUS IMMUNOGLOBULIN

IVIG is a passive immunotherapy form that also acts on multiple processes in the immune system. The immunomodulatory activity of IVIG involves different pathways grouped into the Fc-dependent pathways and the F(ab')2-dependent pathways (Figure 1.2). The Fc-dependent pathways act by binding antigens and include reduction of the Th17 cellular activity, upregulation of the Fc-inhibitory receptors, inhibition of signal pathways of the Fc-activating receptors, expansion of Tregs, and binding to and blocking immune complexes and some of the activating receptors on leukocytes. The F(ab')2-dependent pathways include neutralization of antibodies and cytokines, support to antibody-dependent cytotoxicity by, e.g., NK cells, and blockage of the anaphylatoxins C3a and C5a, which inhibits the anaphylatoxins' ability to activate and attract (chemotaxis) leukocyte, upregulate phagocytosis, and trigger degranulation of mast cells, endothelial cells, and phagocytes [254]. These actions are just some of the many actions of IVIG therapy. However, the passive treatment competes with a highly dynamic immune system that can readily counteract or compensate for such immunoglobulin extension. Therefore, assessing the treatment dose and intervals required for achieving the full effect is complicated.

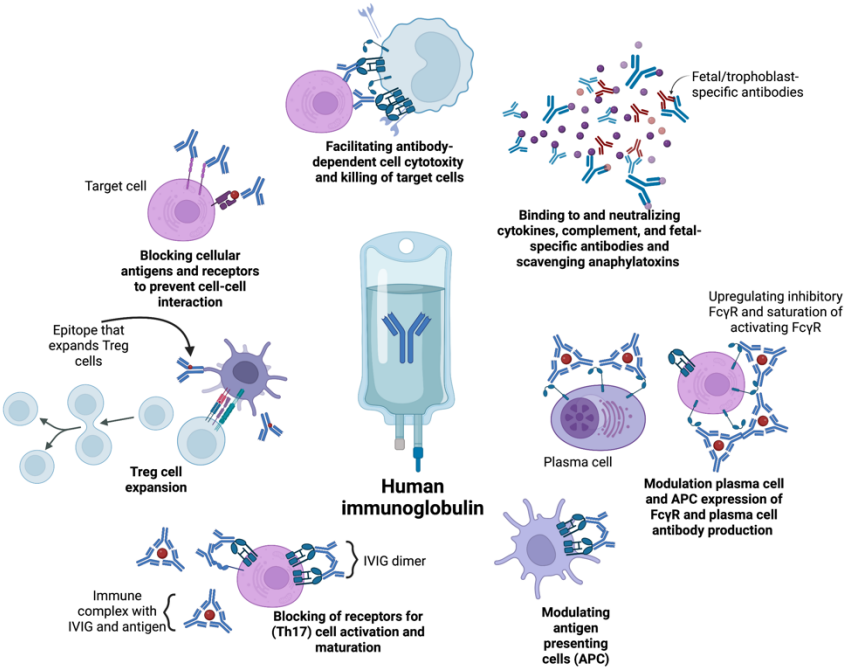

Figure 1.2: An overview of some of the immunomodulatory effects of intravenous immunoglobulin*. The upper mechanisms include the Fc-dependent pathways, and the lower mechanisms include F(ab')2-dependent pathways.*

The most recent meta-analysis on 15 (supposedly) RCTs testing IVIG versus placebo, including 902 RPL patients, found a significantly increased LBR in the total RPL sample (odds ratio [OR] 2.30; 95% confidence interval (CI) 1.23-4.30); however, the authors must have overlooked that four of the included studies were indeed not randomized [255]. A similar meta-analysis that included 11 RCTs found a marginally significant effect of IVIG on LBR in RPL patients (RR 1.25, 95% CI 1.00-1.56), which was more pronounced when treatment started before conception (RR 1.67, 95% CI 1.30-2.14) [256]. However, whether two of the included trials were, true RCTs is questionable. Another meta-analysis, only partly overlapping with the former meta-analysis, included 11 true RCTs comparing IVIG with placebo or "treatment as usual" and found no significant effect on the frequency of no live birth in RPL patients (RR 0.92, 95% CI 0.75–1.12) but a subgroup analysis showed a significant borderline effect in sRPL patients (RR 0.77, 95% CI 0.58–1.02, p=0.06) [257].

No meta-analysis has included the results from the most recently published Japanese RCT on IVIG, which included 99 uRPL patients with four or more clinically confirmed pregnancy losses [258]. In this trial, treatment with 400 mg/kg IVIG or 8 mL/kg saline was initiated six days after identification of the gestational sac by

ultrasonography (GW 4-6) and continued for five consecutive days. The study found IVIG to be associated with a significantly increased LBR (OR 2.60, 95% CI 1.15-5.86), and in sub-analyses, this effect was even more pronounced when treatment was initiated in GW 4-5 (OR 4.85, 95% CI 1.74-13.49) while insignificant when treatment was initiated in GW 6 (OR 0.66, 95% CI 0.14-2.76). Moreover, the effect was also more pronounced in patients with six or more previous pregnancy losses (OR 7.20, 95% CI 1.35-38.32) than in patients with four to five previous losses (OR 1.79, 95% CI 0.69-4.61) [258]. An updated meta-analysis may produce a more robust and significant result of IVIG for uRPL.

Nevertheless, the selection criteria in the RCTs were highly heterogeneous. Therefore, identifying patients with the highest chance of gaining a beneficial treatment effect remains up for discussion, which is important due to the risk of adverse effects that could be avoided in patients not eligible for treatment. The recent RCT reported that the gestational age at delivery and birth weight were significantly lower in the active treatment group. In contrast, the frequency of preterm delivery, fetal growth restriction, and small for gestational age was significantly higher [258].

A clinical study in RPL patients with elevated percentage and/or cytotoxicity levels of NK cells in peripheral blood found an increased LBR in patients treated with IVIG compared to untreated patients. Furthermore, reduced NK cell percentage and cytotoxicity from pre- to port-treatment were found in IVIG-treated patients, while both parameters remained unchanged in untreated patients [259,260]. This result follows the findings in a meta-analysis of eight non-randomized controlled trials that selectively included RPL patients with an immune aberration, including elevated NK cell percentage, elevated Th1/Th2 ratio, or an autoimmune disease. The LBR was significantly increased after immunomodulatory treatment (OR 1.98; 95% CI 1.44-2.73), and the treatment efficacy was even more pronounced in the subgroup of RPL patients with elevated NK cell percentage (OR 2.32, 95% CI 1.77-3.02) and when the treatment was initiated before conception (OR 4.47, 95% CI 1.53-13.05), respectively [260].

Overall, the selection criteria and the treatment protocol differ substantially between the studies testing IVIG in RPL patients [256,257,260]. The evidence from the observational studies may have a high risk of being seriously biased, especially by sampling bias, confirmation bias, outcome reporting bias (P-hacking), the Hawthorne effect, and publication bias.

No RCTs have so far included blood or tissue analyses of immunological biomarkers before and during treatment. The biomarkers that have been suggested to identify patients with an immunological disturbance contributing to the RPL pathogenesis and that will benefit from IVIG therapy are based solely on low-quality, observational studies with a high risk of bias. Thus, no high-quality evidence exists on which biomarker(s) can be used to identify the RPL subgroup most likely to benefit from

IVIG – or, for that matter, from any other immunotherapy to RPL patients. All uRPL patients do not have a causative immunological aberration, and IVIG cannot neutralize all immune dysregulations; however, based on the sub-analyses from the meta-analyses according to timing and patient characteristics, one may question whether we with the optimal timing, treatment regimen, and appropriate patient selection will find IVIG to be a highly successful RPL treatment.

While international guidelines that do not recommend IVIG were based on findings from the meta-analyses that do not include the latest findings [15,16], the updated ESHRE guideline from 2022 included a conditional recommendation of IVIG for uRPL patients with four or more losses based on the most recent findings [13,258].

CHAPTER 2. CONSEQUENCES

RPL has been described as one of the most complex and challenging conditions within reproductive medicine [261]. When women/couples with RPL attend the specialized RPL clinic, they often seek an explanation for their pregnancy losses. However, one or more of the evidence-based factors associated with RPL can be found in only approximately 40%, and these cases are often referred to as “explained” RPL. The remaining subgroup of patients is categorized as “unexplained.” However, the “explained” cases may not be explained by the presence of the specific risk factor since, for example, treatment of such risk factor, if available, far from always improves the prognosis for the patient’s subsequent pregnancy outcome. Also, for some factors considered risk factors for RPL, no association with the number of pregnancy losses or the prognosis has been found, raising questions on the causal link. Such factors include, for example, sperm DNA damage, PCOS, and subclinical hypothyroidism [13]. Consequently, the lack of explanation and effective treatments leave the patient and the physicians frustrated and disheartened.

The prognosis of RPL is generally considered good, especially if the female age and number of prior pregnancy losses are low [13]. However, patients often find it hard to believe that the next pregnancy may succeed without intervention. Indeed, the prognosis of RPL may be overestimated. Previous studies evaluating the prognosis for a live birth in RPL patients may be biased since these studies are often based on small samples containing solely couples with pregnancy after admission. Thus, couples who keep trying are included, while those who give up are not. As the couples who give up may represent patients with the worst prognosis, this may bias the findings by overestimating the suggested general prognosis for RPL [262]. Furthermore, the studies evaluating RPL prognoses rarely include internal or external validation.

The emotional impact of a pregnancy loss on the couple, possibly predominantly the woman [36,263,264], is highly significant and may stop them from attempting once more. The feelings of loss and grief may intensify with each loss [226,265], while with social support, a new pregnancy may alleviate such feelings [265]. However, couples rarely announce a pregnancy before the second trimester, so the social network is not in a position where they can provide the desired support [265,266]. Moreover, besides the increased risk of serious psychological disorders associated with miscarriages, the events also put significant stress on the relationship and put the couple at risk of separation [261,266]. Thus, RPL patients often need interventions that integrate multiple disciplines within medicine.

Some of the therapeutic drugs suggested for unexplained RPL patents described previously, for which sufficient evidence of their effectiveness was lacking, may prove to be effective in the future. Until then, the physician's job separating patients

with a non-modifiable cause (i.e., patients whose pregnancy losses were due to cytogenetic aberrations) from patients with an underlying modifiable cause for whom an intervention is crucial for succeeding the subsequent pregnancy is intricate. Thus, when making the treatment plan, the physician is often left with a dilemma of whether to await the documentation or follow his/her instincts with the best intention.

Not only is the number of prior pregnancy losses considered a major determinant for the risk of a new pregnancy loss, but a history of RPL also increases the risk of obstetric complications, including placental abruption, preterm birth, low birth weight, and stillbirth. In addition, RPL is associated with health risks later in life, including cardiovascular complications and venous thromboembolism [19]. Thus, RPL may be one of the most complex and challenging conditions within reproductive medicine [261], and further research in risk factors and underlying causes. Therefore, treatments are highly needed.

Chapter 2. Consequences

CHAPTER 3. DEFINITION OF THE KNOWLEDGE GAP

As described, it is clear that RPL is a devastating condition that can rarely be explained. Even in most RPL cases with risk factors, the underlying pathophysiological patterns remain unknown. In approximately 60% of patients, no risk factor can be found (unexplained RPL), and the research on these patients has predominantly focused on immune system aberrations and immunomodulatory treatments. The current scientific insight into RPL immunopathology has left several questions to be answered before clinical implementation of immune examinations and treatments can be recommended:

- Which susceptibility, diagnostic, predictive, and response biomarkers can be used to identify patients with an underlying immunological etiology?
- Which treatments can counteract, reduce, or correct such diagnostic immune aberrations?
- Which biomarkers can be used to identify individuals with favorable responses to immunomodulatory drugs?
- Which biomarkers can be used to confirm that a beneficial response has occurred in the individual exposed to such treatment?

This viewpoint implies that the underlying premise of an immunological disturbance is of clinical significance in the RPL pathogenesis for some patients. The majority of research on immune system aberrations in RPL has been performed in patients with unexplained RPL. However, the pathophysiologic mechanisms that explain the association between RPL risk factors and pregnancy losses are largely unknown. Therefore, using the term "unexplained" (or "idiopathic") exclusively for RPL patients with no presence of risk factors may be misleading. "Unexplained" may apply to more patients than the practice today, which may bias the research on causative factors of RPL. For example, excluding patients with positivity for ANA, TPO antibodies, and/or low/moderate titer of aPL in studies of uRPL may introduce bias to the findings as we still do not know how autoantibodies are involved in the RPL pathogenesis. On the other hand, investigating the prevalence of immune system aberrations or efficacy of immunomodulatory treatments in all RPL patients (i.e., no exclusion of patients with well-described causes like anatomic or genetic abnormalities) or RPL patients selected due to the presence of immune biomarkers of unproven relevance may cause even more bias. Thus, the current need for further knowledge on RPL pathophysiology and the different use of inclusion criteria put the studies in RPL patients at high risk of bias.

In order to answer the first question listed above, it is essential to investigate differences in the immunological mechanisms important for reproductive functioning between RPL patients and women with no reproductive disorders. The prognostic impact of such factors associated with RPL would be of interest. However, a risk factor may not always have a detectable prognostic impact, i.e., if the factor is rare, since one risk factor may contribute to the pathogenesis at a similar level as other risk factors do, in which case little or no difference on the reproductive prognosis would be found in regression models when comparing RPL patients positive for each of these specific factors. Nevertheless, if the factor fulfills several of Hill's 'viewpoints' for causation [267], one may expect it to be causative. Subsequently, the relevance of the risk factor as a target for therapeutic drugs or as a biomarker for therapeutic drug effectiveness would be of interest.

My research has focused on identifying biochemical and clinical markers to be used as markers for susceptibility, prognostic, predictive, and pharmacodynamic response in patients with RPL. This research included MBL, major and minor histocompatibility antigens, autoantibody positivity, and distribution and activity of leukocyte subgroups in peripheral blood. The study designs detailed cross-sectional studies in finding associations with RPL and susceptibility markers for RPL, cohort studies to assess the prognostic value of such markers for the reproductive outcome, and an RCT for evaluation of treatment effect and related prognostic, predictive, and pharmacodynamic response markers [268]. The research was performed in cooperation with the Department of Clinical Immunology at Aalborg University Hospital.

CHAPTER 4. STUDY AIMS AND HYPOTHESES

STUDY I

Study I was a combined cross-sectional and cohort study that aimed to investigate whether respectively low, intermediate, and high p-MBL level was associated with RPL, whether low p-MBL was a risk factor for subsequent pregnancy loss, and whether low p-MBL was associated with adverse perinatal outcomes before and after RPL.

We hypothesized that a low p-MBL was a risk factor for RPL and subsequent pregnancy loss. An additional investigation of the association between low p-MBL and perinatal outcomes was performed for exploratory purposes [1].

STUDY II

Study II was a cross-sectional study that aimed to explore the distribution of women with RPL who had an older brother and a previous birth of a boy, respectively and combined, in comparison to the expected distribution among women with no history of RPL and whether the association was more pronounced in pRPL or sRPL patients.

We hypothesized that male microchimerism is a risk factor for RPL, especially sRPL. We, therefore, expected the prevalence of RPL patients with a family history, including a firstborn boy, an older brother, or both, to be higher than expected and that the association was more pronounced in sRPL patients [2].

STUDY III

Study III was a combined cross-sectional and cohort study that aimed to replicate a previous study [166,170] that was the first to explore whether the sex ratio of children born prior to sRPL patients differed from the sex ratio of newborns in the Danish background population and whether it was associated with a subsequent negative reproductive outcome with and without stratification for maternal carriage of an HYr-cII allele. We also aimed to further explore the factors by evaluating whether the prognostic impact remained after adjustment for confounding variables, whether carriage of an HYr-cII allele was a risk factor for a subsequent negative reproductive outcome in pRPL patients, and whether the combination of a firstborn boy and carriage of an HYr-cII allele was associated with adverse perinatal outcomes prior to RPL and the sex of the child born after RPL in comparison to patients with no firstborn boy and/or no carriage of an HYr-cII allele.

We hypothesized that the prevalence of a firstborn boy was higher in sRPL patients than in the general Danish background population and that a firstborn boy in combination with maternal carriage of an HYr-cII allele was associated with an increased risk of adverse perinatal outcomes before sRPL, a reduced chance of a successful pregnancy after sRPL, and a reduced male- to- female sex ratio of children born after sRPL when compared to sRPL with a firstborn boy and no maternal carriage of HYr-cII alleles. Furthermore, we hypothesized that maternal carriage of HYr-cII alleles was not associated with a reduced chance of successful pregnancy or the sex ratio of children born after referral in sRPL patients with a firstborn girl nor in pRPL [3].

STUDY IV

Study IV was a cross-sectional study that aimed to explore the association between the presence of autoantibodies in RPL patients and three immunogenetic susceptibly markers, including carriage of HLA-DRB1*03, carriage of HLA-DRB1*07, and the presence of a low p-MBL level.

We hypothesized that the prevalence of patients with one or more autoantibodies was higher among patients carrying one or more of the listed RPL susceptibility markers than among patients who did not [4].

STUDY V

Study V was a protocol for an RCT that aims to investigate the effect of treatment with IVIG and prednisolone in combination on the chance of an ongoing pregnancy compared to a placebo in the following ART cycle in patients with a history of RPL after ART. The trial also aims to explore the safety of such treatment, how it affects immune biomarkers, and whether an immune biomarker can predict which patients benefit from the immunomodulatory treatment.

We hypothesize that the treatment combination increases the chance of an ongoing pregnancy after embryo transfer in RPL patients [5].

CHAPTER 5. METHODOLOGY – THE DATABASE

The Center for Recurrent Pregnancy Losses of Western Denmark (CRPLWD) is one of two specialized public centers for RPL patients in Denmark. Patients with RPL, defined as three consecutive pregnancy losses before GW 22, can freely choose between these two clinics. The CRPLWD also allows the referral of patients with two consecutive pregnancies if one occurred after GW 12 or if both pregnancy losses were achieved by ART and occurred before GW 10 for the intention of participating in the RCT (study V).

All patients admitted to CRPLWD undergo a routine diagnostic workup and receive medicinal counseling financed as part of the national health insurance, while prescribed drugs are at the patient's own cost except for IVIG treatment. This approach may contrast with other countries with high costs for such counseling and treatment.

At the CRPLWD, treatment plans are made based on the findings in the diagnostic workup. Therefore, upon referral, patients receive a letter with a recommendation not to get pregnant before the diagnostic workup. After the plan is made, patients are advised to contact the CRPLWD as soon they have a positive pregnancy test to monitor the serum hCG titer and schedule the next consultation. Besides the national health care offer to pregnant women in Denmark, the CRPLWD offers all RPL patients so-called "tender loving care," which refers to psychological support with ultrasonography and medical examinations weekly in the first trimester and two to four times in the second and third trimester, respectively. Patients receive an individualized treatment plan, and the following description of the therapeutic regimens offered at the CRPLWD is representative for our clinical practice but to some degree over-simplified. Patients with APS or hypothyroidism are treated following the ESHRE guideline [13]. Furthermore, the majority of RPL patients with ≥3 pregnancy losses are prescribed vaginal progesterone. Most patients with uRPL and the presence of autoantibodies are prescribed oral prednisolone at a dose of 5 mg/day before pregnancy or embryo transfer. At this time, the dose increases to 10 mg/day until GW 8-10. Patients with ≥4 pregnancy losses in whom the described treatments have been tried but have not been successful may be offered treatment with 400 mg/kg IVIG once before pregnancy and again during pregnancy, where the number of infusions and time interval depending on whether the patient has a history of late pregnancy losses or not. The IVIG treatment may be combined with the prednisolone treatment.

During my PhD, I developed a research database in which I manually typed in data registered in patients' medical records admitted to the CRPLWD after each diagnostic

workup and follow-up consultation if the patient consented. Written informed consent was obtained during the diagnostic workup. The database contains data solely on routine investigations. The database was approved by the regional research unit at the Region Nord (Approval number: 2018-5), and all data is stored in Microsoft Access®. We assume that the database contains information on a representative sample of Danish women who experience RPL as more than 99% of referred patients consent, the Danish healthcare system offers equal and universal access for all residents, and the treatment of RPL is roughly limited to solely two RPL clinics in Denmark which have similar referral criteria and clinical procedures.

The data collected in the diagnostic workup includes information on the patient's personal identifier (CPR number), age, BMI, smoking and alcohol habits, the patient's history of prior pregnancies, current pregnancy status, comorbidities, medical treatments prescribed by clinicians at our clinic or other clinics, and the number of siblings with a common mother and their age differences. Furthermore, results from the routine blood analysis on the female patient are registered, including p-MBL concentration, CRP, presence of autoantibodies (ANA, aPL [anti-cardiolipin, beta-2-glycoprotein-1 (β2-GPI), lupus anticoagulant], and TPO antibodies), homocysteine level, anti-müllerian hormone level, Factor II and V gene variants and HLA-DRB1 and HLA-DQB1 genotype (HLA-DQB1 only from October 2019) as well as chromosomal analysis (Array-CGH) on the female patient and her male partner (if any). All patients are examined for uterine malformations by 3D ultrasound scan, hysterosalpingography, or hysteroscopy.

Information on perinatal outcomes before and after RPL includes the date of birth, sex of the newborn, birth weight, gestational age at birth, delivery method, volume of peripartum hemorrhage, presence of preeclampsia, and presence of congenital malformations on all live births and stillbirths delivered after GW 22.

Information on subsequent pregnancy losses includes the date, the number, and the GW of all pregnancy losses after admission and until the first live birth after referral. Biochemical pregnancies with serum hCG over 5 IE/l are counted as pregnancy losses, while confirmed molar and ectopic pregnancies are not.

In principle, equal access to the clinic allows admission independent of the patient's socioeconomic status strengthening the database's generalizability to the general population of patients with RPL in Denmark. This database is fundamental for quality control of the Center's procedures and research in RPL etiology, treatment, and prognosis.

In studies I-IV [1–4], the sample consisted of RPL patients registered in this database.

Chapter 5. Methodology – the database

CHAPTER 6. STUDY I

PLASMA LEVEL OF MANNOSE-BINDING LECTIN IS ASSOCIATED WITH THE RISK OF RECURRENT PREGNANCY LOSS BUT NOT PREGNANCY OUTCOME AFTER THE DIAGNOSIS

INTRODUCTION

MBL is a C-type lectin that binds to carbohydrates and activates the complement system through the lectin pathway. MBL is produced mainly in the liver by hepatocytes, but it can also be produced by placental tissue [77,269].

The MBL2 gene encodes for the MBL protein and consists of four exons separated by three introns. Exons 1 and 2 encode the signal peptide with a cysteine-rich domain followed by the collagenous region, exon 3 encodes the neck, and exon 4 encodes the carbohydrate-binding domain. The MBL monomer is a homotrimer of three identical polypeptides that self-associate by disulfide bonds forming a collagen triple helix [270].

The MBL protein structure can comprise different oligomeric orders depending on the number of homotrimers in the structure. The high-order oligopeptides circulate typically in complexes with three MBL-associated serine proteases (MASP), which are crucial for MBL to activate the lectin pathway upon binding to carbohydrates or acetylated patterns. Alternatively, MBL form complexes with the two non-enzymatic MBL-associated proteins acting as competitive MASP inhibitors [270,271].

MBL2 gene polymorphism affects the plasma concentration and the capacity to form stable higher-order oligomers [270]. The p-MBL level in individuals with the same *MBL2* genotype can vary up to 10-fold and the ranges of p-MBL concentrations associated with each *MBL2* genotype are highly overlapping [272,273]. Thus, it is not possible to deduce the genotype from the plasma level or vice versa [270,273]. In addition, MBL is considered an acute-phase reactant whose responsiveness seems to depend on the *MBL2* genotype [274,275]. However, the change in p-MBL concentration during, e.g., an infection remains relatively small and often insignificant [276–278].

Although the binding is non-selective, the pattern recognition receptor allows MBL to distinguish self from non-self and altered-self structures [279]. MBL has a high

affinity to the oligosaccharides on the surface membranes of, e.g., pathogens, infected cells, and apoptotic bodies. This opsonization facilitates phagocytosis, complement activation, cell lysis, oxidative burst, leukocyte recruitment, etc., which are important innate immune functions. While the affinity to carbohydrates seems to be independent of the number of peptides in the MBL oligomer, the higher-order oligomers have higher ligand avidity. Furthermore, they are the only forms able to create complexes with MASPs [270,273].

The innate immune defense is the fast but non-specific first-line response, but the power is limited compared to the powerful, specific adaptive immune response that, however, is a slow responder. MBL deficiency is the most common immunodeficiency [280], but in the general Caucasian population, low and high MBL level is normally clinically silent. However, the significance of MBL deficiency is considered greater when adaptive immunity is compromised [281,282]. It may predispose to or be protective against some diseases, including recurrent viral, bacterial, and fungal infections, autoimmune diseases, and ischemic heart disease [283,284]. The same applies to high p-MBL levels in relation to other disorders [284].

Some studies found an association between RPL and MBL deficiency [174–176,179,182], while others found no association. However, the studies that did not find any association mainly defined MBL deficiency based on the *MBL2* gene polymorphism rather than the plasma concentration [174,180,182]. Only one cohort study investigated the impact on the reproductive outcome in RPL patients and found a slightly higher risk of a new miscarriage and a slightly lower mean birth weight (-287g) of neonates born at term. In addition, the study found a correlation between the prevalence of low p-MBL levels and the number of previous pregnancy losses [174]. Moreover, another study found a significantly lower transcription of the *MBL2* gene by placental tissue from spontaneous abortions than those from early elective terminations [269]. However, the studies of p-MBL levels concerning the risk of RPL used different cut-off values to define MBL deficiency, and no study has evaluated the association between RPL and high p-MBL levels. This information is highly relevant since high levels have been associated with preeclampsia and intrauterine growth restriction [285–288].

Thus, the small number of studies, their small sample sizes, and the inconsistent findings call for more evidence to clarify the association between RPL and MBL. The present study aimed to elaborate on the association between high and low p-MBL levels and RPL and the outcome after diagnosis, including successful pregnancy and perinatal outcomes.

METHODS

Patients with ≥3 consecutive pregnancy losses consecutively admitted to the CRPLWD between January 2016 and March 2020 were included if they had a regular menstrual cycle, no uterine malformations, and no parental chromosomal aberration. They were followed until March 2021. The outcome of the first pregnancy after admission was divided into two categories for the logistic regression analysis of the impact of p-MBL level on the reproductive outcome: a negative outcome included a pregnancy loss, while a positive outcome included an ongoing pregnancy beyond GW 12 or a live birth at the time of final follow-up. Perinatal data from all deliveries after GW 22 were collected.

p-MBL level was measured in a group of 187 healthy female blood donors of reproductive age which was used as a reference.

The p-MBL level was measured at the first consultation using an enzyme-linked immunosorbent assay with biotin-conjugated monoclonal anti-MBL antibodies and mannan-coated wells.

A low p-MBL level was defined as a plasma level of ≤500 μg/l and a high level of >3000 μg/l [1].

RESULTS

In total, 267 RPL patients were included. The prevalence of p-MBL ≤500 μg/l was significantly higher in the 18 pregnant patients (before GW 8) at the time the blood sample was collected in comparison to the remaining non-pregnant patients (77.8% vs. 44.2%, p=0.003).

The prevalence of p-MBL ≤500 μg/l was significantly higher in RPL patients than the reference group (44.6% vs. 24.9%; prevalence proportion ratio (PPR): 1.79, 95% CI: 1.34–2.38) while the prevalence of p-MBL level >3000 μg/l was significantly lower in RPL patients (17.2% vs. 30.8%, PPR: 0.56, 95% CI: 0.40–0.79).

No correlation between the prevalence of low p-MBL level and the number of previous pregnancy losses was found, as 44.9% of patients with three pregnancy losses and 44.1% of patients with four or more pregnancy losses had a low p-MBL level (PPR: 0.98, 95% CI: 0.75–1.29).

The p-MBL level did not significantly influence the risk of a pregnancy loss after admission when adjusted for maternal age, body mass index (BMI), and smoking (aOR: 0.61; 95% CI: 0.33–1.15; p=0.13) and neither on gestational age and

birthweight of children born before and after RPL. However, sRPL patients with low p-MBL levels had a significantly higher frequency of a firstborn boy than patients with p-MBL levels >500 μg/l [1].

DISCUSSION

Following previous studies measuring p-MBL levels in RPL patients [174,177,179,182], a significantly higher prevalence of a low p-MBL level was found in the present study. In addition, this study is the first to examine the frequency of a high p-MBL level in RPL patients, and interestingly, a high p-MBL level was significantly less frequent in the RPL patients compared to the reference group [1].

The definition of a low p-MBL level and a high level differs between the studies [174,177,179,182]. In the present study, MBL deficiency was defined as a p-MBL level ≤500 μg/l since it is the definition of low p-MBL levels in the Danish departments of clinical immunology since it is similar to the cut-off used in several studies on autoimmune diseases [289–292] and since it was considered the optimal cut-off level for functional MBL deficiency according to predict the risk of infections after allogeneic stem cell transplantation [272]. However, previous studies in RPL patients defined low p-MBL levels by a lower cut-off value between 50 and 200 μg/l [174,177,179,182]. The various definitions limit the comparability of study findings. Therefore, we advocate that the optimal cut-off for the p-MBL level below which the complement system is compromised should be determined. However, it may require comprehensive studies of the functional capacity of MBL. Furthermore, the optimal cut-off level may differ between diseases, making the determination even more complicated.

The p-MBL level had no influence on the miscarriage rate nor perinatal outcomes, including birth weight and gestational age of neonates born before sRPL and after RPL, respectively, in the present study [1]. In addition, no association between low p-MBL level and preterm birth was found in the present and the previous studies in RPL patients [1,174]. In contrast, the findings from studies in women with no reproductive complications were contradictory regarding which *MBL2* genotypes conferred an increased risk of preterm birth [178,184,293]. In contrast to our findings, Kruse et al. (2002) found a higher miscarriage rate and a 287g lower mean birth weight in neonates born at term by RPL patients with a low p-MBL level (<100 μg/l) compared to patients with higher p-MBL levels [174]. However, the findings should be interpreted with caution for several reasons. For example, the lower birth weight solely applied to neonates born at term, which may be a chance finding since it seemed to derive from a posthoc subgroup analysis as no such subgroup analysis was performed on any other perinatal outcomes; the analysis included perinatal data from a very small number of patients with a low p-MBL level; and a lower male : female

sex ratio of children born by sRPL patients with low p-MBL in comparison to women with normal p-MBL level was found (sex ratio: 0.91 vs. 1.24) [174]. Overall, the inconsistent findings on the association between MBL and perinatal outcomes may be due to chance findings in the present or previous studies or a result of different laboratory methods and cut-off values used in these studies. In conclusion, there is currently no evidence supporting that p-MBL level plays an important role in the perinatal outcome.

Previous studies in healthy pregnant women suggested that the p-MBL level is elevated throughout pregnancy, which suggests an important functional role of MBL in maintaining a healthy pregnancy [177,184]. In contrast, the present study found a significantly higher frequency of low p-MBL levels in the small sample of pregnant patients compared to non-pregnant patients. Since we have no p-MBL measurement before the onset of pregnancy in these patients, we do not know if their p-MBL level had changed with conception or represented their habitual level. The conflicting findings could be due to differences in the time these samples were collected since the referred studies measured p-MBL later in the first trimester than the present study did. A reduced p-MBL level in very early pregnancy could potentially reflect a sudden increase of MBL utilization during early implantation, which the woman will try to compensate for by upregulating MBL production, e.g., through the addition of placental MBL production [269], which causes an elevated p-MBL level in GW 12 and onwards [184]. Alternatively, it could suggest that the response to pregnancy following regulating MBL production differs between RPL patients and healthy women, or it could simply reflect a chance finding. It would therefore be of great interest to investigate whether changes in p-MBL level from before conception to each trimester in RPL patients differ from healthy women and whether the dynamic changes have a prognostic impact on the chance of ongoing pregnancy. This issue could possibly explain why the present study found no significant impact of pre-conception p-MBL level on subsequent risk of pregnancy loss.

The association between MBL deficiency and RPL may reflect the importance of complement recognition factors for the efficient clearance of apoptotic cells and cell debris from the circulation. The clearance is critical to prevent the accumulation of immunogenic material and the development of mature, immunogenic cellular immune responses. Indeed, MBL deficiency has been associated with an increased level of apoptosis and macrophage tissue infiltration and a reduced phagocytosis rate and clearance of apoptotic cells [101,102,172]. During apoptosis, a striking reorganization occurs in which membrane, cytoplasmatic, and nuclear components appear at the surface as highly immunogenic neoantigens. With time, the apoptotic cells may become secondary necrotic cells leaking such antigens, which is even more immunogenic. In case of a delayed or inefficient clearance, which is seen in MBL and C1q deficient patients, the normal tolerogenic response from the immature phagocytes to self-antigens in/on apoptotic cells may switch into an immunogenic response by mature phagocytic cells [294,295] which could include excessive

inflammation, antibody production, and development of immunological memory prepared for subsequent exposures (e.g., pregnancies) [170,295–297]. This theory has been suggested to explain the strong association between MBL deficiency and several immunologically determined conditions like systemic lupus erythematosus (SLE) and allograft survival [102,284,298]. The nearly 100% association between SLE and deficiency of C1q – and, to less extent, MBL - reflects the plausibility and relevance of the present hypothesis [299]. Like RPL, SLE is an immunogenic condition characterized by frequent positivity for autoantibodies to nuclear antigens and apoptotic cell surfaces antigens like cardiolipin, phospholipids, and C1q [13,294,299–302]. During pregnancy, the high cell turnover from the placenta causes excessive pressure on the mechanisms clearing maternal circulation for apoptotic trophoblasts. Insufficient clearance may deviate the immune response to fetal alloantigens towards sensitization rather than tolerance which may end up causing pregnancy complications or, ultimately, fetal demise. However, this hypothesis needs to be further investigated in RPL patients.

Besides binding to apoptotic cells and pathogens, MBL can also bind to healthy autologous (immune) cells, including monocytes, B cells, and NK cells. However, this binding was only detectable at higher concentrations of MBL (>600 μg/l) [303]. Such binding may affect cell functioning since a study found an almost two-fold increase of IgG antibodies and a three-fold expansion of B cells after Group B streptococcus vaccination in MBL-deficient mice compared to wild-type mice [304]. Such findings may suggest that MBL at sufficient levels has profound effects on the adaptive immune system due to an inhibitory impact on antibody production. Also, these mechanisms may explain the association between MBL deficiency and the development and severity of several autoimmune diseases [292,305,306] and support our hypothesis that impaired clearance in MBL-deficient women may cause fetal cell sensitization due to elevated levels of immunogenic fetal alloantigens and subsequently, loss of tolerance, antibody formation, and ultimately fetal demise.

sRPL patients with a low p-MBL level had more frequently given birth to a boy than a girl compared to sRPL patients with higher p-MBL levels [1]. This significant finding could be by sheer coincidence. However, it could also indicate that a combination of p-MBL deficiency and a firstborn boy may act synergistically on the immune system: i.e., a stronger potentiation of the susceptibility to have pregnancy losses after a birth when they act concurrently than separately [1]. In a continuation of the theory presented before, H-Y antigens are known to be highly immunogenic to the female immune system [307,308]. The required accumulation of such highly immunogenic antigens before the immune response deviates towards sensitization may therefore be lower than for other less immunogenic antigens, i.e., X-chromosome and autosomally encoded antigens [170,295–297]. Consequently, susceptibility for sRPL may be higher after the birth of a boy if the woman has a low clearance capacity due to, e.g., MBL deficiency [170,295–297].

Another way MBL may affect reproductive outcomes is through its ability to bind to the aPL β2-GPI, which can activate the complement system and thrombus formation [309]. Studies in mice have suggested that β2-GPI has a higher affinity to decidual endothelial cells and EVT than to endothelial cells located elsewhere [310]. The thrombin production upon MBL binding to β2-GPI in the decidua may explain the increased risk of pregnancy loss in patients with APS [309]. This mechanism is promoted in a pro-inflammatory environment with high levels of the Th1 cytokine, TNF-α [309], which has been often found elevated in RPL patients in several studies [133,311]. The dependence of complement in the APS pathogenesis was supported by a study in complement-deficient rodents that found a failure of the aPL to cause vascular thrombus formation and pregnancy loss [310,312]. Overall, these findings suggest that complement deficiency would reduce the risk of pregnancy loss in patients with obstetrical APS. Thus, one may speculate if the impact of the p-MBL level on reproduction depends on other biochemical factors, for example, the presence of autoantibodies, immune cell aberrations, or change in hormone levels, which complicates the investigation of the prognostic impact of high or low p-MBL.

Variations in the *MBL2* promotor region and exon 1 can alter the transcriptional level, the MBL degradation rate, the capability of the cells to secrete MBL, and the functional capacity of MBL by causing conformational changes that interfere with the ability to form high-order oligomers that can bind MASPs [273,279,313]. These characteristics are relevant when deciding which measurement method to use in studies on the clinical impact of MBL deficiency. The lack of *MBL2* genotyping in the present study may, at first sight, appear as a limitation since insight into the functional capacity associated with each *MBL2* allele is then missed. However, the mannan-binding ELISA used in the present study makes the most reliable, direct quantification of the functionally active MBL in plasma (i.e., the level of oligomerized MBL molecules) according to a study comparing several MBL assays [273]. With the wide range of plasma concentrations associated with each genotype, the functional capacity may also vary greatly between patients with the same genotype. Genotyping was therefore considered a less sensitive approach than mannan-binding ELISA, and the latter has therefore been recommended over the former when searching for relationships between MBL and diseases [273]. Indeed, including the plasma concentration and the fraction of functionally active MBL in the measurement method is important when searching for an association and causal link between MBL deficiency and RPL. Only with a reliable and accurate measure of the functional MBL capacity can we make such a hypothesis on the pathophysiologic role of MBL in RPL, as presented above.

In continuation hereof, studies that found a significant association between MBL and RPL measured the plasma levels [174,176], while studies that determined the *MBL2* genotype found no significant association [180,181] except from one study finding a significant association between the *MBL2* genotype and recurrent late pregnancy loss (≥2 losses after GW 14) [179]. These inconsistent findings between studies using

different methods may be explained by the lower sensitivity of genotyping than the mannan-binding ELISA for measuring the functional p-MBL multimer level. Indeed, using genotypes would be comparable to dichotomizing continuous variables, which often dilute associations and cause false negative findings [174,314].

A limitation of the study is the small sample of patients giving birth within the follow-up period. Although the sample was larger than the previous study [174], the prevalence of perinatal outcomes like preterm birth and low birth weight was small, and the study may not have been powered for analyzing the impact of p-MBL level on these outcomes. However, the prevalence of these adverse perinatal outcomes in the present study corresponds to what was found in RPL patients in a large Swedish register study [315]. Since MBL is an acute phase reactant that may respond to physical stressors like pregnancy or infection, another limitation of the present study may be that a small fraction of the RPL cohort was pregnant when the blood sample was collected, and no information on infection was collected. However, since the association between low p-MBL and RPL remained significant after the exclusion of pregnant RPL patients [1] and the MBL acute phase response is small and most often insignificant in patients with low p-MBL levels [177,184,274,277,316,317], we expect that it had no significant impact on the study findings.

Furthermore, we have no information on the reproductive history of the reference group. However, if any of the references had a history of RPL, this would not have weakened but rather strengthened our results.

One of the strengths of the present study is the comparison of RPL patients and the reference group on the prevalence of p-MBL level divided into small intervals [1]. No previous study has published their data allowing such an informative insight into the association between MBL concentrations and RPL. Interestingly, the prevalence of intermediate levels was comparable to the reference group, while the prevalence of the high MBL levels was significantly lower. These novel findings may suggest that high p-MBL levels may protect against RPL, while low levels may increase susceptibility to RPL. This result seems opposite to what has been found in patients with cardiovascular disease [284,318–320]. Since the present study is the first to publish data on the prevalence of intermediate and high p-MBL levels, and the number of patients with high p-MBL levels was small, the finding should be interpreted cautiously and reexamined to evaluate such a theory.

In the future, studies exploring the dynamic and functional mechanism of MBL on reproduction could potentially help explain why low pre-conception p-MBL level is associated with RPL but not the subsequent prognosis. It has been suggested that MBL deficiency alone is rarely symptomatic, but it may intensify and manifest if associated with other immune deficiencies such as IgG deficiency [321,322]. Thus, the possible requirement of concurrent immune deficiencies to reach clinical relevance may explain our findings and should be further investigated.

CHAPTER 7. STUDY II

WOMEN WITH RECURRENT PREGNANCY LOSS MORE OFTEN HAVE AN OLDER BROTHER AND A PREVIOUS BIRTH OF A BOY: IS MALE MICROCHIMERISM A RISK FACTOR?

INTRODUCTION

sRPL patients have previously proven their reproductive fitness by completing a pregnancy beyond 22-24 GWs. Therefore, the distribution of RPL risk factors in sRPL patients may differ from patients with pRPL. For example, hereditary intrauterine malformations, parental chromosomal abnormalities, thrombophilia, and (subclinical) thyroid disease may be expected to have complicated previous pregnancies too, and therefore less prevalent in sRPL patients, in contrast to acquired structural and/or histologic abnormalities in the uterine cavity (after, e.g., a complicated birth) and advanced age. However, one study comparing pRPL and sRPL found no such differences but solely a significantly higher mean age and frequency of gynecologic surgery and a lower frequency of elevated prolactin levels in sRPL patients. All other examinations in the thorough diagnostic workup were comparable [323]. The higher age may explain the significantly higher frequency of aneuploid fetal losses in sRPL patients than in pRPL and healthy controls, respectively, found in another cohort study [324].

However, a potentially important difference between the two subgroups is the exposure to large amounts of fetal cell-free DNA and intact fetal cells transferred to the mother and vice versa during especially third trimester in a sRPL patient's prior pregnancy. Microchimerism is the presence of foreign cells or DNA in a genetically distinct individual. The fetal microchimeric cells appear to have pluripotent stem cell-like properties, including longevity and the ability to differentiate into a wide range of cell types [325,326]. Microchimeric cells are often cleared from the circulation rapidly after delivery but can persist for decades [327]. These cells present allogeneic surface proteins encoded by the paternally derived autosomes or sex chromosomes to the maternal cellular immune system and trigger an immune response.

A previous study found that sRPL patients more often have given birth to a boy than a girl compared to the background population [166]; prior pregnancy with a boy compared to a girl was associated with increased prevalence of obstetric complications [325,328,329] and anti-H-Y antibody positivity [330]; and that sRPL

patients with a firstborn boy had a reduced LBR and elevated risk of obstetric complications in a subsequent pregnancy in comparison to patients with a firstborn girl [166,330,331]. These findings may indicate that a male-specific factor like H-Y antigen exposure can trigger an aberrant immune response and memory formation that, in case of re-exposure, e.g., a subsequent pregnancy, may initiate a rapid, robust immune response against the fetus.

However, sRPL patients with a firstborn boy may not be the only patients with H-Y antigen sensitization. Previous studies have detected male DNA and male microchimeric cells in nulliparous women, prepubertal girls, newborn girls, female stillbirths, umbilical cord blood, and in fetuses after elective termination [325,328,329], suggesting that the source of male specific microchimerism can be other than a prior pregnancy. Transplacental transfer of microchimeric cells present in the proband's mother, originating from, for example, the proband's older brother, could explain these findings.

Thus, as male microchimerism is detected in women with no prior pregnancy, and exposure to H-Y antigens may be a risk factor for pregnancy loss, we aimed to investigate if a history of an older brother and/or a firstborn boy was more frequent in RPL than expected and whether it was more pronounced in pRPL or sRPL patients in comparison to the expected distribution.

METHODS

RPL patients with ≥3 consecutive pregnancy losses consecutively admitted to the CRPLWD between January 2016 and March 2021 were included if they had no uterine malformations, no parental chromosomal aberration identified, and no missing information on their family history of siblings and previous childbirth. Only information on older siblings of the patient with a common mother was accounted for following the theory of transplacental transfer of cells [2].

Patients were divided into four groups according to the sex of their older siblings:

I. Patients with an older brother(s) only
II. Patients with an older sister(s) only
III. Patients with both older sisters and older brothers
IV. Patients with no older siblings

Patients were also divided into two groups based on the risk of H-Y exposure from transplacental cell transfer:

I. Patients with an older brother and/or prior birth of a boy were considered to have a high risk of male microchimerism.
II. Patients with no older brother and no birth of a boy were considered to have a small risk of male microchimerism.

The male-to-female sex ratio of births in the Danish background population in 2017 was 1.04 (51:49) [332], which was used as the expected sex ratio in binomial tests. The examination of sex distribution of the patients' older siblings and firstborn child, respectively, included solely patients who had only brothers or sisters and only boys or girls.

As a reference for the prevalence of at least one older brother and/or birth of a boy, the male-to-female sex ratio of the patients' younger siblings and the Danish background population were used. A detailed elaboration on the mathematical equation can be found in the published article [2].

RESULTS

In total, 383 RPL patients were included, among whom 47.5% had previously given birth after GW 22.

Baseline characteristics, including age, BMI, smoking habits, and number of prior pregnancy losses, did not differ between the four RPL subgroups that were based on the sex of the older siblings.

The male-to-female sex ratio of older siblings was significantly higher in all RPL than expected patients (1.49 vs. 1.04, p=0.027) and even more pronounced in the subgroup of sRPL patients (1.79 vs. 1.04; p=0.019). In contrast, the sex ratio of younger siblings of RPL patients did not differ from the expected sex ratio (0.86 vs. 1.04).

The male-to-female sex ratio of the firstborn child delivered before RPL differed significantly from the expected (1.51 vs. 1.04; p=0.026). The difference was even more pronounced in children of sRPL patients with older sisters (2.50 vs. 1.04) but less prominent in children of sRPL patients with older brothers (1.33 vs. 1.04). However, these sex ratios did not differ significantly from the expected sex ratio.

In total, 79.1% had at least one older brother and/or firstborn boy before sRPL, while 20.9% had only older sister(s), no older siblings, and/or a firstborn girl, and this distribution differed significantly from the expected frequency in the reference group (79.1% vs. 69.8%, p= 0.041). The distribution in the total RPL sample did not differ

significantly from the expected (Prevalence of women with an older brother, a firstborn boy, or both: RPL=53.5% vs. expected=49.0%, p=0.247) [2].

DISCUSSION

The percentage of sRPL patients who were considered to have a high risk of male microchimerism was significantly higher than expected. These patients had an older brother and/or had given birth to one or more boys before their pregnancy losses. The sex ratios of older siblings to all RPL and to sRPL patients as well as the sex ratio of prior births to sRPL patients, respectively, differed significantly from the sex ratio in the Danish background population (see Table I in the published article). No difference was found among older siblings to pRPL patients and neither among younger siblings. These findings follow our hypothesis based on the theory of maternal sensitization to H-Y antigens being a pathogenic factor in the development of unexplained sRPL. Since H-Y exposure can come from male microchimeric cells acquired during the proband's fetal life via the transplacental transfer of male cells present in the circulation of the mother of the proband (Figure 7.1) [333], it is possible that H-Y immunity may be a response to cells from older brothers and not only the proband’s own male pregnancies.

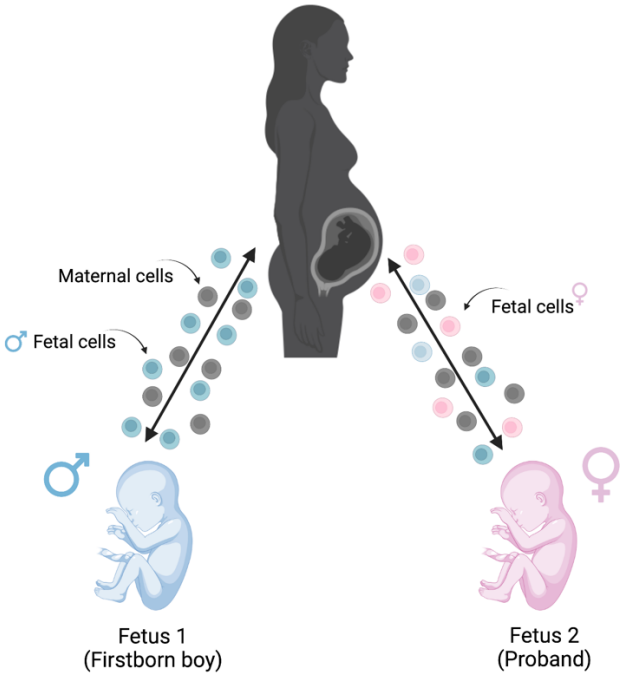

Figure 7.1: Transmaternal sibling cell trafficking*. The pink fetus represents the proband/patient with recurrent pregnancy loss, the blue fetus represents the proband's older brother, and the grey woman represents the proband's mother. Male microchimeric cells flow from the older brother to their common mother during her first pregnancy. Then in a subsequent pregnancy, the male microchimeric cells flow from the mother of the proband across the placenta to the proband.*

A potential critical limitation of the study is the need for a more relevant reference group. The expected percentage of patients considered to have a high risk of male microchimerism was based on both the distribution of sex among younger siblings to the RPL patients and the sex ratio of newborns in the Danish background population. However, the representativeness of this expectation is speculative, especially since the sex ratio of younger siblings was (insignificantly) lower than that of the Danish background population (0.86 in all RPL and 0.92 in sRPL) and, therefore, also differed significantly from that of the older siblings (p=0.014).

An alternative calculation of the expected number of healthy women having an older brother and/or firstborn boy could be based solely on the sex ratio in the Danish population and number of patients having an older sibling (Figure 7.2). This method may be more representative since the sex ratio (1.04) of firstborn children also applies

to older siblings in Danish women [332]. However, the result from this alternative calculation is similar to the expected number included in the study since the first calculation expects 126 women to be at risk of H-Y antigen exposure while the latter expects 127. Therefore, this calculation would not have changed the difference between the observed and expected risk of H-Y antigen exposure in sRPL patients in the study.

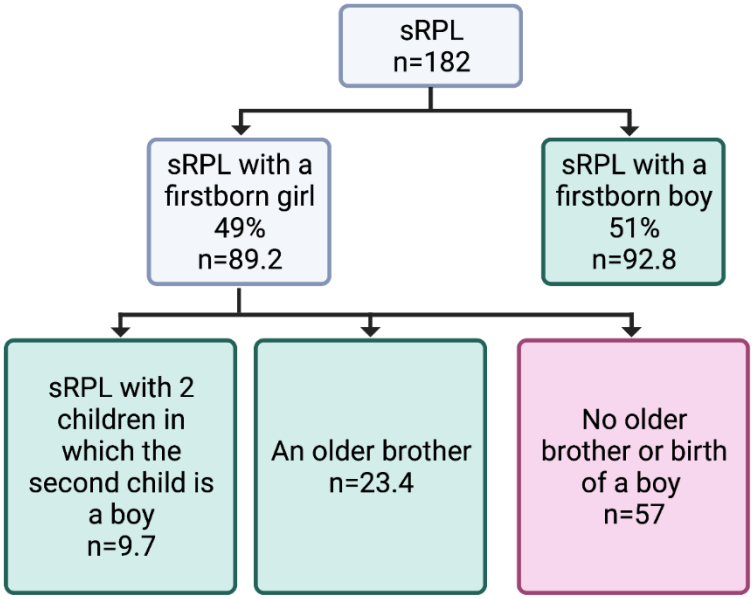

Figure 7.2: A new theoretical reference group. *The alternative calculation for the expected number of women with a firstborn boy and/or older brothers is as follows: we assume that the sex ratio of older siblings and firstborn child is 1.04 (51:49). Thus, we expect 51% of 182 sRPL patients to have had a firstborn boy (0.51*182=92.8). Among the remaining 89 patients with a firstborn girl, we expect 21.4% would have had ≥2 prior births based on the frequency observed in sRPL patients. The second child would expectedly be a boy in 51% of cases (0.214*0.51*89=9.7). That is, 102 sRPL patients (92.8 + 9.7) have expectedly given birth to a minimum of one boy prior to RPL. Among the remaining 80 patients with the birth of only girls, 57.7% are expected to have an older sibling based on the frequency in sRPL patients. The older sibling would expectedly be a boy in 51% of cases (0.577*0.51*80=23.4). In total (green boxes), 102+23=126 (69 %) would expectedly have been at risk of exposure to H-Y antigens from an older brother and/or a firstborn boy.*

In addition, following the study's publication, we have collected information on the number and the sex of siblings to women giving birth at the maternity ward at Aalborg

University Hospital. Based on 180 women with a history of a minimum of one livebirth and no known pregnancy losses, 127 (70.6%) had a minimum of one older brother and/or given birth to a boy. The difference between this group and the group of sRPL patients in the study was borderline significant (p=0.060). Thus, the three different reference groups all suggest that sRPL patients may be at a higher risk for exposure to H-Y antigens from prior births or older siblings than healthy women. This data supports the hypothesis of H-Y immunity as a possible cause of sRPL.

One month after the present study was published, a Dutch study investigating the prevalence of male microchimerism by a sensitive qPCR method detecting the DYS14-gene in blood samples from 152 female twins with same or opposite-sex twin pairs, their singleton non-twin sisters, and their mothers. The prevalence of male microchimerism in the total sample was 27% and did not differ between monozygotic twins, dizygotic same-sex twins, dizygotic opposite-sex twins, and singleton female siblings. Notably, there was a trend towards a higher prevalence of male microchimerism if the female had an older brother compared to those without (RR 1.31; p=0.09), and also if the female's mother was male microchimerism positive compared to females with male microchimerism negative mothers (RR 1.52; p=0.08). These findings support our theory of the transplacental flow of microchimeric cells present in the mother's circulation to the fetal circulation. However, the study found no difference in the prevalence of male microchimerism between women with and without the previous birth of a boy, but a significant, positive correlation was found between the woman's age and the presence of male microchimerism (p=0.02) [334]. These findings may suggest that women acquire and potentially accumulate male cells from one or more sources during a lifetime other than solely prior term pregnancies and that pregnant women with persistent microchimerism may be an important source of microchimerism in their offspring.

Nevertheless, the findings were not significant, and such a theory should be considered cautiously. However, the present and previous findings support the theory that the H-Y antigen exposure may derive from several generations back, including older brothers, rather than only the probands' own pregnancies. Other routes than transplacental flow for acquiring microchimeric cells have been suggested, including breast milk, blood transfusion, and sexual intercourse, which routes are relevant for further investigations.

An important limitation of the majority of studies on microchimerism is that they focus solely on detecting male microchimerism in a female by Y chromosome-specific techniques without making unequivocal matching with suspected sources. This constrains our ability to assess the results since these data do not reveal any information about the number of sources, the definite sources, or the frequency and concentration of microchimerism in general when female microchimerism is neglected.

Only a few small studies have used methods like HLA-matching or insertion/deletion (InDel) polymorphism methods to determine the definite source. Maternal DNA has been detected in 55% and 57% of female adults and fetal cord blood, respectively, and grandmaternal DNA in 18% of fetal cord blood [335,336] by HLA-specific qPCR techniques. Furthermore, fetal DNA has been detected in 37% of 51 parous, non-pregnant women up to 17 years after delivery with a more than 2-fold higher prevalence and 5-fold higher mean concentration in mother-daughter pairs than mother-son pairs [337]. In addition, the persistency of microchimerism positivity and microchimeric cell concentration was significantly higher in maternal blood in the presence of feto-maternal HLA-A, HLA-C, and HLA-DR compatibility than incompatibility [337,338].

No study has to our knowledge, performed such specific examinations of female blood for the presence of microchimerism from sources other than the proband's mother, grandmother, and children. Therefore, we still need definite confirmation whether other family relatives, like older brothers, are true sources of H-Y antigen exposure. We are currently performing a pilot study on a cohort of ten women with sRPL having older brothers in which detection of InDels and TSPY1-gene (chromosome Y) by quantitative PCR is used to search for DNA from siblings and offspring in the patient’s peripheral blood. The aim is to evaluate whether the specific InDel method can identify microchimerism from an older brother and children independent of sex.

Overall, the presence of microchimerism has been associated with both positive, neutral, and negative effects [339]. The immune response to microchimerism may mitigate both immune tolerance and allo-sensitization and consequently cause different diseases. Whether sensitization or tolerance develops depends on several factors, including the dose; the duration and route of exposure; the stage of life when exposed for the first time; the antigen immunogenicity; the immunoregulatory gene polymorphisms (including the HLA haplotype); and the immune environment present at the time and place of exposure [340]. For example, a significantly higher level of Treg cells is present in fetal and maternal blood during a normal pregnancy compared to non-pregnant adults, which creates an environment promoting tolerance rather than sensitization in response to foreign antigens [93].

In conclusion, the present study finds that the frequency of women who have given birth to a boy and/or have an older brother is higher than expected, and it may indicate a higher risk for H-Y antigen exposure. Both the present findings and the results from previous studies suggest H-Y immunity as a risk factor for RPL, but further studies on the importance of male microchimerism in the RPL pathogenesis are needed, including the differential impact from acquiring microchimerism during the fetal or adult stage of life, respectively.

CHAPTER 8 STUDY III

MATERNAL CARRIAGE OF H-Y RESTRICTING HLA CLASS II ALLELES IS A NEGATIVE PROGNOSTIC FACTOR FOR WOMEN WITH RECURRENT PREGNANCY LOSS AFTER THE BIRTH OF A BOY.

INTRODUCTION

Pregnancy is a state of immunological tolerance in which the placenta confers a "semipermeable" fetomaternal barrier defining what can be transferred from mother to fetus and the reverse. The fact that a woman can carry a semi-allogeneic organism for nine months – and even more extraordinary that she can bring multiple pregnancies with the same partner successfully to term – is the diametrically opposite to almost any other situation where the immune system is exposed to allogeneic or pathogen/exogenous antigens. This phenomenon is called "the immunological paradox of pregnancy" [61].

During a normal pregnancy, the maternal immune system acquires a tolerable phenotype due to several adaptations in the innate and adaptive immune system [96]. It has been speculated that aberrancies in such essential, pregnancy-induced regulatory mechanisms that prevent unrestrained fetus-specific immune responses may be a cause of RPL. It is possible that tilting the balance between sensitization and tolerance by, for example, environmental, genetic, or endogenous pro-inflammatory mediators acting regionally or systemically will cause fetal rejection.

H-Y antigens are ubiquitously expressed by all male cells, including the trophoblast from male pregnancies, but not by any female cells. Thus, female lymphocytes are not introduced to chromosome-Y-encoded mHA during central tolerance development in the primary lymphoid organs [308].

H-Y immunity after the birth of a boy has been suggested as a potential cause of fetal rejection in subsequent pregnancies [166,331] based on the study findings described in the introduction to study II. Furthermore, a study found that a history of a firstborn boy combined with maternal carriage of an HYr-cII allele was associated with a poorer pregnancy prognosis and elevated risk of pregnancy complications in sRPL patients compared to patients who did not carry such alleles and to patients who had

a firstborn girl [166,170,331,333]. These findings suggest that a firstborn boy is an sRPL risk factor only when combined with maternal carriage-specific HYr-cII alleles,

The authors hypothesized that H-Y immunity could be induced by maternal exposure to a large quantity of apoptotic syncytiotrophoblast debris shred from the placenta during late pregnancy with a boy which would be phagocytized by placental APCs, transported to local lymph nodes, and presented to maternal lymphocytes [170,330]. Only if the mother carries HYr-cII alleles, H-Y antigens are presented. Subsequently, maternal sensitization to H-Y and HLA antigens may develop, especially if the presentation occurs in a pro-inflammatory environment. Alternatively, it could be induced by an increased quantity of fetal microchimeric cells entering the maternal circulation during a complicated pregnancy with a boy [170].

This hypothesis is supported by transplantation research finding H-Y antigens to be highly immunogenic compared to autosomally encoded mHAs [341–343]. The H-Y antigen exposure can elicit plasma cell formation with antibody specificity to H-Y antigens, which is hypothesized to be a major cause of graft rejection because the use of a parous female hematopoietic stem cell donor increases the risk for chronic GVHD in both a male and a parous or nulliparous female recipient in comparison to using a male or nulliparous female donor [343–345]. Furthermore, H-Y antibody positivity is associated with acute graft rejection [307], and H-Y mismatch between the donor and recipient of a hematopoietic stem cell transplantation have a significantly higher incidence of acute and chronic GVHD as well as reduced overall survival and disease-free survival which did not apply mismatch in autosomally encoded mHAs [341,342].

However, the aforementioned hypothesis should be considered with caution for several reasons. The study's regression analysis estimating the impact of HYr-cII alleles and firstborn boy on pregnancy outcome was not adjusted for history of obstetric complications or age despite such factors differed between women with a firstborn boy and girl in their studies and influence on pregnancy outcome. Thus, such variables may have confounded the results [167,170,330]. In general, both the design of the studies and the sparse information on baseline characteristics of patients included in these studies put the results at risk of bias. In addition, the definition of which HLA alleles are H-Y restricting cannot be considered exhaustive due to the high number of combinations between the highly polymorphic HLA genes and chromosome Y-encoded peptides. For example, after their first publications on HYr-cII alleles, a study demonstrated that the HLA DRB1*0701 allele is able to present RPS4Y-encoded peptides [167,169]. This allele was then included in a subsequent study evaluating the long-term prognostic impact of the sex of the firstborn child and HYr-cII alleles in the Danish cohort, which found equal prognostic value between DRB1*0701 and the previously defined HYr-cII alleles [169]. Furthermore, the findings are all based on data from the same cohort of Danish female sRPL patients and have not been reexamined in independent cohorts from different environments and clinical settings.

These limitations of previous studies call for replication studies. Replication studies, especially new findings of genes associated with diseases, are highly important since these are rarely consistent in replication studies [346–348]. Despite the lack of relevant replication studies, the updated ESHRE guideline on RPL now includes a conditional recommendation for investigating HLA class II genes in sRPL patients after the birth of a boy for prognostic purposes as well as investigations for HLA genes in RPL for research purposes [13].

We found the hypotheses presented by Nielsen et al. plausible but simultaneously concerning that their findings are used to argue for recommending HLA screening in all sRPL patients with a firstborn boy for providing prognostic information to the clinician and the patient despite these relevant limitations. The present study therefore aimed to elaborate the hypothesis by examining whether the sex ratio of a firstborn child in sRPL is different from the background population, whether a firstborn boy affects the pregnancy outcome negatively after adjustment for relevant confounding variables, and whether the carriage of HYr-cII alleles influences this impact.

METHODS

RPL patients with ≥3 consecutive pregnancy losses consecutively admitted to the CRPLWD between January 2016 and October 2022 were included if they had no uterine malformations, no parental chromosomal aberration identified, no prior birth of both a boy and a girl after GW 24, and if the HLA-DRB1 genotype was determined during the diagnostic workup.

The cross-sectional sample consisted of all RPL patients, while the prospective cohort only included patients with a minimum of one pregnancy after admission. The reference group consisted of anonymous Danish bone marrow donors in whom data on sex, age, and high-resolution HLA-genotype were known. The male-to-female sex ratio in the Danish background population is 1.04 (51:49) and was used as a reference [332].

The HLA genotyping was performed at a 2-digit level using the FluoGene system and a modified TaqMan probe system (inno-train Diagnostik GmbH).

The HYr-cII alleles were defined as DRB1*07 and DRB1*15, as well as DRB1*01, DRB1*10, and DRB1*16, among which the latter three alleles were considered proxies for carriage of the HYr-cII alleles DQB1*0501/0502 in patients with no HLA-DQB1 analysis. The definition was based on findings from previous studies showing that only these specific HLA-DRB1 and -DQB1 alleles possess the ability to present H-Y peptides, and that strong positive linkage disequilibria exist between specific HLA-DRB1 and -DQB1 alleles [3,349].

RESULTS

In total, 582 RPL patients were included in the cross-sectional sample, among whom 391 were included in the prospective cohort. The smaller prospective cohort did not differ from the cross-sectional sample regarding baseline characteristics, sex ratio of firstborn children, and the proportion of patients carrying each HYr-cII allele.

Among the 269 sRPL patients in the cross-sectional sample, 61.0% had a firstborn boy. The male-to-female sex ratio for the firstborn child was 1.56, which differed significantly from the Danish background population (p=0.001). The frequency of giving birth to a boy after primary and secondary RPL did not differ from each other or from the Danish background population. Also, when sRPL patients were stratified by the carriage of HYr-cII alleles and sex of the firstborn child, no significant differences in sex ratios of children born after RPL were found between each subgroup and the reference. The highest sex ratio was 1.54, which was found in patients with a firstborn boy carrying no HYr-cII allele, while the lowest sex ratio was 0.90, which was found in patients with a firstborn girl carrying one or more HYr-cII alleles [3].

The prevalence of RPL patients carrying at least one HYr-cII allele was 63.4% which did not differ from the reference group (63.6%). No significant differences in the prevalence of the HYr-cII alleles separately and together, respectively, were found when comparing the RPL subgroups with each other or with the reference group, except for the HLA-DRB1*15 allele, which was found significantly more often in sRPL patients with a firstborn girl than sRPL with a firstborn boy (p=0.018). Also, 71.4% of sRPL patients with a firstborn girl carried ≥1 HYr-cII alleles, while only 60.1% of sRPL patients with a firstborn boy did, which difference was borderline significant (p=0.059).

Perinatal data on previous births after GW 24 was reported for sRPL patients stratified by sex of the firstborn child and carriage of HYr-cII alleles. The mean birthweight of the firstborn boy was 242 g lower in boys from sRPL patients carrying HYr-cII allele(s) compared to boys from patients carrying no HYr-cII alleles (3537g vs. 3295g, p=0.030). No difference was seen in gestational age and frequency of emergency cesarean section between subgroups. Also, no mean birthweight or median gestational age differences were seen between firstborn girls from mothers carrying 0 or ≥1 HYr-cII alleles. The frequency of preeclampsia was significantly higher in sRPL patients with a firstborn boy in those carrying ≥1 HYr-cII alleles than in those carrying 0 HYr-cII alleles (0% vs. 10.5%, p=0.004). At the same time, no difference was seen in patients with a firstborn girl (12.5% vs. 4.8%, p=0.213).

The frequency of a successful reproductive outcome in first pregnancy after admission was significantly lower in sRPL patients with a firstborn boy compared to sRPL patients with a firstborn girl (56.9% vs. 81.2%, OR 0.41, 95% CI 0.20–0.83,

p=0.012). The difference was more pronounced among patients carrying ≥1 HYr-cII alleles (60.0% vs. 83.3%, OR 0.30; 95% CI 0.13–0.71; p=0.005) and those carrying 2 HYr-cII alleles (53.3% vs. 84.6%; OR 0.21; 95% CI 0.02-1.58; p= 0.077) but these findings were insignificant. There was no significant difference in patients carrying no HYr-cII alleles (69.6% vs. 73.3%, OR 0.83; 95% CI 0.23–3.07; p=0.781). After adjustment for BMI, maternal age, and smoking, the association found between the sex of the firstborn child of sRPL patients carrying ≥1 HYr-cII alleles and successful reproductive outcome notably remained significant (aOR 0.37, 95% CI 0.18–0.80, p=0.011).

The success rate of the final reproductive outcome determined at the time of final follow-up did also differ significantly between sRPL patients with a firstborn boy and those with a firstborn girl in the subgroup of patients carrying ≥1 HYr-cII alleles (90.7% vs. 72.9%, OR 0.27, 95% CI 0.09–0.79, p=0.017). The highest success rate was found in sRPL patients with a firstborn girl carrying no HYr-cII allele (93.3%), while the lowest success rate was found in sRPL patients with a firstborn boy carrying >1 HYr-cII allele (72.9%).

The secondary infertility rate was defined as the percentage of patients with no pregnancy after admission with more than six months follow-up. When compared to sRPL patients with a firstborn girl carrying no HYr-cII, the secondary infertility rate was significantly lower in sRPL patients with a firstborn girl carrying ≥1 HYr-cII alleles (OR 0.36, 95% CI 0.13–0.98, p=0.042) and sRPL patients with a firstborn boy carrying no (OR 0.29, 95% CI 0.10–0.86, p = 0.021) or ≥1 HYr-cII alleles (OR 0.27, 95% CI 0.10–0.74, p=0.011), respectively [3].

DISCUSSION

The present study found that the male-to-female sex ratio of the first-born child was significantly higher in sRPL than in the Danish background population. sRPL patients with a firstborn boy had a significantly poorer reproductive prognosis in first pregnancy after admission as well as on long-term. The aOR for a successful reproductive outcome found in the present study was comparable to previous findings (aOR 0.37; 95% CI 0.1–0.7, p=0.001) by Nielsen et al. [166]. Thus, the findings were consistent despite the data being collected from two different clinics with an approximately 20-year time gap. Moreover, both the present and the previous study [170], respectively, found a negative but insignificant correlation between the number of maternal HYr-cII alleles and the chance of successful reproductive outcome in sRPL patients with a firstborn boy and a positive correlation for those with a firstborn girl. Yet, the present study only included 28 patients who carried two HYr-cII alleles, and according to previous findings, with an alpha level of 0.05, and a power of 0.80, the required sample size for this comparison was 42 patients. Thus, it is possible that the present study was underpowered to perform such an analysis.

However, when we combine data from the present study [3] with data from the prior study by Nielsen et al. [170] (Table 8.1), a significant negative correlation was found between the chance of a successful pregnancy outcome and the number of maternal HYr-cII alleles in sRPL patients with a firstborn boy (OR 0.57, 95% CI 0.39-0.83, p=0.002) while no significant correlation was found in sRPL patients with a firstborn girl (OR 1.48, 95% CI 0.88-2.49, p=0.143). Nevertheless, the two trendlines were significantly different (p=0.002).

Table 8.1: Success rate in the subsequent pregnancy according to the sex of the firstborn child and carriage of HYr-cII alleles*. Combining results from the present study with previous results on the percentage of patients with a successful pregnancy outcome in first pregnancy after admission according to the sex of the firstborn child and number of maternal HYr-cII alleles*

Successful pregnancy outcome	Boy & 0 HYr-cII	Boy & 1 HYr-cII	Boy & 2 HYr-cII	Girl & 0 HYr-cII	Girl & 1 HYr-cII	Girl & 2 HYr-cII
Study III						
N/N_{total}	32/46	34/55	8/15	11/15	34/41	11/13
%	69.6	61.8	53.3	73.3	82.9	84.6
Previous study N/N_{total}	51/77	35/75	4/14	40/58	41/48	10/14
%	66.2	46.7	28.6	69.0	85.4	71.4
Combined						
N/N_{total}	83/123*	69/130*	12/29*	51/73	75/89	21/27
%	67.5	53.1	41.4	69.8	84.3	77.8

*The data presented originate from the previous article by Nielsen et al. [170] and the present study III by Nørgaard-Pedersen et al. [3]. *Test for trend: p=0.002. HYr-cII: male-specific minor histocompatibility antigen restricted human leukocyte antigen class II.*

No sample size calculation was reported in the published article, but it was described in the protocol published on ClinicalTrials.gov, which was made publicly available before the final data collection. The primary outcome analysis (comparing pregnancy outcomes between sRPL patients with a firstborn boy carrying ≥1 HYr-cII alleles with

those with a firstborn girl) included 124 sRPL patients, while only 48 patients were required.

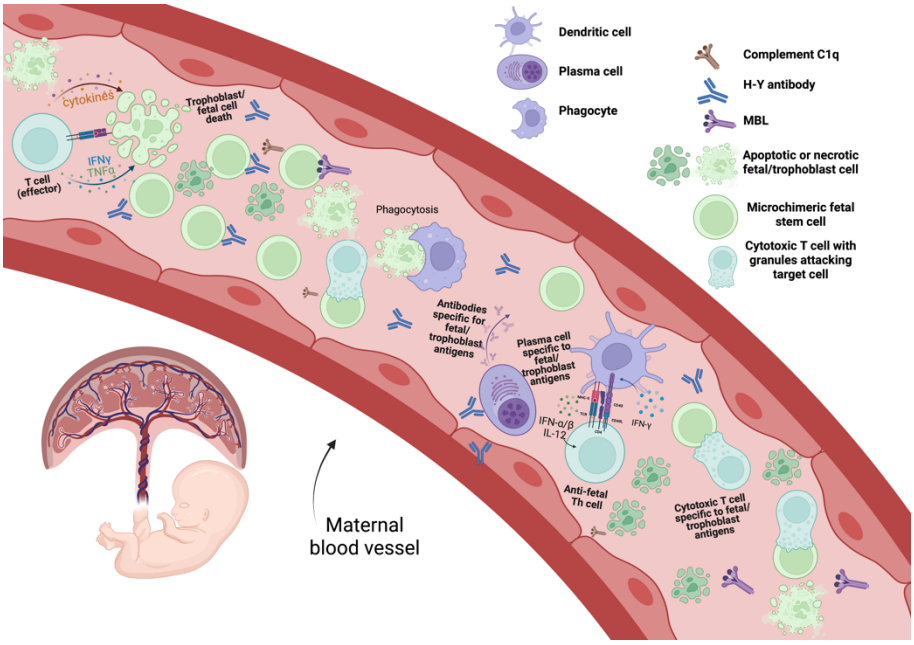

Figure 8.1: Maternal immunization against fetal or trophoblast alloantigens. *The figure shows a maternal blood vessel with an accumulation of fetal cells in the maternal blood, exaggerated due to MBL deficiency. Maternal immune cells respond to the fetal/trophoblast alloantigens (e.g., H-Y antigens) with maternal immunization, which may cause fetal/trophoblast cell death, and subsequently increased accumulation of antigenic material in the maternal circulation, activation of anti-inflammatory responses, and ultimately fetal rejection.*

As a new finding, the prevalence of preeclampsia was significantly increased in sRPL patients with a firstborn boy carrying ≥1 HYr-cII alleles compared to sRPL patients with a firstborn boy carrying no HYr-cII alleles. This finding is interesting because previous studies have demonstrated elevated shred of syncytiotrophoblast cells and microvesicles into the maternal circulation in pregnant women with preeclampsia which cells and vesicles expressed mHAs (such as H-Y antigens) and HLA-DR molecules on the surface membranes in contrast to those shred from healthy control females with uncomplicated pregnancies [350,351]. The expression of paternal mHAs and HLA-DR may activate T lymphocytes and subsequently facilitate a destructive cellular immune response similar to that seen in GVHD. Thus, preeclampsia is associated with pathogenic changes that involve the mechanism theorized in the present study to predispose to RPL. However, since this study is the first to make such findings; since the finding originated from explorative analyses; and since no events occurred in one of the groups, this finding is at noteworthy risk of bias and needs to

be re-examined before any conclusive remarks can be made on whether women who carry an HYr-cII allele and experience preeclampsia during pregnancy with a boy are more susceptible to subsequent sRPL.

H-Y-specific T cells with a potent functional capacity and male microchimeric cells have been detected as early as the first trimester, only 4-5 weeks after fertilization [352–355], and the cell levels increase steadily until parturition [355]. However, the H-Y specific response is tightly controlled by the pregnancy-related regulatory mechanisms promoting tolerance in a normal pregnancy. We hypothesize that a potent sensitization to H-Y antigens may only occur in situations where the capacity of these mechanisms to ensure fetal tolerance is exceeded [89,352,356]. This may be the situation during late pregnancy or during birth where Treg levels have gradually declined from their peak in the second trimester [87,88,357] and where a major exposure to immunogenic antigens (e.g., H-Y antigens) on microchimeric cells, microvesicles, and placental apoptotic bodies occur [85,358]. An excessive load challenging the pregnancy-related regulatory mechanisms may break the balance, cause activation, stimulation, and proliferation of paternal-antigen-specific lymphocytes, and establish an organized, sensitized immune cell population with the functional capacity to reject subsequent fetuses. Thus, the small alloantigen exposure and the common, controlled immunization that often occurs in the first trimester may not give rise to an equally potent sensitization as that during late pregnancy (Figure 8.1). Therefore, the influence of an early pregnancy loss of a male fetus may not be comparable to the delivery of a boy at term; hence, we did not attach the sex of miscarried fetuses to any value in the study. However, it is possible that in some pregnancies with a male fetus terminated before GW 24, a similar response could be activated.

We have previously found that p-MBL deficiency is associated with the birth of a boy before RPL [1]. Since MBL plays an important role in the clearance of apoptotic and necrotic cells, one may speculate if a state of p-MBL deficiency contributes to elevated alloantigen exposure due to reduced clearance that will consequently predispose the patient to more easily exceed the capacity of the pregnancy-related tolerance [101,102,294,300,301]. Thus, p-MBL deficiency may increase the risk of a potent sensitization to the fetus, as theorized in the present and former studies [1,3].

If the suggested sensitization in sRPL patients remained solely and specifically directed against H-Y antigens, a decreased male-to-female sex ratio in subsequent successful pregnancies would be expected in sRPL patients with ≥1 HYr-cII allele and a firstborn boy. In Figure 4 in the published article [3], a typing error occurs in the column presenting the percentage of sRPL patients with ≥1 HYr-cII allele and a firstborn boy. The numbers were oppositely reported since 56.1% had given birth to a girl after sRPL (sex ratio 0.78). This sex ratio is similar to the sex ratio (0.76) found among children born after the birth of a boy in sRPL patients in a previous study which differed significantly from healthy controls [359]. However, in the present

study, the sex ratio in the subsequent birth in this subgroup did not differ significantly from that in the background population (p=0.536) nor from that in sRPL patients with no HYr-cII allele and a firstborn boy (p=0.144) which could be due to the small sample size. The correct diagram is shown in Figure 8.2.

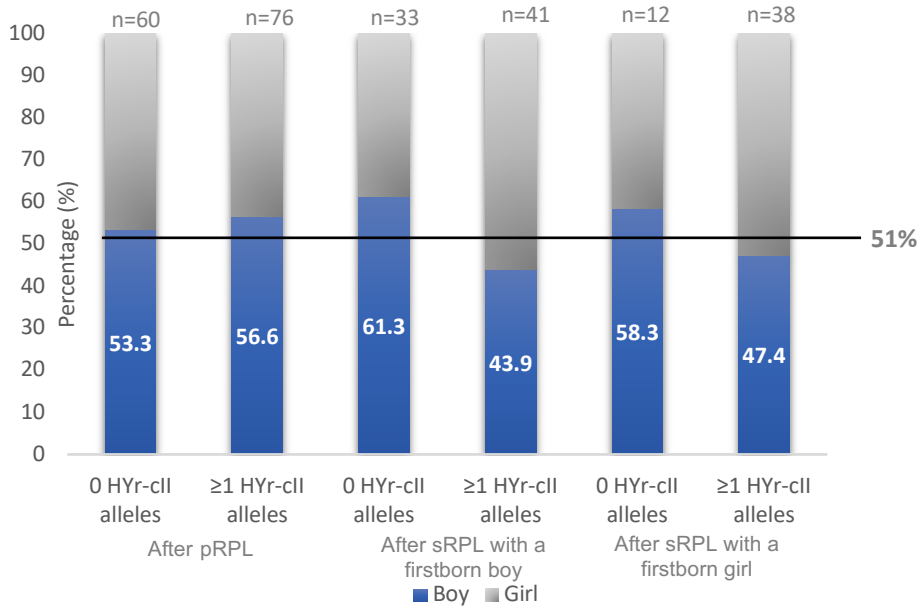

Figure 8.2: The percentage of patients giving birth to a boy after RPL in subgroups based on carriage of HYr-cII alleles and sex of the child born prior to RPL*. The diagram is similar to Figure 4 in the published article by Nørgaard-Pedersen et al. (Study III) [3] but with a correction in column no. 4. None of the sex ratios differed significantly from the Danish background population. HYr-cII: male-specific minor histocompatibility antigen restricted human leukocyte antigen class II.*

The relatively normal sex ratio in subsequent birth among patients with a firstborn boy carrying HYr-cII alleles may suggest that the risk of fetal rejection is not increased for male fetuses. This may be attributed to the "determinant spreading" phenomenon known as a mechanism often occurring in autoimmune diseases and chronic inflammatory conditions [360–362]. H-Y antigens may be the initial trigger of a potent immune response, but during the relatively long period of exposure to increasing titer of the highly immunogenic H-Y antigens intermolecular epitope spreading, molecular mimicry, or cross-reactivity to the fetus' autosomal minor- or major histocompatibility antigens or placental-specific antigens may occur. The maternal exposure may terminate simultaneously with delivery if the maternal blood is cleared for fetal-specific antigenic material. However, microchimeric cells from the

fetus may persist for decades [327,335] and continue to stimulate the maternal lymphocytes, potentially allowing epitope specificity to diversify from the initial dominant epitope-specific immune response. Moreover, T cell specificity for one epitope of a multi-antigenic complex can activate B cells with specificity to other epitopes of that complex. Alternatively, molecular mimicry with cross-reactivity could explain the broadening from a chromosome Y-encoded to an X-encoded or autosomal mHA [362]. Since only a few amino acid residues distinguish H-Y proteins from their homologous H-X proteins, the H-X proteins and their derived peptides are strong candidate targets for immune responses that originally were specific for H-Y antigens. For example, the amino acid sequence of the DBX protein is 91% identical to the DBY protein [363], and molecular mimicry between DBY- to DBX peptides may occur. Thus, the development of fetus-specific immunity to non-H-Y antigen targets may explain why the sex ratio of children born after sRPL did not differ significantly from that in the background population.

Supporting such theory is the increased risk of preterm birth, lower birth weight, and stillbirth in second pregnancy after a firstborn boy, irrespective of the sex of the second fetus found in the Danish population [364–366].

Study III has two unexpected findings. First, a borderline significantly increased prevalence of HYr-cII alleles was found in sRPL with a firstborn girl compared to a firstborn boy (71.4% vs. 60.1%) [3]. In contrast, in the previous study the prevalence did not differ between groups and was lower than in the present study (51.7% vs. 53.6%) [170]. This unexpected finding could be a chance finding since multiple comparisons of the prevalence of the HYr-cII alleles were performed for explorative purposes without performing adjustments for multiple comparisons. Another highly hypothetical explanation of the finding could be selection bias due to the significantly increased risk of secondary infertility of sRPL patients with a firstborn girl carrying no HYr-cII alleles. Since infertile women are not referred to the CRPLWD, this may consequently give rise to an increased frequency of sRPL patients with a firstborn girl carrying ≥1 HYr-cII alleles. Thus, the explanation of the unexpected finding may not be that the HYr-cII alleles predispose to sRPL if the firstborn child is a girl but rather be a consequence of selection bias in this study. However, as it is a new and interesting finding, the association between HLA alleles, sex of the firstborn child, and secondary infertility needs further investigation.

The second unexpected finding was the high frequency of a firstborn boy in sRPL patients with no HYr-cII alleles (67.7%) [3]. In the previous study, the sex ratio of the firstborn child did not differ between patients with and without HYr-cII alleles [170]. This unexpected finding challenges the hypothesis of H-Y sensitization as a cause of sRPL, and it highlights the need for further investigation into the cellular and molecular processes occurring in these patients before making any conclusions.

The ESHRE guideline development group justified the ESHRE RPL guideline recommendation of including HLA-DR and -DQ determination in the workup of Scandinavian women with sRPL after a firstborn boy for explanatory and prognostic purposes as it considered the association found by Nielsen et al. was strong [166]. The guideline group only called for further confirmation in non-Scandinavian women [13].

The authors of this study did, however, consider the recommendation was based on rather sparse documentation, also in Scandinavian women, since only a single cohort of 358 patients constituted the basis for this investigation and recommendation. Therefore, we aimed to replicate the study and expand the horizon by further clinical investigations, including the perinatal data from firstborn children, the impact on secondary infertility, and the sex ratio of the second children according to RPL subgroups, and by improving the adjusted analyses. While some of the findings in the present study support the usage of HLA class II determination for prognostic purposes in sRPL patients, other findings challenge the suggested theory and, consequently, the clinical usage.

The present study was limited by the lack of HLA-DQB1 determination in 46.3% of patients and of HLA-DRB3 determination in all patients. However, the lack of HLA-DRB3 determination may only have had a minor role in the present findings due to the low cell expression of DRB3 molecules [367] and the lack of association with autoimmune diseases [368] and RPL prognosis [170]. The strong positive linkage disequilibrium between HLA-DRB1 and -DQB1 alleles [349] only leaves a minor risk for false classification of HYr-cII carriers by our method. We estimate that a maximum of two HLA-DRB1*01, -DRB1*10, or -DRB1*16 positive patients in our cohort have falsely been assigned an HYr-cII allele.

Future studies should focus on the immune cell responses to H-Y antigen exposure in RPL patients. A study similar to that of Lissauer and colleagues [352] would provide valuable information on the underlying mechanism proposed to give rise to recurrent fetal rejection. A study comparing the immune response to H-Y antigens in sRPL patients with a firstborn boy with those with a firstborn girl, in pregnant and non-pregnant states, and subgroups divided by carriage of HYr-cII alleles could provide valuable insight into the sRPL pathophysiology and potentially confirm or deny the proposed theory.

CHAPTER 9. STUDY IV

A COMBINATION OF THE HLA- DRB1*03 PHENOTYPE AND LOW PLASMA MANNOSE-BINDING LECTIN PREDISPOSES TO AUTOANTIBODY FORMATION IN WOMEN WITH RECURRENT PREGNANCY LOSS

INTRODUCTION

RPL is associated with several autoimmune diseases, including SLE [369], APS [370], thyroid autoimmunity [195], and undifferentiated connective tissue disease [371]. Also, the presence of autoantibodies without clinically overt autoimmune disease is associated with RPL. However, understanding how these conditions are related remains unknown [372]. One may speculate whether RPL and these autoimmune diseases shares an (at least partly) common pathway but are otherwise independent of each other, whether pregnancy losses can cause autoimmunity, or whether RPL is a consequence of an autoimmune response. The autoimmune diseases associated with RPL are more often diagnosed in women than men and often strike at reproductive age; thus, the conjuncture of pregnancy and disease onset challenges the feasibility of determining the sequence of the causal pathway. Nevertheless, it is well documented that parity is associated with an increased risk of female-predominant autoimmune diseases [373] and that autoimmune disease activity before conception is highly related to pregnancy outcomes [369,371,374].

Despite the major clinical heterogeneity among autoimmune diseases, they share several risk factors and pathophysiologic mechanisms. Such risk factors include specific genetic and environmental factors that mutually affect the innate and adaptive immune systems and may contribute to developing autoimmune diseases. The strongest association among most autoimmune diseases is specific HLA alleles encoded by the highly polymorphic HLA region on chromosome 6, especially certain HLA-DRB1 alleles [375,376]. Moreover, autoantibody positivity is also associated with specific HLA alleles. Among the non-HLA genetic susceptibility biomarkers are the *MBL2* variant alleles associated with both low p-MBL levels and the development and severity of several autoimmune diseases. Besides the association with autoimmune diseases, specific HLA class II alleles and MBL deficiency have been associated with RPL [1,3,159,174,175,333]. The HLA-DRB1 alleles found at an increased frequency in Caucasian RPL patients include HLA-DRB1*03 and

DRB1*07. HLA-DRB1*03 is highly associated with several autoimmune diseases [376,377], including autoimmune diseases associated with positivity for the autoantibodies examined in the present study [376,378,379]. In contrast, HLA-DR7 has been associated with an increased risk for APS [380] and celiac disease [381] but a reduced risk of some other autoimmune diseases such as Graves' disease, Hashimoto's thyroiditis, and rheumatoid arthritis [382,383]. We, therefore, speculate whether immune-mediated RPL is genetically determined and whether autoantibody development is an intervening variable.

The present study aimed to investigate if the presence of autoantibodies in RPL patients is associated with three genetically determined susceptibility factors for RPL: HLA-DRB1*03, HLA-DRB1*07, and low p-MBL [4].

METHODS

Patients with ≥2 consecutive pregnancy losses consecutively admitted to the CRPLWD between January 2016 and August 2022 were included if they had no chromosomal abnormalities, no uterine malformations, and no missing data on the presence of autoantibodies, p-MBL level, and HLA-DRB1 genotype. The autoantibodies included aPL, ANA, and TPO antibodies. APS diagnosis required positivity for lupus anticoagulant or an anti-cardiolipin or β2-GPI antibody level ≥35 kU/I detected twice three to four weeks apart, and TPO antibody concentration above 60 kU/l was considered positive while any detection of ANAs (titer ≥1) were considered positive. p-MBL levels ≤500 μg/l were considered low while p-MBL levels >500 μg/l was considered normal [4].

Three separate subgroupings of patients were made based on the following:

I. Low or normal p-MBL level
II. Carriage or no carriage of HLA-DRB1*03
III. Carriage or no carriage of HLA-DRB1*07

In addition, patients were separated into four subgroups based on

I. Presence of autoantibodies and low p-MBL level.
II. Presence of autoantibodies and normal p-MBL level
III. Absence of autoantibodies and low p-MBL level
IV. Absence of autoantibodies and normal p-MBL level

HLA-DRB1 phenotype frequency from a group of Danish bone marrow donors was used as a reference to the phenotype frequency of HLA-DRB1 in RPL patients, which reference group has previously been used [161].

RESULTS

In total, 644 patients with RPL were included, in which the majority had ≥3 consecutive pregnancy losses (88.7%) and conceived naturally (63.8%). The inclusion of 663 patients was mistakenly noted twice in the published article as a typing error, but the remaining results are correctly noted. Baseline characteristics, including age, BMI, number of previous pregnancy losses, and frequency of previous live birth, did not differ within each of the three subgroupings based on p-MBL level, maternal carriage of HLA-DRB1*03, and carriage of HLA-DRB1*07, respectively.

The frequency of HLA-DRB1*03 in RPL patients was significantly lower than in the Danish bone-marrow donor cohort (OR 0.79, 95% CI 0.64-0.98; p=0.03) while HLA-DRB1*07 in RPL patients were similar to the reference (OR 1.08, 95% CI 0.87-1.35; p=0.53). The frequency of autoantibody positivity separately or combined did not differ between patients with low or normal p-MBL levels, carrying the HLA-DRB1*03 or not, nor between patients carrying the HLA-DRB1*07.

The study found a non-significantly higher frequency of positivity for two or more autoantibodies in patients with low p-MBL level compared to patients with a normal p-MBL level (p=0.14) and in patients carrying an HLA-DRB1*03 allele compared to patients not carrying an HLA-DRB1*03 allele (p=0.11). Also, a non-significantly lower frequency of positivity for aPL in patients carrying an HLA-DRB1*07 allele compared to patients carrying no HLA-DRB1*07 (p=0.08) was found.

Patients positive for a minimum of one autoantibody and carried an HLA-DRB1*03 allele more often had low p-MBL levels than patients who carried no HLA-DRB1*03 allele and were positive for a minimum of one autoantibody (OR 2.4, 95% CI 1.2-5.0; p=0.01). No other significant associations were found between HLA-DRB1 phenotype frequency, positivity for autoantibodies, and p-MBL levels. Similarly, the frequency of HLA-DRB1*03/X genotypes did not differ between these subgroups based on positivity for autoantibodies and p-MBL levels.

The frequency of ANAs was significantly higher in patients with the combination of HLA-DRB1*03 and low p-MBL level compared with those without this combination (OR 2.0; 95% CI 1.0-3.9; p<0.05). In contrast, the frequency of TPO antibodies and aPL were not significantly higher in patients with the combination of HLA-DRB1*03 and low p-MBL level compared with patients without this combination (OR 1.7; 95% CI 0.8-3.2; p=0.17 and OR 1.5; 95% CI 0.6-3.6; p=0.41, respectively) [4].

DISCUSSION

The present study found that positivity for ANA, TPO antibodies, and aPL was non-significantly elevated in RPL patients with a low p-MBL level and in patients carrying an HLA-DRB1*03 allele, respectively, compared to their counterparts with a normal

p-MBL level and not carrying an HLA-DRB1*03 allele, respectively. Moreover, patients carrying both HLA-DRB1*03 and a low p-MBL level showed the highest susceptibility to antibody production with ORs ranging from 1.5 to 2.0 in comparison to patients carrying one or none of these factors (shown in Table 3 and Table 5 in the published article of study IV). However, ANA production was the only autoantibody significantly elevated in this subgroup. A significant association was found between carriage of an HLA-DRB1*03 allele and a low p-MBL level among patients positive for a minimum of one autoantibody. These findings align with previous findings of higher frequency of autoantibody positivity in SLE patients carrying an *MBL2* gene variant than SLE patients carrying the *MBL2* wildtype genotype [299]. Indeed, SLE is also a condition highly associated with the HLA-DRB1*03 allele [384,385].

Based on previous studies in patients with clinical autoimmune diseases finding a strong association between antibody positivity and MBL deficiency or specific HLA alleles, in particular HLA-DRB1*03, it may seem surprising that no significant association between HLA-DRB1*03, HLA-DRB1*07, or MBL deficiency and autoantibody positivity was found in the present study. Clinically overt autoimmune diseases may involve more pronounced autoimmune reactions than those in otherwise healthy RPL patients which could explain the findings in the present study.

It is complicated to compare the prevalence of each autoantibody positivity in the present study with the prevalence found in meta-analyses due to the great heterogeneity in laboratory methods, the specific autoantibodies tested for in the ANA and APS analyses, the cut-off values, the RPL definition, and the exclusion criteria. Nevertheless, in the present study, ANA was found in 12.7%, TPO antibodies in 13.4%, and aPL in 7.7% of patients, while meta-analyses have reported an approximate prevalence of ANA to 22.0% [386], TPO antibodies to 15-39% [198,387,388] and APS to 18-55% [389,390]. Thus, an overall lower prevalence was found in patients included in this study than previously reported, which could possibly explain the weaker associations between genetically determined factors and autoantibody positivity found in the present study than in previous studies of other autoimmune diseases. However, the consistent finding of slightly elevated frequency of autoantibody positivity – separately and combined - in RPL patients carrying an HLA-DRB1*03 allele and/or a low p-MBL level may indicate that an association does exist but that the study was not sufficiently powered to show it in patients carrying only one of these two factors.

On the contrary, the findings of HLA-DRB1*03 carriage being an RPL protective rather than a susceptibility gene in this cohort and the relatively small differences in frequency of antibody positivity between HLA-DRB1*03 carriers and non-carriers may suggest that no or only a weak HLA-related susceptibility to RPL exists. The non-significant difference in the frequency of patients with autoantibodies between patients with and without the immunogenetic susceptibility factors reduces the probability that autoantibody positivity is a mediator variable between the

immunogenetic susceptibility factors and RPL. However, further studies are needed to verify these findings due to the consistent but weak tendency to a higher prevalence of several autoantibodies in RPL patients with ≥1 HLA-DRB1*03 alleles and/or MBL deficiency.

Among studies comparing HLA-DR phenotype frequency between Caucasian RPL patients and a reference group, only one Danish case-control study group found a significantly elevated phenotype frequency of HLA-DRB1*03 [159]. The association was not confirmed in the two subsequent case-control studies by the same group of investigators [160,161]. Their most recent study found an elevated allele frequency of HLA-DRB1*07 in RPL patients in comparison to a reference group, but the phenotype frequency was not significantly elevated in RPL when correction for multiple comparisons was undertaken [161]. While the phenotype frequency was considered the primary outcome for the former studies due to a recommendation to use phenotype frequency to determine disease associations [391], the latter study reported the allele frequencies as the main outcome [161]. Moreover, the correction for multiple comparisons was based on only the number of alleles rather than the total number of comparisons (alleles and phenotypes), as recommended [391]. Thus, the finding could potentially be a chance finding.

Most studies searching for associations between RPL and HLA antigens excluded patients positive for autoantibodies [159]. Arguments exist for and against excluding autoantibody-positive RPL patients when searching for RPL susceptibility genes. As RPL is a condition associated with positivity for several autoantibodies without necessarily fulfilling diagnostic criteria for an autoimmune disease [159,371], the exclusion in studies searching for RPL susceptibility genes may cause significant selection bias. Differences in selection criteria may explain the inconsistent findings on RPL susceptibility genes between the present and previous studies [159–161,163,392–394]. Overall, the heterogenous study methods and inconsistent findings between these studies call for further studies of higher quality as no conclusion can be made on HLA susceptibility genes for RPL based on the current evidence.

The susceptibility to developing autoreactive immunopathologic responses in RPL patients may be influenced more by lifestyle, environmental exposures, and life events like pregnancies, infectious diseases, and iatrogenic injuries than by inherited susceptibility. A pattern of temporally changed effects of some susceptibility HLA alleles in other diseases, including type I diabetes mellitus, has been described. With the simultaneously increasing population incidence of type I diabetes mellitus, investigators have suggested an increasing role of environmental and lifestyle factors in the pathogenesis [4,395,396]. The same explanation may apply to RPL, for which an increased incidence has also been found [20]. Moreover, environmental exposures and susceptibility genes may act synergistically rather than additively, as is reported for DRB1*1501, smoking, and overweight in patients with the inflammatory disease multiple sclerosis [397].

Limitations of the present study involve the small sample size that may have caused insufficient statistical power to examine the association between genetic factors and autoantibody positivity in RPL patients. Also, the present study did not correct for multiple testing since we specifically a priori hypothesized to find an association between low p-MBL, HLA-DRB1*03, and -DRB1*07 and autoantibody positivity. However, correction should only have been applied to analyses with explorative, multiple testing with no predetermined hypothesis [398], which in the present study would apply the multiple comparisons presented in Tables 3 and 4 in the published article except for the comparisons between the patients carrying HLA-DRB1*03 and -DRB1*07 [4]. Since none of these comparisons were statistically significant before correction, adding corrected values was not considered valuable for the reader.

In conclusion, we found an association between autoantibody positivity and the combination of MBL deficiency and the HLA-DRB1*03 allele in RPL patients. This is new and may suggest a possible genetic susceptibility to autoantibody formation. However, the remaining non-significant findings suggest that if such susceptibility exist, it is weak. Thus, the study did not have the power to verify whether the effect of autoantibodies on the RPL pathogenesis is an intermediate variable on the causal path between immunogenetic susceptibility and RPL rather than an independent risk factor variable and therefore further prospective studies are needed.

CHAPTER 10. STUDY V

INTRAVENOUS IMMUNOGLOBULIN AND PREDNISOLONE TO WOMEN WITH UNEXPLAINED RECURRENT PREGNANCY LOSS AFTER ASSISTED REPRODUCTIVE TECHNOLOGY TREATMENT: A PROTOCOL FOR A RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED TRIAL

INTRODUCTION

Assisted reproduction technologies have improved remarkably during the last decades simultaneously with increasing demand; however, many couples still fail to achieve a child [399]. The clinical pregnancy rate is approximately 36% per embryo transfer performed at Danish public and private IVF clinics, among which 99% are single embryo transfers. In Denmark, public health care offers three cycles of IVF (controlled ovarian stimulations with the associated number of fresh and frozen embryo transfers) to couples with no common children. However, only 64% of these couples achieve a live-born child [400]. Despite using PGT and imaging techniques to select euploid and morphologically normal embryos, a large proportion of the embryos fail to implant. This is possibly due to multiple interconnected factors affecting fertility, such as the clinical and laboratory performance and procedures; male-related factors including sperm quality; female-related factors including maternal age, hormonal levels, endometrial conditions, endometrial receptivity, and the immunological milieu in the endometrial lining; and embryo-related factors like cleavage rate and embryo quality [224]. Thus, ART treatment is far from a guarantee for infertile couples.

The incidence of RPL among couples undergoing ART is unknown; however, the majority of the remaining 36% of patients with an unsuccessful ART outcome after three IVF cycles are expected to suffer from recurrent implantation failure or RPL [400]. The pregnancy loss rate per clinically confirmed pregnancy after ART has been estimated to be slightly higher than that after spontaneous conception [401], and the proportion of couples with RPL and with the need for ART, respectively, is rising [20,402,403]. Therefore, the group of patients needing counseling on both RPL and infertility is expected to increase.

A major effort has been put into determining the RPL pathogenesis since no evidence-based risk factors for RPL can be identified in approximately 60% of patients [33,323]. Moreover, the presence of these RPL risk factors does not necessarily lead to RPL, and even after treatment of RPL patients with these risk factors, when possible, the pregnancy loss rate remains relatively high [13]. Consequently, it is difficult for the patient to understand RPL and accept it when no explanation can be found, and for the same reason also difficult for the physician to approach and treat RPL.

A wide range of immunological markers has been investigated in patients with uRPL, including the concentration and functional activity of leukocyte subsets, proteins, and signal molecules. An increased prevalence of immune aberrations has been found in uRPL patients compared to healthy fertile women, but such findings often derive from low-quality studies. Also, the findings are rarely incontestable and unambiguously confirmed in subsequent independent, high-quality studies. Lack of compelling proof for the actual role of these immunological markers in RPL pathogenesis call for controlled studies testing a wide range of these measures in one cohort to elucidate and compare their diagnostic accuracy and predictive value. Moreover, since not just one but rather a wide range of immune aberrations have been found in the uRPL patients, studies on immunomodulatory agents acting broadly on the immune system have been hypothesized as potentially effective treatments for these patients [404,405]. IVIG and corticosteroids are some immunomodulatory treatments suggested to affect LBR in uRPL patients [257,406,407]. However, evidence for using IVIG and corticosteroids – or immunotherapy in general - on patients undergoing ART with and without a history of RPL is sparse [408].

The treatment regimens of IVIG to RPL patients differ considerably between studies. IVIG is commonly used for diseases considered to be caused by autoimmune responses to self-peptides destructing otherwise healthy tissue like endothelial cells (Kawasaki's disease) [409], nerve cells (Guillian-Barré and chronic inflammatory demyelinating polyneuropathy) [410], platelets (immune thrombocytopenia) [411], and red blood cells (warm autoimmune hemolytic anemia) [412] as well as for primary immunodeficiency [413]. The exact mechanism causing most of these diseases is not fully described, as is the case for RPL, which may complicate the decision on the optimal treatment regimen. Indeed, the recommended treatment regimens for such diseases differ significantly between the diseases and between the national or local recommendations for each disease. The time of treatment initiation, duration, dose, and time interval between administrations are all adjustable factors and possible determinants of the clinical outcome.

Two meta-analyses on the effect of IVIG in RPL patients trying to conceive primarily naturally included 11 RCTs, among which 8 RCTs recurred in both meta-analyses and used random-effect models. While Egerup et al. [257] found no difference in the frequency of no live birth (40.4% in the IVIG group and 42.5% in placebo/"usual

treatment" group, RR 0.92, 95% CI 0.75-1.12), Wang et al. [256] did find a marginally significant effect on LBR (68.0% vs. 53.0%, RR 1.25, 95% CI 1.00-1.56). However, a cumulative meta-analysis and trial sequential analysis in the latter meta-analysis revealed that a borderline significance was reached only after the inclusion of the last published RCT [258] and that the diversity-adjusted required information size was not reached. Overall, this suggests a high risk for false positive results. Unfortunately, two of the trials [414,415] included by Wang et al. [258] were Chinese and not accessible in international scientific literature databases. According to the quality assessment by Wang et al. [258], the Chinese studies [414,415] did not describe their random sequence generation, allocation concealment, blinding, sample size calculation, or baseline comparability, and it is, therefore, uncertain if they were indeed RCTs or not. Besides lack of transparency, these two trials contributed with the highest risk ratios to the meta-analysis, which adds further to the uncertainty about the results in the meta-analysis [258] and the results should therefore be interpreted with caution. Furthermore, the significant heterogeneity between the RCTs included in the meta-analysis by Wang et al. [258] (I^2=62%) contrasts the low heterogeneity in those included in the meta-analysis by Egerup et al. [257] (I^2=0%), which supports cautiousness.

The two meta-analyses also made several subgroup analyses in which no significant effect of IVIG on reproductive outcome was found when analyses were restricted to pRPL or sRPL patients, patients with 2, 3, or ≥4 prior pregnancy losses, or to a high or low IVIG dosage [256,257]. However, Wang et al. [258] did find a significant effect when treatment was initiated before conception (RR 1.67, 95% CI 1.30-2.14) but not after conception (RR 1.10, 95% CI 0.93-1.29). As one of the studies that used pre-conception treatment was inaccessible and comprised the largest sample of studies finding pre-conception treatment initiation beneficial [414], this finding should be interpreted cautiously.

More than two decades ago, three small RCTs tested IVIG in women with IVF failures with or without RPL, in which studies IVIG was initiated before implantation [416–418]. A meta-analysis of these three RCTs found a significantly higher LBR in the group treated with IVIG compared to placebo [418], supporting the importance of pre-conception treatment. However, again the result should be interpreted with caution. Firstly, a description of selection criteria, randomization, treatment protocol, and blinding in these trials was deficient or missing in the meta-analysis, and no quality assessment was described. Secondly, concomitant treatments, such as heparin and LDA, were given in one of the trials that only included patients positive for thyroid autoantibodies [417], which may introduce bias in the effect estimates. Thirdly, the calculated effect on LBR was flawed by including the implantation rate from one of the RCTs [416].

The only well-described placebo-controlled RCT of IVIG in patients undergoing ART was performed by Stephenson and Fluker [419]. In this study, treatment with

500 mg/kg BW IVIG was compared to saline given on the day of embryo transfer and again every four weeks during pregnancy in 51 patients with a history of two or more embryo transfers that resulted in implantation failure or pregnancy loss before GW 9+0. An LBR of 15% and 12% was found in the IVIG and placebo group, respectively, which did not differ significantly. The RCT aimed to examine the effect of IVIG on LBR in patients with a history of repeated unexplained IVF failures. Therefore, it was not powered to detect whether IVIG increases the chance of pregnancy maintenance after a positive pregnancy test. However, less than 30% of patients became pregnant after embryo transfer. In a post-hoc analysis of their data, I found that 4/5 (80%) in the IVIG group and 3/7 (43%) in the placebo group with a positive pregnancy test after embryo transfer had a live birth (OR 1.9; 95% CI 0.2-18.6). Thus, it is possible that IVIG does not increase the chance of becoming pregnant, but if pregnancy is achieved, IVIG may be beneficial for successful placentation and pregnancy maintenance.

Overall, the trends found in the RCTs testing IVIG in both patients undergoing ART and conceiving naturally, respectively, suggest a beneficial effect of IVIG when administered pre-conception and repeated in early pregnancy and highlight an important knowledge gap that incited and motivated us to make the present RCT.

In the present RCT, IVIG is combined with a low dose of the glucocorticoid prednisolone. Prednisolone is a widely used drug in patients with RPL or recurrent implantation failure after ART but well-controlled, sufficiently powered clinical trials examining the effect of prednisolone in these patient populations are lacking. Moreover, the mechanisms by which prednisolone acts on female fertility and the developing embryo/fetus are not fully determined. The rationale behind prescribing prednisolone to improve RPL prognosis is that a higher frequency of increased NK cell levels and/or NK cell cytotoxicity has been found in the endometrium and peripheral blood in uRPL patients compared to fertile women; the presence of these NK cell parameters is associated with a poorer RPL prognosis [133,142,146,420]; and that prednisolone reduced NK cell level and cytotoxicity in RPL patients [245,246]. However, evidence for the premise of this deduction (increased NK cell level or cytotoxicity cause RPL) is insufficient. Furthermore, no study has monitored both pregnancy outcome and NK cell parameters before and after treatment with prednisolone in the same cohort of women. Thus, prescribing prednisolone solely based on this deduction is not sufficiently consolidated [421].

Although 90% of prednisolone is inactivated by placental metabolism and only a slight amount of active drug is transferred to the fetus, there is little evidence that low-dose prednisolone is associated with a minimally increased risk of side effects in the mother and fetus like cleft lip with or without palate, preterm birth, and preeclampsia [422]. Therefore, it remains a subject of intense debate whether it is justifiable to use prednisolone in patients with and without autoantibodies and/or autoimmune conditions.

Only a few and mostly small, low-quality studies have evaluated the clinical effects of corticosteroids on reproductive outcomes in RPL patients, and therefore most hypotheses on prednisolone efficacy on RPL prognosis are based on findings in other populations or in combination with other drugs.

In placebo-controlled RCTs in RPL patients conceiving naturally, no significant difference in LBR was found in patients positive for autoantibodies who were given prednisolone (0.5-0.8mg/kg/day) in combination with LDA (OR 1.5, 95% CI 0.8-2.6) [250] nor in a small feasibility trial in patients with an elevated uNK cell density who were given 20 mg/day prednisolone alone (OR 1.5, 95% CI 0.8-2.9) [251].

A Cochrane meta-analysis of 14 small RCTs showed no overall effect of peri-implantation administration of glucocorticoid compared to no glucocorticoid or placebo on LBR or ongoing pregnancy rate when tested in non-selected infertile patients undergoing ART treatment. However, a subgroup analysis of women undergoing IVF, rather than ICSI, found a significantly positive effect solely on the pregnancy rate (OR 1.50, 95% CI 1.05-2.13) [423]. Also, several RCTs have examined the effect of corticosteroids in combination with LDA. Some found an improved pregnancy rate and LBR in infertile women with an estimated good prognosis and in RPL patients [248,424], while others did not [425]. Besides an improved pregnancy rate and LBR in infertile patients undergoing ART who were positive for autoantibodies [426,427], an RCT found an increased number of recovered oocytes [424], while a cohort study found an elevated percentage of good-quality embryos in patients treated with prednisolone and LDA in comparison to no supplemental treatment [427].

Based on these findings, we may question whether IVIG may mainly support the maintenance of pregnancy while prednisolone may (also) improve the chance of conceiving in RPL patients undergoing ART. However, since the study findings are inconsistent, and the quality of the methods is mainly low, further studies of higher quality are urgently needed.

In continuation of our pilot study [406,407] and the presented knowledge gap, we decided to combine IVIG with prednisolone in the present trial and to accompany the trial with a comprehensive analysis of a wide range of immune biomarkers in peripheral blood, including the level of several leukocyte subsets before and after treatment [5].

METHODS

In a 1:1 randomized, double-blinded, placebo-controlled trial, the effect of prednisolone and IVIG on reproductive outcome after embryo transfer and on relevant immune biomarkers will be examined in a sample of 74 female RPL patients

undergoing ART treatment. Participants are recruited from the CRPLWD at Aalborg University Hospital [5].

Women are eligible for inclusion if they have a history of ≥2 consecutive early (≤10 GW) pregnancy losses in pregnancies achieved through ART and apply to the following exclusion criteria:

I. Body mass index ≥35 kg/m^2
II. Age ≥41years
III. Significant uterine malformation
IV. Known parental chromosomal abnormality.
V. ≥2 previous pregnancies with embryos/fetuses with known ab-normal karyotype
VI. IgA deficiency, IgA-autoantibodies, or hyperprolinaemia
VII. Anti-Müllerian hormone ≤4 pmol/l (for patients not using an oocyte donor*)
VIII. Treatment with medication interacting with prednisolone.
IX. Patients with moderate/severe hypertension, diabetes mellitus, heart insufficiency, severe mental disorders, Cushing syndrome, myasthenia gravis, ocular herpes simplex, pheochromocytoma, systemic sclerosis, and moderate/severe renal dysfunction
X. Patients with a clinical or biochemical profile indicating the need for heparin or levothyroxine treatment during pregnancy, including APS and overt hypothyroidism.
XI. Previous treatment with IVIG
XII. Known allergy to prednisolone and/or IVIG.

For patients using an oocyte or sperm donor, the previous two pregnancy losses must also be from pregnancies achieved by gamete donation but using the same donor in all three cycles is not required [5].

An informed consent form is signed after eligible patients have been informed according to national guidelines (written and oral). The blinded investigators randomized the participant within one week before estimating menstrual bleeding. Non-transparent paper bags with a tablet container prepared by an independent pharmacist are numbered from ID-number 1 and forward and are given in continuous order to the participants. Bags, containers, and tablets have identical external appearances. Participants also receive a written information folder containing a summary of known adverse reactions related to the study drugs, contact information in case of emergencies, a scheme with ticking boxes to tick off after daily tablet administration, and a diary to write down any adverse events and adverse reactions happening during the period where study drugs are administered [5].

The randomization list is made before first inclusion by the Hospital Pharmacy North Denmark Region, which is an externally located and independent pharmacy. The computer-generated, simple, 1:1 randomization list is kept confidential to

investigators, participants, personnel collecting or analyzing the participants' blood samples and study data, and all personnel in contact with the center's RPL patients. The randomization blocks are arranged in different sizes, assuring a 1:1 ratio at a time for interim analysis, and the sizes are unknown to the blinded personnel. The randomization list will not be disclosed before 74 participants have completed the study according to the criteria for per-protocol analysis and the pre-analysis blinded data review has been performed. Individual code-breaking in case of serious medical illness will be performed with support from the pharmacy if principal investigators deem it necessary [5].

The study intervention starts from the first day of menstrual bleeding in the cycle with expected embryo transfer. Oral administration of one tablet in the morning continues until the day of embryo transfer. On this day, the daily dose increases to two tablets in the morning and continues until a negative pregnancy test, a pregnancy loss, or GW 8+0. Intravenous infusion is administered once at the time of embryo transfer (from 5 working days before to 2 working days after embryo transfer) and repeated in GW 5, 6, and 7 if the participant is pregnant. The tablets contain 5 mg prednisolone or placebo, and the infusions contain approximately 4 ml/kg body weight of 10% IVIG or 5% human albumin. A blood sample is collected on the day of the first infusion and again approximately 3-4 weeks later (if pregnant: right before initiating the third infusion). After GW 8+0, the participant receives an online questionnaire right after the nuchal translucency scan (GW 11-13) and after the due date, which collects information on adverse events, pregnancy, and perinatal outcomes. In case of a missed abortion, the investigators will apply for chromosomal examination of fetal tissue whenever possible and if accepted by the participant (Figure 10.1) [5].

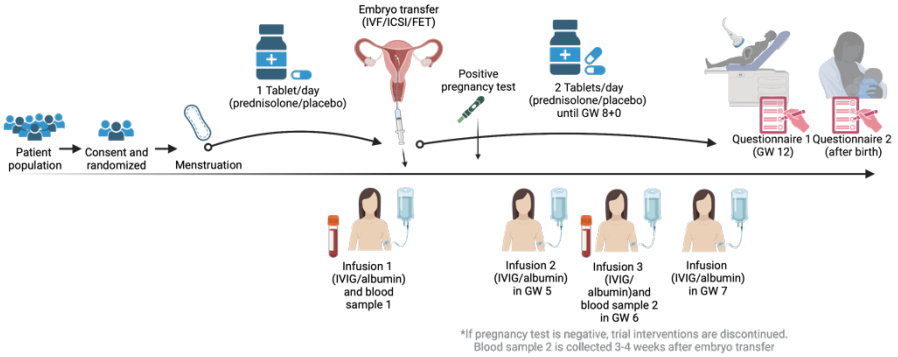

Figure 10.1: The treatment protocol in the randomized controlled trial (study V). *The treatment with prednisolone (or the comparator: placebo tablets) is initiated on the first day of the menstrual cycle and continued until gestational week (GW) 8+0+. The treatment with intravenous immunoglobulin (IVIG) (or the comparator: human albumin) is initiated close to the time of embryo transfer (from 5 working days before to 2 working days after) and repeated in GW 5, 6, and 7. Follow-up questionnaires are sent after the nuchal translucency scan and the due date. A blood sample is collected before the first and third infusions.*

The study participation does not regulate the patient's ART treatment. The individual fertility clinics decide the stimulation protocols, the transfer day, and the dose and timing of hormone replacement therapy. Medical supplements after embryo transfer and before GW 8 that have not been used in previous ART cycles and immunological drugs prescribed for improving fertility are not accepted [5].

Data are collected by the principal investigator in an electronic case report form with an audit trail. The electronic case report form is continuously reviewed and validated by an independent Good Clinical Practice board having no competing interest and who has access to the trial master file and medical records of all participants. All data are protected according to the General Data Protection Regulation (GDPR), the Danish Data Protection Act, and the Danish Health Act. The study is approved by the North Denmark Region Committee on Health Research Ethics and the Danish Medicine Agency, which have unrestricted access to monitor, audit, and inspect the source data [5].

Data on blood sample analyses are not reviewed by investigators or personnel in contact with participants to remain fully blinded until study completion, as their results may indicate allocation group. The Department of Clinical Immunology personnel are blinded, perform all blood sample analyses, and manage the research biobank. Flow cytometry analyses on fresh EDTA blood samples are made within six

hours measuring the total leucocyte cell count and the fraction of several leucocyte subsets. In a subgroup of 25 participants, a TruCulture analysis is performed to explore the relevance of measuring stimuli-specific cytokine production. The biobank material contains EDTA plasma, serum, and citrate plasma and is stored in a freezer for future research [5].

A thorough statistical analysis plan is published with all predetermined analyses described [5]. A pre-analysis blind review of all predetermined outcomes will be performed before unblinding.

The primary endpoint is the percentage of participants with ≥1 normal, viable fetus at nuchal translucency scan among all patients included in each allocation group and in all patients with a positive pregnancy test in each group. The RR, absolute risk reduction, and adjusted risk ratio will be reported based on these endpoints. The primary outcome is the RR. One sub-analysis will exclude the pregnancies with a confirmed chromosomal abnormality, and one sub-analysis will stratify participants according to their history of pRPL or sRPL.

The secondary endpoints are the incidence of adverse events/reactions, pregnancy complications, and perinatal outcomes. The tertiary endpoints include the explorative analyses of leucocyte subset distribution in relation to treatment and reproductive outcome [5].

The sample size calculation is based on previous studies in a similar population reporting LBR after prednisolone and IVIG treatment [406] and after no intervention [419]. LBRs of 40% and 12% are expected in the active treatment and placebo groups, respectively, and with an α-level of 0.05 and β-level of 0.20, each group should contain 37 participants.

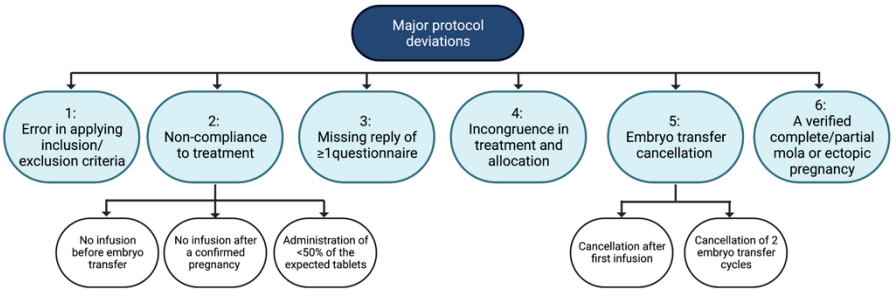

Figure 10.2: The criteria for major protocol deviations in the randomized controlled trial (study V). *ET: embryo transfer, IV: intravenous.*

The intention-to-treat analysis will include all randomized participants, while the per-protocol analysis will exclude participants with major protocol deviations described

in Figure 10.2. A blinded interim analysis is performed by an independent, blinded statistician who receives a non-identifiable labeled randomization list (i.e., ID numbers and treatment coded A/B) from the pharmacy after 38 participants have completed their participation with no major protocol deviations and the ID list with participants and their primary outcome of the study was prepared by the principal investigator. The interim analysis ensures no major deviation in the incidence of suspected unexpected serious adverse reactions (SUSARs) occur between the allocation groups. In addition, the OR for ongoing pregnancy is calculated in the interim analysis, but the study will not terminate in case of futility or implement any protocol modifications based on these results due to the insufficient dataset [5].

The study inclusion is expected to be complete in November 2023.

RESULTS

The RCT is in the recruitment phase. Therefore, no data are available for analysis or publication except from the results from the interim analysis performed on November 28, 2022, at what time no SUSARs or serious adverse events or adverse reactions had been registered, and eight participants had passed nuchal translucency scan with ≥1 apparently healthy fetus. The blinded analysis showed that in the intention-to-treat analysis, 6/20 (30.0%) and 2/21 (9.5%), respectively, were pregnant beyond the nuchal translucency scan (RR 1.8, 95% CI 1.0-3.1, Fisher's exact test: p=0.13), while in the per-protocol the numbers were 6/19 (31.6%) and 2/19 (10.5%) in each of the two groups, respectively (RR 1.7, 95% CI 1.0-3.1, Fisher's exact test: p=0.23).

DISCUSSION

This RCT is the first to test the effect of prednisolone and IVIG on both reproductive outcomes and immunological biomarkers in RPL patients undergoing ART.

When the RCT protocol was developed, experiences and results from the previous trials described in the introduction were taken into consideration. The protocol development to the present RCT was, however, complicated due to the considerably differing trial protocols according to both the selection criteria (e.g., the minimum number of prior pregnancy losses and embryo transfers, the inclusion of biochemical pregnancies and late pregnancy losses, and the definition of unexplained RPL) and the treatment procedure (e.g., the choice of comparator, product manufacturer, time points for treatment initiation and termination, time intervals between administrations, and dose schedules) as well as the differing findings even when comparable protocols were used. In continuation hereof and due to our hypothesis

that only patients with specific immune aberration can be expected to benefit from immunomodulatory treatment, monitoring immunological effects was considered of great value in providing further insight into which subset of patients the treatment with prednisolone and IVIG might benefit.

However, the RCT has some limitations that may affect its ability to accurately determine the effect of prednisolone and IVIG treatment on the reproductive outcome and the immune system.

Since the participants are admitted to various public and private ART clinics, the outcomes in the RCT will be affected by the difficulty of isolating a single variable for examination. In the setting of ART, multiple, partly uncontrollable factors affecting the ART outcome exist, including technical, laboratory, and interpersonal differences, various practical know-how/expertise levels, and differing medicinal products and treatment protocols used for each participant, which is all entwined. Such heterogeneity may introduce bias. However, we considered that with the presented pragmatic trial protocol, the study will reflect the real world in which the prednisolone and IVIG treatment will be used, and due to random allocation, variability in the above-mentioned factors is expected to be canceled out. Therefore, we believe that the study protocol is appropriate for answering the primary research question and providing results that are generalizable to the daily clinical practice.

An inevitable issue associated with examining therapeutics for pregnancy maintenance administered pre-conception is the low pregnancy rate per embryo transfer. Thus, the relatively large proportion of the randomized sample could not be expected to benefit from a treatment that mainly promotes pregnancy maintenance if no pregnancy is achieved. Indeed, previous studies found that the clinical pregnancy rate was approximately 66% in the first embryo transfer but only 35% in the embryo transfer following three failed embryo transfers independent of the use of PGT [428,429]. This information should be considered when calculating the required sample size, and therefore, we used pregnancy rates from studies of patients undergoing ART. The result from the interim analysis is relatively comparable to the expected numbers if we assume that the group with the highest success rate received active treatment (Active: 31.6% vs. 40% – placebo: 10.5% vs. 12%). However, this assumption cannot be confirmed because the randomization list has not been disclosed. Furthermore, the approach to sample size calculations often differs between researchers and is often up for discussion with the benefit of hindsight when results have been published. We expected an LBR of 12% in the placebo group based on the LBR in the RCT by Stephenson and Fluker [419]. In that trial, the patients had undergone a mean of 3.2 previous embryo transfers, while the corresponding number was 6.4 among patients undergoing ART referred to the CRPLWD between 2016 and 2021. The LBR in the active treatment group was expected to be 40% based on the findings from our pilot studies [406,407]. Thus, we considered these expectations for LBRs to be realistic.

The present RCT includes ART cycles independent of the use of PGT. The use of PGT has increased dramatically, especially in the US, where the percentage of PGT cycles is now more than 30% of all cycles [430,431]. In contrast, less than 2% of embryo transfers in Denmark in 2018 were from PGT cycles, and only 15.6% of these cycles were PGT-A [432,433]. A Cochrane meta-analysis found no change in LBR or miscarriage rate between ART cycles with and without PGT-A [434]. Therefore, the Danish public ART clinics offer PGT solely to couples with at least one partner carrying an inheritable, serious disease or structural chromosomal aberration. Only one private clinic offers PGT-A as a treatment adjunct at the couple's own costs. Thus, although the inclusion of merely PGT-A screened embryos would minimize bias from aneuploid embryos in the present RCT, it would also limit the external validity of the results, especially in relation to the current Danish standard treatment. Since the euploidy rate mainly depends on maternal age at the time of oocyte retrieval and not the patient's reproductive history (e.g., the previous number of failed IVF cycles, implantation failures, miscarriages, and livebirths) [429], the number of participants with a failed cycle or pregnancy loss resulting from an aneuploid embryo is expected to be equal in the two allocation groups if the mean age is comparable. To minimize the impact of chromosome abnormalities, participants were screened for chromosomal aberration as part of the routine diagnostic workup upon referral to the RPL clinic. In addition, the RCT investigators strive to analyze embryonal/fetal tissue for aneuploidy in case of a miscarriage in the study cycle since one of the effect measures will exclude these pregnancies, as described in detail in the statistical analysis plan. However, the aneuploidy rate of embryos failing to implant will remain unknown.

A major strength of this RCT is the initiation of IVIG treatment near the time of embryo implantation. In the majority of studies on IVIG for RPL patients, initiation of IVIG treatment occurred after pregnancy had been confirmed (GW 6-8) [256,257]. Post-implantation treatment initiation will inevitably cause a delay between the first time the maternal immune system is exposed to the fetal alloantigens and until the immune response is modulated by the exogenous immunoglobulin supply. It is believed that the foundation for a successful pregnancy is established during the most critical stages of placental development, i.e., during blastocyst implantation, cytotrophoblast invasion, and spiral artery remodeling initiation, which occur before clinical confirmation of a pregnancy by ultrasound is possible [435]. Thus, we speculate whether initiation of IVIG treatment after clinical confirmation of pregnancy has little if any influence on pregnancy outcome since the embryo has already survived the most critical stages of early pregnancy, including the extremely harsh conditions with severe hypoxia, lack of vascularization, and interchangeable inflammatory environments [435]. Passing this phase may be a manifestation of the embryo's viability, vitality, and independence of immunomodulatory support. In contrast, biochemical pregnancies that may have had the potential to succeed only if supported are already lost in the peri-implantation period. Indeed, the risk of miscarriage decreases from approximately 22% when confirmed by the hCG test to

11% in GW 6 and 1.5% in GW 8 [436–438]. This may indicate that the pregnancies supplemented with IVIG in previously published RCTs testing post-conception therapy were pregnancies having a good prognosis if left untreated. Moreover, based on the known importance of early IVIG treatment for the prognosis of autoimmune and infectious diseases [439–441], the most critical phase with the need for IVIG may be at the time of initial exposure, where the immune system determines whether to mount a defensive or regulatory response. In case of immune intolerance, early fetal demise may occur; while in case of immune tolerance, the immune response must be preserved for the maintenance of pregnancy. If this hypothesis is true, initiating immunomodulation right before first exposure to the fetus is crucial to support the development of immune tolerance. Moreover, it may also explain the lack of significant findings in most RCTs with post-conception treatment initiation.

The hypothesis is supported by previous findings from RCTs with post-conception IVIG for RPL, suggesting that the earlier IVIG is administered in pregnancy, the higher the success rate [258,442]. One of these trials was published during the recruitment phase of the present RCT [258]. The RCT included 102 pRPL patients and compared 400 mg/kg IVIG to saline with treatment initiated in GW 4-6 and administered for five consecutive days. The ongoing pregnancy rate in all participants was significantly higher in the IVIG group than in controls (OR 3.1, 95% CI 1.4-7.0). In a sensitivity analysis, the ongoing pregnancy rate did not differ between IVIG and placebo groups when treatment was initiated in GW 6 (OR 0.6, 95% CI 0.1-2.8), contrary to the significantly improved reproductive outcome when treatment was initiated in GW 4-5 (OR 6.3, 95% CI 2.2-17.8) [443]. These findings support the theory of treatment timing being essential for treatment efficacy.

The hypothesis assumes that immune aberrations play a major role in biochemical pregnancies. However, very little is known about the underlying causes of biochemical pregnancies due to the limited availability to study such pregnancies. However, the high failure rate after the transfer of euploid embryos in otherwise healthy women suggests that other causes of biochemical pregnancies than chromosomal abnormalities could be equally or even more frequent [429].

Thus, to sum up, we hypothesize that an appropriate patient selection and treatment timing are crucial factors determining the success of treatment, and therefore, great effort was put into designing and running an RCT that optimized the timing of IVIG and prednisolone treatment and that provided information that could be helpful for future selection of patients for such therapy.

In an additive, independent study, 37 healthy, fertile women, 18-40 years old, who had no known fertility or immunological complications were recruited. This group had a peripheral blood sample taken at the same time in their menstrual cycle (the WOC) and at the same time of the day (at 7-9 AM) as for the RCT participants. The recruitment and analysis of their blood sample were performed using the same

laboratory protocol and personnel and occurred concurrently with the RCT. Also, as in the RCT, extra biological material was collected from this group and stored for future research. This will allow us to compare RPL patients with non-pregnant, healthy women. Such analyses are expected to add valuable information to the results in the RCT as they can be the foundation, e.g., identifying differences in immune cell distribution between healthy women and RPL patients undergoing ART and determining whether the immunomodulatory treatment changes the immune system towards "normal."

The explorative examination of relevant immune biomarkers in peripheral blood is also a major strength of the present RCT. It will be the first RCT to evaluate the impact of the immunomodulatory treatment on both clinical outcomes and immune biomarkers in the same sample of patients.

The multiple blood analyses included in the study have different effect sizes, but since they were included as explorative objectives, they were not considered in the sample size calculation. The power of each one of these analyses may therefore vary. Nevertheless, the exploration of findings from these analyses may provide information that expands our knowledge on the role of the immune system in the RPL pathophysiology and may have a potentially pivotal role in our understanding of the pharmacodynamics of IVIG and prednisolone as well as which parameter(s) could be used for selective identification of RPL patients eligible for such treatment.

As an alternative, immune biomarkers from analyses on endometrial biopsy material have been widely investigated. However, since studies have shown a high inter- and intra-individual variability on the biomarkers during and between menstrual cycles and the endometrial tissue is inaccessible during the WOC and early pregnancy, a biomarker in peripheral blood is highly preferable [55,252]. Furthermore, the biopsy method is associated with greater psychological stress, pain, and risks than a blood sample.

There is considerable debate in the literature about whether the uterine compartment is the only meaningful compartment to examine and whether immune biomarkers in the two tissues correlate [252,444–447]. Some authors argue that this debate makes no sense since one cannot with any certainty compare the number of different cell types in different tissue compartments analyzed by different laboratory methods [253]. Moreover, regardless of such a correlation between peripheral blood and the uterine compartment, assessing leukocytes in peripheral blood may still be useful since the immunomodulatory treatment is systemic and may affect immune cells in both tissues in a similar direction.

Using, e.g., pNK/uNK cell levels or NK cell cytotoxicity as an indicator of whether immune therapy is beneficial is widely used despite a lack of scientific evidence. The rationale for such practice is based on the widespread belief that using these

biomarkers to identify patients that may benefit from immunotherapy is better than using empirical immune therapy without any investigation. However, it may be misleading since the scientific foundation for the suggested biomarkers and their associated cut-off values are based on low-quality studies not appropriately designed for such evaluation [146,147,448,449]. Moreover, the subsequent studies using these suggested biomarkers as selection criteria did not necessarily use similar laboratory methods to measure these biomarkers, which makes the validity of their results questionable. Therefore, evidence of higher quality for which biomarkers are qualified to select patients for the specific immunomodulatory treatment is required before this clinical approach can be fully endorsed [55].

RPL patients represent a relatively large subgroup of patients undergoing ART and are often exposed to several diagnostic interventions and adjuvant treatments that have not proven effective. This clinical practice may be a response to emotional patients who request further examinations and interventions even without scientific evidence after numerous failed embryo transfers and/or pregnancy losses [253,450,451]. The current practice of unselective use of these tests and treatments could potentially be harmful and costly and reduce the patient's chances of a live birth, as we can only expect an effect of treatment if it acts on the pathology causing the disease. This calls for high-quality studies that provide further insight into the pathophysiology of uRPL and the therapeutic targets and clinical effects of, e.g., immunomodulatory treatments. This information could potentially be generated in this RCT testing an immunomodulatory treatment that we deem to have a high potential to help patients if administered to a selected eligible group with the right timing and dose interval. The findings will hopefully aid in heightening the reproductive success rate in RPL patients undergoing ART in the future.

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CHAPTER 11. GENERAL DISCUSSION

The effect associations in all studies can be affected by type I and II errors and several types of bias, including confounding, selection bias, and information bias. In observational studies like study I-IV assessing RPL risk factors [1–4], it is particularly important to consider which covariates may impact the association measured between exposure and outcome.

TYPE I AND II ERRORS

The genuine ambition of most, if not all, researchers is to conduct a study with an adequate sample size and power to generate conclusions that can be applied to the background population with ample confidence. The adequate sample size depends on the expected effect size, which is the minimum clinically relevant difference in effect between exposed and non-exposed on the outcome variable, and the alpha- and beta-levels which describe the probability of type I and II errors, respectively. Thus, any analysis searching for statistical significance has a risk of type I and type II errors. Type I errors occur when a significant difference is found by chance, even when no true clinically relevant difference exists. At the same time, type II errors occur when a truly significant difference is not shown, as can be a consequence of, e.g., an insufficient sample size. Since a study should aim to conduct valid research with minimal risk of type I and II errors and without experimenting on more patients than necessary, the sample size calculation should not include too optimistic expectations of the effect difference but neither underestimate it [452].

The probability of type I error is often set at 5%, while the probability of type II error is often set at 10-20%. As the probability of error applies to each statistical analysis, the probability of the presence of errors in a study increases with the number of analyses included [452].

In our research, as with any other, we cannot rule out the probability that type I and II errors affect our findings, and such speculations should always be kept in mind. Indeed, studies I and III were conducted to replicate previous studies and observe if the prior findings could be reproduced and also to further extend our understanding of the role of MBL and H-Y immunization as we acknowledge both the novelty and potential importance of the previous findings but simultaneously question the validity without further evidence.

Due to publication bias, type I errors are expected to be more frequent than type II errors in published articles. Such bias has damaging effects on the integrity of

knowledge [453]. It is therefore considered good practice to publish aims and hypotheses before final data analysis in public registers and to publish both positive and negative findings. These principles were, in general, included in the research practice in my PhD by, e.g., registration of all prospective trials in ClinicalTrials.gov, publishing the RCT protocol and a thorough statistical analysis plan for the RCT, and publication of study IV, which reported several non-significant findings as well as findings opposite to our prior findings (e.g., the prevalence of HLA-DRB1*03 in RPL patients). However, we cannot rule out the probability that study IV did not have sufficient statistical power to test the study hypotheses and that type II errors may occur. On the contrary, the implications of multiple comparisons in the study are an increased risk of type I errors and, consequently, the need for considering correction by, e.g., Bonferroni adjustments. Nevertheless, the study was explorative and no information from previous similar trials was available for sample size calculation but with our current knowledge it is now apparent that a much larger sample size would be required for such analyses due to the small effect size and the high number of comparisons included in study IV.

Further discussion on sample size, type I and II errors, and multiple testing are included in the previous study discussions.

CONFOUNDING AND EFFECT MODIFICATION

CONFOUNDING

A confounding variable is an ancestor of both the exposure and response/outcome variable, but it must not be affected by the exposure or outcome variable in the source population. Therefore, it cannot be on the causal pathway between the exposure and outcome variable. The assessment of confounding variables should rely on knowledge of their causal relationship with the exposure and outcome variable and the assessment of potential confounding variables may be visualized by drawing a directed acyclic graph (DAG). Depending on the researcher's knowledge, the decision about which confounding variables exist in a model is a subjective judgment. Since it is not always clear whether and how these different variables are related and not all information can be obtained, the confounding variables may differ between studies examining the same relationship in different populations or during different periods. This highlights the necessity for a critical assessment of which confounding variables were included in prior studies and the underlying explanation of why. However, it is not uncommon to find articles where the arguments for selecting potential confounders included in the adjusted analyses are missing or false [454,455].

Frequent causes for incorrect inclusion of covariates include misunderstanding confounding effects, which may be manifested by:

I. Researchers believing that lack of adjustment for certain variables can cause rejection for publication.
II. Researchers including an adjustment for variables based solely on which variables were (falsely) adjusted for in previous studies investigating similar topics.
III. Researchers' assessment of covariates being based solely on automated statistical procedures like forward entry or backward elimination.

If the interpretation of the relationship between the covariate and the exposure and outcome variable under study is confused, the inclusion of a covariate that is not a confounder but is on the common path of the exposure and outcome variable may introduce bias into the model rather than eliminate it [456]. Covariates should, therefore, preferentially be included in the stage of planning the study methods or, at minimum, before the final collection of data and data analysis to minimize bias from confounding; however, this is not always possible. During the planning phase, restriction, matching, and randomization are different methods to handle confounding [457].

EFFECT MODIFICATION

Effect modification is fundamentally different from confounding, but the two concepts sometimes need clarification due to the seemingly analogous evaluation methods. While confounding variables distort an association, which may obscure whether the exposure is a cause of the outcome being studied, effect modifiers are variables that affect the magnitude of the association between the exposure and the outcome variable. The information about different effect sizes of an exposure on an outcome in different subgroups is valuable to the analysis of interest and, if true, reflect aspects of the true characteristic of the relationship in contrast to confounders which we strive to eliminate [457].

Investigation of presence of confounders and effect modifiers involves data analyses with stratification and adjustment for the covariates suspected to influence the association between exposure and outcome. After stratification, effect modifiers will manifest as differing sizes of the associations between exposure and outcome across strata, while confounders will manifest as (substantial) changes between unadjusted and adjusted association measures but not after stratification. Thus, the interpretation of the size of the association after stratification and adjustment can aid in differentiating between variables that act as confounders and effect modifiers [457].

The observational studies in this thesis included adjusted and/or stratified analyses of the association of exposure variables (p-MBL level and a firstborn boy) with the

outcome variable (first pregnancy outcome after admission). In the following, the thoughts behind deciding which variables to adjust for in these studies will be elaborated.

STUDY I

The impact of MBL on the reproductive outcome after RPL was assessed in a multivariable model that included maternal age, BMI, and tobacco smoking habits as confounding variables.

It is much debated what factors affect the p-MBL level. *MBL2* gene polymorphism is proposed as the main determinant for the p-MBL level. However, high variability in plasma concentrations exists in each genotype, and the ranges between genotypes overlap [273,274,458]. The *MBL2* genotype is not a confounding variable but rather an ancestor of the exposure. Furthermore, MBL is considered an acute phase reactant. However, previous studies suggest the response to stressful events like surgery and infection depend on the *MBL2* genotype since no significant increase in the p-MBL level was observed in individuals with coding mutations. In contrast, some individuals homozygous for the *MBL2* wild-type variant showed small acute decreases or increases in p-MBL concentration [274,277,317]. Moreover, changes in p-MBL seem to be independent of changes in CRP level [277]. Since the p-MBL level in our study was measured on the day of the diagnostic workup, no patients were severely ill at the time of sampling, and potential confounding from this factor could therefore be considered as controlled by restriction. Other factors suggested to affect the p-MBL level are TSH level and those included as confounding variables in the study: maternal age, BMI, and tobacco smoking.

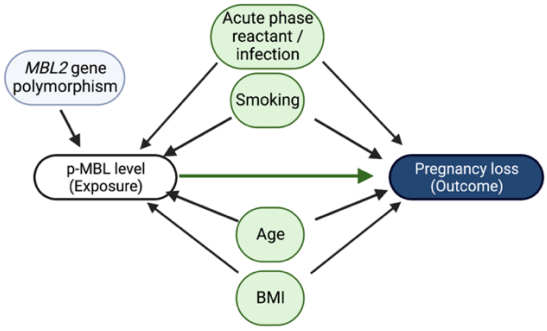

Figure 11.1: Confounding variables in the study I illustrated in a directed acyclic graph. *Confounding variables in green circles.*

Three prior studies found a significant association between p-MBL level and age in healthy Caucasians and Chinese people [459–461]. Another study found no association between p-MBL levels in Danish blood donors and age [462]. Even so, the three first studies were possibly more well-designed than the latter, and therefore age was included as a potential confounding variable.

Evidence of an association between p-MBL and obesity and smoking is very sparse. An association between p-MBL level and respective BMI and smoking has been suggested since chronically elevated levels of acute phase reactants [463–465] and some complement factors [77,459,466] have both been associated with elevated BMI and smoking. However, the associations are inconsistent. Studies designed to search for an association between p-MBL and BMI percentiles or smoking habits did find significant associations [467–469], but the direction of the correlation with smoking was conflicting [468,469]. Other studies did not find a difference in baseline characteristics that included BMI and/or smoking when comparing patients with high and low p-MBL concentrations or wildtype and mutant *MBL2* genotypes [284,319,470–472].

The evidence for the effect of age, BMI, and smoking on reproductive success rate is well-established [24,473–475], while the evidence for whether these factors affect p-MBL levels is less clear. However, based on the low-quality evidence that suggested potential associations between p-MBL levels and these factors exists, the authors decided to include age, BMI, and smoking as confounding variables (Figure 11.1). Data on these three variables reflected the time of admission where p-MBL (the exposure variable) was measured, which differed from when pregnancy outcome (i.e., the outcome variable) was evaluated. However, the authors considered that smoking habits, BMI, and age are relatively consistent variables over the relatively short period from admission to first pregnancy in the cohort, and we would therefore not expect significant bias from such potential changes over time.

Furthermore, as the thyroid hormone level affects the p-MBL level [476,477], undetected thyroid disease the diagnostic workup at the CRPLWD may have biased some p-MBL measurements. However, the associated treatment was initiated immediately in the case of thyroid disease, and subsequently, the patient was monitored closely. Therefore, we considered the effect on the subsequent pregnancy outcome would be small or insignificant, and consequently, the same applied the residual confounding effect on the association between p-MBL level and pregnancy outcome.

STUDY III

Study III examined the effects of a firstborn boy on the reproductive prognosis in RPL patients. The analysis was adjusted for maternal age, BMI, and smoking, acted as confounding variables, and stratified for carriage of an HYr-cII allele that acted as an effect modifier (Figure 11.2).

If a factor should act as a determinant of the sex ratio in newborns, it should affect either the gametes pre-conceptionally, the periconceptional period, or fetal mortality. Studies have confirmed that the proportion of chromosome X and Y-bearing spermatozoa does not differ, nor does their potential to fertilize an egg [478,479]. Previous studies have suggested that increasing maternal age is associated with a decreasing proportion of male births [480–484]. Also, maternal weight [485–489] and smoking [480,486,490–493] have been suggested to influence the sex distribution at birth. However, some studies report an association with increased male-to-female sex ratio, and other studies report the opposite.

While the effect of these three variables on the secondary sex ratio is considered minor and the quality of evidence is low, the evidence regarding the effect on reproductive success rate after RPL is considered clinically relevant, statistically significant, and of high quality [19]. Previous simulations studies have recommended adjusting for such covariates as the potential adverse impact on the estimation of the exposure-outcome association is minimal when adjusting for (what may turn out to be) a non-confounder that was believed to be a confounder based on little or no theoretical background knowledge [494]. Thus, maternal age, BMI, and smoking habits were included in the adjusted analyses in study III.

Race/ethnicity was also considered to impact the secondary sex ratio and risk of early pregnancy loss [21,484], and this was partly taken into account by restriction since all patients were Danish citizens and approximately 98% were Caucasians; thus, only small numbers were of a different race or ethnicity.

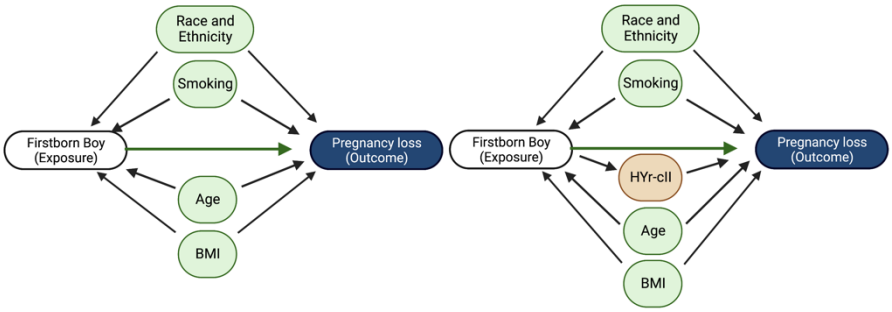

Figure 11.2: Confounding variables in study III illustrated in a directed acyclic graph (DAG). *Potential confounding variables (green) illustrated in a DAG with (right) and without (left) the inclusion of the effect modifier (orange: carriage of H-Y restricting HLA class II alleles [HYr-cII]).*

Other variables may also confound the association between a firstborn boy and subsequent pregnancy outcome. Maternal psychological stress may have had an impact as some previous, low-quality studies have suggested that peri-conceptional stress is associated with fetal wastage, especially in male fetuses, and increased risk of miscarriage [19,495]. Other factors that may affect both the exposure and outcome variables include socioeconomic status and periconceptional hormone levels [495]. However, the evidence of the impact of these factors on the secondary sex ratio comes from small, very low-quality studies, and the confounding effect is therefore questionable. We have no information on the patient's psychological health, social class, or periconceptional hormone level, and it was, therefore, not possible to make an adjustment for these variables in a sensitivity analysis. However, we do not consider the current evidence sufficient to assess whether and/or how the variables are associated. Therefore, adjusting for these potential covariates would probably introduce bias rather than reduce it.

The HYr-cII carriage, maternal age, BMI, and smoking variables were determined at the time of admission, which time point is after the pregnancy with her firstborn child (i.e., the exposure variable) and before the time of her pregnancy after admission (i.e., the outcome variable) was evaluated. As age, BMI, and smoking status may differ considerably between these three time points, adjustments for these variables are subject to some degree of uncertainty. However, BMI and smoking habits are considerably stable during fertile age [211,496] and the time interval between the firstborn and the wish of a second child varies relatively little between the majority of Danish couples [497]. The influence is therefore expected to be relatively equal for all patients included. Therefore, the impact of changes in these factors over time would be very small and insignificant.

STUDY V

Study V is an RCT whose design is the gold standard to compare different interventions/treatments as it (if performed correctly) balances the baseline characteristics of known and unknown factors between treatment groups, eliminates confounding, and ensures an unbiased estimate of a given intervention effect. Therefore, we did not consider matching for any baseline characteristics between groups to be necessary, but we did plan a sensitivity analysis of the primary outcome that adjusts for age, BMI, and current smoking habits and one that also adjusts for parity, presence of autoantibodies, and low p-MBL level in the data analysis phase. However, we expect that the random allocation of participants will avoid confounding and minimize the risk of bias in this RCT.

During the planning phase of the RCT, the investigators discussed whether the exclusion criteria should include patients with the presence of autoantibodies. No study has found treatment with prednisolone nor IVIG alone or in combination to increase LBR in RPL patients with the presence of autoantibodies in comparison to those without autoantibodies or in healthy fertile women [250,498]. Only a few very low-quality studies have suggested a beneficial effect of glucocorticoids combined with LDA on pregnancy rate after ART in infertile patients with the presence of autoantibodies [426,427], while others did not find a significant effect in infertile [499]. As described in the introduction, the presence of aPL and ANA is associated with RPL, but there is no evidence for using the presence of autoantibodies or any other immune biomarker as an indicator (i.e., effect modifier) for a beneficial effect of prednisolone, IVIG, or any other treatment except for the possible beneficial effect of anticoagulant treatment to patients with high titer of aPL and ≥3 pregnancy losses based on very low-quality studies [191,224]. Thus, we could not find evidence suggesting that including patients with autoantibodies without an overt autoimmune disease would introduce bias to the study. Therefore, we decided to allow inclusion of patients with ANAs, TPO antibodies (if thyroid stimulating hormone level was within normal range), and patients with low aPL titers. To evaluate the potential effect modification of the presence of autoantibodies, it is described in the statistical analysis plan that the table with baseline characteristics will include the prevalence of autoantibody positivity in each treatment arm and that a sensitivity analysis of the modified Poisson regression analysis of the primary outcome will be made with stratification for the presence of minimum one autoantibody.

SHOULD TREATMENT BE INCLUDED AS A CONFOUNDER?

The rationale for the treatment offered to patients at CRPLWD is described in the section “Methodology – the database.” As it is individualized, it is not possible to include treatment as a confounding variable in the logistic regression analyses in

studies I and III. Instead, in an attempt to include some information on treatment, we dichotomized patients into those who did or did not receive immunomodulatory treatments and underwent ART, respectively, and reported the frequency of each variable in the tables with baseline characteristics. At the CRPLWD, treatment with immunomodulatory therapeutics is not planned based on p-MBL levels nor the sex of a child born before RPL, as highlighted by the non-significant differences in the tables of baseline characteristics in the two cohort studies. In addition, no significant difference in the proportion of patients undergoing ART was seen according to exposure status. However, treatment with immunomodulatory therapeutics and ART, respectively, was more frequent in pRPL than sRPL patients in study III which could have confounded the exposure-outcome relationship in the comparison of pRPL and sRPL patients. As no high-quality evidence suggests ART to be beneficial to RPL patients in general terms and neither to be more beneficial to either sRPL or pRPL patients, we do not consider it a significant confounding variable in study III. The same applies to the variable on the use of immunomodulatory treatments at the CRPLWD; thus, it is neither considered a potential confounding variable.

In conclusion, we cannot rule out that ART or immunomodulatory treatment affected the pregnancy prognosis, but since the evidence of such impact on LBR is inconclusive and the distribution of these two covariates did not differ significantly by exposure status in studies I and III, we did not consider these variables to be confounders in these studies.

IS THE NUMBER OF PREVIOUS PREGNANCY LOSSES A CONFOUNDER?

In both cohort studies, a sensitivity analysis of the main regression analysis was performed, including the above-mentioned confounding variables and the number of previous pregnancy losses. International societies in reproductive medicine agree that maternal age and the number of previous pregnancy losses are “conventional predictors” for reproductive outcomes [500]. These two factors are often included as confounding variables in adjusted analyses examining the association between an exposure and the pregnancy prognosis; however, their relationship to the exposure variable is rarely described. Since age and number of previous pregnancy loss are not ancestors to all conceivable exposure variables associated with pregnancy prognosis, the variables do not inevitably meet the criteria for confounding variables. When they are not ancestors to the exposure, they may act as mediators or competing variables in the model. Mediators are descendants of the exposure and ancestors of the outcome variable, while competing variables are ancestors of the outcome but neither an ancestor nor descendant of the exposure variable. Inclusion of such covariates in the model would introduce bias rather than control for it.

Prior studies examining the effect of an exposure, e.g., sex of the firstborn child, on the reproductive prognosis in RPL patients have often included age and number of

previous pregnancy losses as confounding variables in their adjusted regression models [171,331]. However, an important question is whether the inclusion of age and number of previous pregnancy losses in these models fulfill criteria for confounding and whether their inclusion reduces or increases bias in these models.

The negative effect of increasing age on reproductive outcome has been suggested to be mediated through a long list of biological factors gradually deteriorating with age, including, but not limited to, declining egg reserve, lower ovulation rate, decreased endometrial receptivity, increased embryo aneuploidy rate, decreased oocyte and embryo quality, and changes in sex hormone levels [501]. Thus, high-quality evidence for age being an ancestor of the outcome variable (pregnancy outcome) exists, but age is only sometimes an ancestor of the exposure variable of interest. Accordingly, age should solely be included in the model as a confounding variable when age is an ancestor of both the exposure and outcome variable.

Several studies investigating predictors of pregnancy outcome in RPL patients have found a significant positive correlation between odds for a new pregnancy loss and the number of previous pregnancy losses: both when the latter variable was included as a continuous and as a categorical variable; and when tested in a univariable and a multivariable logistic regression model [24,231,500,502]. Specifically, the registry-based cohort study by Kolte et al. [24] showed the additive negative effect of increasing number of prior pregnancy losses to maternal age on the reproductive prognosis illustratively. Thus, the theory of a dose-response effect between the number of previous pregnancy losses and the risk of a new pregnancy loss is widely accepted, but the underlying mechanisms are unclear.

One may consider that such an association requires accumulation of destructive and lasting changes after each pregnancy loss that increase the risk of a new pregnancy loss for each event. An example of such an additive, negative impact of prior pregnancy losses is the theory presented in the section “Endometrial hyper-receptivity,” which describes the accumulation of chronic senescence cells and prolonged pro-inflammatory, decidual response in the mid-luteal phase causing a prolonged window of implantation and reduced receptivity.

Another explanation for the correlation between the number of prior pregnancy losses and the risk for a new pregnancy loss may be differences in patient characteristics between the patients grouped according to their number of previous pregnancy losses. Let us imagine that RPL could be separated into two groups based on whether a known causative factor or chromosomal aberrations (so-called “bad luck”) could explain all of the women’s pregnancy losses [224,503,504]. The literature has suggested that about 20-30% of pregnancies are lost due to developmental errors in the early embryonic stages. Therefore, the probability of three consecutive pregnancy losses occurring by chance (“bad luck”) would be 0.8-2.7% as the probability is constant. We would expect that the probability of a pregnancy loss in the subgroup of

patients with "bad luck" is constant, i.e., 20-30%. We would expect the probability of a pregnancy loss to be higher but also constant in the subgroup with an underlying disorder, as their probability is affected by both their disorder and the universal risk for chromosomal aberrations. Based on this assumption, we would expect that the proportion of patients with an underlying disorder in the whole sample of RPL patients would increase with an increasing number of pregnancy losses while the number of "bad luck" patients (black dots in Figure 11.3) would gradually decrease. This would consequently make the mean probability of a new pregnancy loss (red columns in Figure 11.3) approximate the probability found in the group with an underlying disorder.

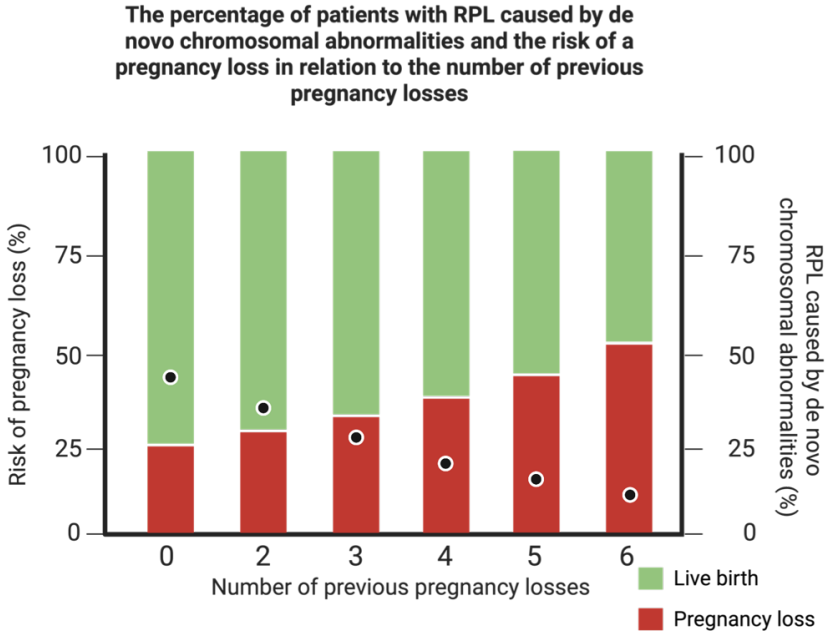

Figure 11.3: A made-up, theoretical diagram explaining the proposed theory of why the risk of a new pregnancy loss increases with each number of pregnancy losses*. In the diagram, patients are grouped based on the number of previous pregnancy losses at a given time, and the x-axis represents these groups. The red columns represent each group's risk of a new pregnancy loss (left y-axis). The black dots represent the proportion of patients in each group with RPL solely due to fetal chromosomal or embryonal developmental aberrations (right y-axis). In contrast, the remaining patients have an underlying constant maternal disorder explaining most, if not all, of their pregnancy losses. When the proportion of patients with RPL due to embryonal chromosomal or developmental aberrations decreases, the probability of a pregnancy loss approximates the probability of a pregnancy loss associated with the underlying maternal/endometrial disorder plus chromosomal aberrations. In the fictive example, that probability is approximately 55%.*

Thus, while the first explanation suggests that the risk of a pregnancy loss increases for each pregnancy loss due to the accumulating detrimental effects of prior pregnancy losses, the second explanation suggests a constant risk of a pregnancy loss but a change in group characteristics that leave patients with the worst prognosis/disorder in the group with the highest number of prior pregnancy losses.

Thus, it may not be a consequence of the event itself but an expression of the change in the characteristics of the patients remaining in each RPL subgroup stratified by number of prior pregnancy losses. If this is the case, then the number of previous pregnancy losses is not a confounder (Figure 11.4 A) but rather a mediator between the exposure and the outcome variable (Figure 11.4 B-C) or a descendant of the exposure with no relation to the outcome variable (Figure 11.4 D) as illustrated on the DAGs. Thus, the exposure variable (in at least some cases, if not the majority) could be considered a risk factor for both prior pregnancies and the current pregnancy. If you assume the previous pregnancy losses are a confounder, the exposure should be a consequence/descendent of prior pregnancy losses since a confounding variable affects the exposure and outcome variables but is not affected by any of them [454,455], which seems less plausible.

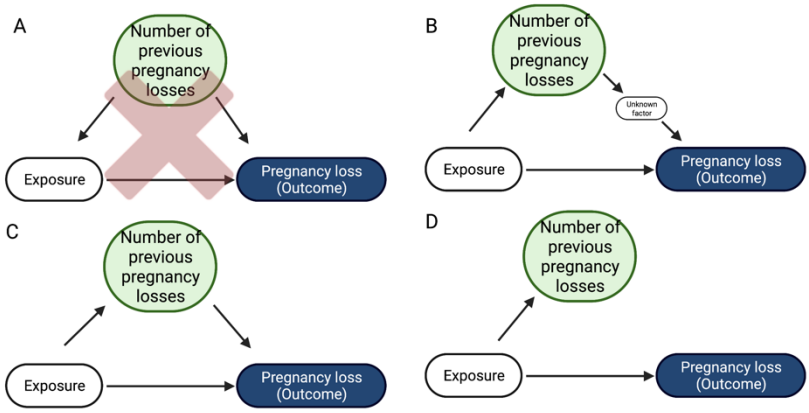

Figure 11.4: Directed acyclic graphs (DAGs) on potential relationships between an exposure and pregnancy loss (outcome). *A illustrates a DAG where the number of previous pregnancy losses is a confounding variable, and the red cross indicates that this may not be the true relationship between the three variables. B and C illustrate a DAG where the number of previous pregnancy losses is a mediator variable with and without an unknown factor in the path. D illustrates a DAG where the number of previous pregnancy losses is a descendant of the exposure and where it has no relation to the outcome.*

Based on this theory, adjustment for the number of prior pregnancy losses in the regression model on equal terms as other confounders would potentially introduce

overadjustment bias rather than eliminate bias. Therefore, this variable was not included in the main adjusted analyses in our cohort studies. It was only included in a sensitivity analysis in each study to accommodate the reviewer's and the general community's wishes and perceptions on this topic.

SELECTION BIAS, INFORMATION BIAS, AND EXTERNAL VALIDITY

SELECTION BIAS IN RELATION TO THE CRPLWD DATABASE

Selection bias is described as an error occurring when the probability of selection or attrition depends on or is related to the exposure and, independently of exposure, to the disease/outcome status. Consequently, the relationship between the exposure and the outcome in the sample does not represent the relationship in the true population and the internal validity, which is a prerequisite for the external validity, is compromised.

As described, the data from study I-IV originated from the CRPLWD database, which we consider a representative sample of Danish women with RPL. To minimize factors influencing biochemical measures from the routine blood analysis, the diagnostic workups occur within the same time frame of the day and preferentially in non-pregnant patients. All patients are advised to attempt to get pregnant after the diagnostic workup and to contact the CRPLWD upon a positive pregnancy test to plan an appointment for early viability ultrasonography. All contacts to the CRPLWD regarding pregnancy status are registered in the database. In 18% of patients, no pregnancy after admission has been registered ≥18 months after admission, and from a spot check of the medical records of 1/3 of these patients, no correction was to be made. Therefore, we consider the data on the outcome of pregnancies after referral is highly valid and has a low "non-response"/"lost to follow-up" rate. However, the representativeness of the sample and "lost to follow-up" rate have not been fully validated. The authors did not have access to the Danish National Patient Register nor the Danish Medical Birth Register which could have enabled us to make a validation of the database.

As the patients included in the four epidemiological studies in this thesis originate from the CRPLWD database counting all patients consecutively admitted to the CRPLWD, we do not consider selection bias to significantly affect the results in these studies. Furthermore, the outcomes used are all fairly frequent. As has been shown, measures of association are less prone to selection bias (compared to measures of occurrence) when the outcomes are frequent [262].

EXTERNAL VALIDITY

While the database may represent Danish patients with RPL, we cannot directly extrapolate findings in this sample to patients from other countries. The presence of confounding variables affecting the relationship between exposure and outcome may differ between countries, and this should be considered when an exposure-outcome association can be found in one population but not in another. For example, with the addition of our findings in study III, the association between HYr-cII alleles, sex of the firstborn child, and pregnancy prognosis in sRPL patients has now been found in two independent studies, but both studies included mainly Danish women. Thus, while it is possible that confounding may affect the external validity, as we cannot rule out that a confounding variable present in Danish women but not in the population of RPL patients, in general, can explain the observed association, selection bias is a less likely candidate to affect external validity. Therefore, clinical studies on RPL patients living outside Denmark will add further to the validity of the findings and indicate if the combination of carriage of an HYr-cII allele and a firstborn boy is a risk factor or whether the association is confounded. We are currently working on an article including data on the sex of firstborn children delivered before RPL, the subsequent pregnancy outcome, and perinatal outcomes from patients living in the Netherlands, United Kingdom, and Denmark. Despite the lack of HLA genotyping, this study may indicate whether a screwed sex ratio of the firstborn child and the association with the pregnancy prognosis also exist in RPL patients outside Denmark.

The statistical analysis plan for study V was published as an appendix to the protocol [5] and described in detail which statistical analyses are planned to assess the primary, secondary, and tertiary/exploratory outcomes.

INFORMATION BIAS IN RELATION TO THE THEORY ON H-Y IMMUNIZATION IN SRPL PATIENTS

Information bias derives from errors in data collection and most often causes misclassification bias.

Study I discussed the different cut-off values used to classify MBL deficiency in prior studies of RPL patients [174]. Previous studies on the risk of infection suggested the cut-off used in the study I to be optimal [272], but until we have further information on how the functional capacity of the plasma clearance mechanisms is affected by different p-MBL concentrations, the validity of the p-MBL cut-off value to classify RPL patients with MBL deficiency remains.

In studies II and III, we hypothesized that the risk of pregnancy to cause maternal immunization against fetal alloantigens differed between pregnancies carried for more or less than 24 GW. We cannot rule out the possibility that women with a firstborn girl or no pregnancies beyond 24 GW (pRPL) had developed H-Y

immunization after a pregnancy with a male fetus lost before GW 24. In that case, misclassifying patients considered at high risk of H-Y immunization in the study would have occurred. We need further studies on markers of maternal immunization to fetal alloantigens before we can minimize this type of misclassification bias.

In study III, the lack of HLA-DRB3 genotyping and consequently missing information about which patients carried the HYr-cII allele HLA-DRB3*0301 may have caused misclassification, too [164]. However, the lymphocyte expression of the HLA-DRB3 molecules is only approximately ¼ of the expression of HLA-DRB1 molecules [367], the association between autoimmune diseases and HLA-DRB genes is (almost) exclusive to DRB1 genes [368], and no significant impact of the HLA-DRB3*0301 on the RPL prognosis was found in the previous study [179]. Thus, despite a possible H-Y antigen presentation in patients carrying the HLA-DRB3*0301 allele, the clinical relevance seems to be minor. Therefore, it may be more misleading to include it as a marker for being at risk for H-Y immunization than to leave it out. Moreover, we cannot rule out that other HYr-cII alleles than the currently known alleles exist since the research on HYr-cII alleles is sparse. The knowledge about the four HYr-cII alleles originates from only four case reports that respectively included one patient who developed acute GVHD after hematopoietic stem cell transplantation and identified one HLA class II allele able to present an H-Y antigen [165,167,168]. Therefore, further studies are needed to verify our findings and the associated theory.

INFORMATION BIAS IN RELATION TO STUDY V

The allocation, concealment, and blinding of patients, data analysts, and investigators in our RCT minimize the risk of information bias. The allocation is concealed to avoid predictability of treatment allocation, and the active drugs and the placebo comparators have identical appearances. The randomization list is only available for the personnel preparing the treatment, which works on different departments locally distant from the CRPLWD. Based on our experience with low-dose prednisolone and IVIG treatment, we rarely hear patients complaining about side effects other than a transient headache in relation to an infusion. However, transient headache is also a common side effect of albumin infusion. Thus, we expect the number of patients with specific side effects, which could make the allocation relatively predictable, will be small. By advising patients to use the side effect diary, the investigators will rarely become aware of undamaging side effects before GW 9 when treatment is complete, and the diary is returned.

The explorative analysis for the distribution of leukocyte subsets may be at risk for information bias due to the markers used to identify each subset. For example, there is yet to be a consensus on which markers should be used to identify Tregs, as some argue for using FoxP3 while others advocate for using CD127. We decided to use $CD4^{+}CD25^{+}CD127^{low}$ since it has been used in several studies to identify functional

Tregs [110,115,505]; it is inversely associated with FoxP3 expression [506] and considered more accurate than using FoxP3 [506–509]. Similarly, the markers used to identify other leukocyte subsets can be discussed. As mentioned previously, it is a major issue that no gold standard or consensus exists for the laboratory methods used to measure immune cells, the markers used to classify each subset, and the outcome to report (e.g., cell number, the proportion of leukocytes or lymphocytes, etc.). Such variability complicates the comparability of study findings and the clinical implementation of the methods.

CHAPTER 12. CONCLUSION

My PhD thesis aimed to elaborate on immunological risk factors to RPL and to test the efficacy of an immunomodulatory treatment with IVIG and prednisolone in treating RPL.

MBL is a plasma protein that supports the clearance of apoptotic cells, cellular debris, and immune complexes from the circulation [510]. While a low p-MBL level was found to be a risk factor for RPL, as hypothesized in study I, the findings of a potentially protective effect of a high p-MBL level and the association between low p-MBL level and a history of a firstborn boy was a new [1]. In continuation hereof, study II found a significantly higher prevalence of sRPL patients who had a firstborn boy and/or an older brother than expected, which may suggest that male microchimerism could be a risk factor for sRPL [2]. Furthermore, study III found that the lowest chance of a successful reproductive outcome after RPL was observed among sRPL patients who had a firstborn boy and carried two HYr-cII alleles (53.3%). In comparison, the highest chance was found among sRPL patients who had a firstborn girl and carried two HYr-cII alleles (84.6%). The chance for a successful reproductive outcome among patients with pRPL irrespective of their HLA class II genotype and among sRPL patients who carried no HYr-cIL alleles irrespective of the sex of their firstborn child was right in-between these two extremities (approximately 70%) [3]. In addition, the prior pregnancy with a boy in sRPL patients who carried an HYr-cII allele was more often affected by preeclampsia than in those who did not carry such alleles, which condition is associated with increased shedding of apoptotic cells presenting immunogenic surface peptides. Study IV found that the association between immunogenetic susceptibility factors and the production of autoantibodies in RPL patients was weak. Furthermore, no HLA phenotype was associated with RPL, suggesting environmental factors may influence a woman's susceptibility to RPL and that genetic susceptibility to RPL, in general terms, is questionable [4].

These novel findings in study I-III [1–3] may suggest that immunization to H-Y antigens as a consequence of compromised clearance mechanisms or increased shredding of immunogenic material from a male fetus into the maternal circulation in predisposed women is involved in the sRPL pathogenesis.

Overall, RPL is a multifactorial disease, and the group of patients is highly heterogeneous. This complicates the search for RPL risk factors, highlighted by the high prevalence of patients with unexplained RPL. Even after decades of research into causes and risk factors in these patients, no major breakthrough has been accomplished.

Study V describes the trial protocol of an RCT that aims to test whether immunomodulatory treatment with IVIG and prednisolone can increase the chance of

a successful pregnancy in RPL patients undergoing ART [5]. The protocol is supplemented with a statistical analysis plan. Publication of study protocol and statistical analysis plans is a practice highly advocated by the authors as it is critically important for appropriate reporting of clinical trials and strengthens the validity, reliability, and transparency of the findings. We hypothesized that the treatment is effective with appropriate timing for treatment initiation and selection of patients. Furthermore, the trial includes thorough explorative investigations of immune markers before and after treatment. This is hypothesized to aid valuable information about the immune aberrations in RPL and selection markers for patients benefitting from the treatment. Hopefully, it will bring us closer to a breakthrough in immunological reproductive medicine.

CHAPTER 13. PERSPECTIVES

RESEARCH PERSPECTIVES

This year marks 70 years since Sir P. Medawar first posited "the immunological paradox of pregnancy," which described pregnancy as a condition where the maternal host is anomalously tolerant and contrives to nourish an antigenically foreign fetus due to immunological adaptations in both the mother and her fetus. He also postulated that the adaptations are not always successful, in which case "*the mother is immunized against the antigens of its foetus, with the consequence that the foetus, or its successors in later pregnancies, is either destroyed or born with the affections that are the more or less immediate outcome of cellular damage*" [61]. Since 1953, scientific research on this paradox has contributed to our understanding of immune tolerance in pregnancy. However, our understanding is still limited, and in this thesis, I presented several findings that underpin Medawar's citation.

The findings from the clinical studies in this PhD thesis suggested that increased exposure to male-specific antigens is a major risk factor for RPL and significantly impacts the RPL prognosis. However, further investigations into H-Y immunity are needed to finally confirm this theory since some unexpected findings contradict the theory.

We advocate further research in HYr-cII alleles to explore whether more HLA class II alleles can present H-Y antigens and whether some combinations are more immunogenic than others. Moreover, H-Y immunization has now been suggested as a risk factor for RPL in two independent cohorts separated by approximately two decades, but both studies originate from Danish clinics. Thus, one may question if confounding variables could explain the findings. We are currently working on an international study including data from the Netherlands, United Kingdom, and Denmark to elaborate on whether the frequency of sRPL patients with a firstborn boy is comparable across borders and whether it affects the pregnancy prognosis after RPL. Although these countries do not routinely perform HLA genotyping in the diagnostic workup of RPL patients, their clinical settings and socioeconomic and environmental factors are relatively similar to the Danish setting, and the findings may therefore aid valuable information.

Despite further research being needed, we find the current evidence strong enough to recommend that future studies should take both the frequency of sRPL and the sex of children from prior births into consideration when reporting, for example, baseline characteristics. This is rarely reported, but it may be valuable information based on our findings. Indeed, the cause of pRPL vs. sRPL, as well as sRPL after the birth of a boy vs. that of patients with a firstborn girl, may be very different from each other.

Based on the high success rate after RPL in patients with a firstborn girl, one may question: What if sRPL after a girl is rarely caused by immune aberrations but rather by repeated chromosomal abnormalities? Then immunomodulatory treatments may do more harm than good in these patients. Indeed, if further analyses can confirm the theory, the next step is to investigate treatment targets or to test the effect of immunomodulatory treatments with a wide impact on the immune system in sRPL patients with a firstborn boy.

We still have much to learn, but with the findings in this PhD thesis, we are one step closer to understanding the immune system's role in the RPL pathogenesis.

CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

The results from the epidemiological studies in this thesis suggest that the immune system strongly influences both the susceptibility to RPL and the pregnancy prognosis. Maternal immunization to fetal antigens from a prior pregnancy, as posited by Medawar, may influence the subsequent pregnancy outcome. Our findings could be used in the clinic during the diagnostic workup for explanatory and prognostic purposes. The inclusion of questions on perinatal outcomes and pregnancy complications of prior births as well as laboratory determination of the p-MBL level and the HLA class II genotype, at least in Scandinavian RPL patients, can potentially support the current guidance of the physician and provide data that could aid future studies as we still have much to learn.

Today, no treatment with a strongly proven beneficial effect for RPL exists even in patients positive for RPL risk factors, and consequently, they are left with the advice to "keep on trying." However, I believe that unsuccessful adaptations of the pregnancy-related immune response can explain a significant proportion of both early and late pregnancy losses and that we in near future will have biomarkers and/or diagnostic tests with high validity for diagnosing immunologically mediated RPL and predicting pregnancy prognosis in these patients. To achieve this goal, we cannot limit our focus to NK cells, but instead, we need more well-planned and -executed studies that explore the widening spectrum of pregnancy-related immune mechanisms that ensure fetal tolerance by the maternal immune system.

RPL patients seek to understand their condition and rarely find the advice of "keep on trying" acceptable. The frustration is shared between the patient and the physician and may lead to a use of questionable interventions. The widespread approach of using Th1/Th2 or NK cell testing to determine whether immunomodulatory treatments can improve the prognosis in patients with repeated unsuccessful pregnancies is far from well-founded and possibly oversimplified and misleading. Reproductive immunology deserves further scientific scrutiny to better understand the RPL pathogenesis and find efficient treatments that will spare physicians and patients from undue costly and time-consuming investigations, treatments,

frustrations, worries, and false hopes. This is the motivation for the RCT included in this PhD thesis, and hopefully, it will bring us further insights that will help improve the RPL treatment for future patients.

However, a significant obstacle is the gender bias affecting research and research funding, clearly demonstrated by the underrepresentation of women in studies of gender-neutral diseases and the underfunding of female-dominant diseases when the funding level is normalized to the disease burden [511]. Furthermore, RPL has been taboo for decades which has affected the patients' help-seeking behavior, the media attention, and the interest from the pharmaceutical industry. These factors are some of the many difficulties that hinder scientific research in RPL and reduce resources allocated to offer specialist treatment to these patients. The increasing number of celebrities sharing their stories of grief after one or more pregnancy losses during the last five years has drawn attention to the field. Hopefully, this will affect the funding sources and right the disproportionate share of resources.

Studies have shown that the participation rates in prospective studies have dropped from approximately 80% to 35% during the last three decades [262], which is a serious problem that I believe can only be solved by increasing the involvement of patients in the research procedures, communicating the importance of their commitment, and promoting patient engagement. During my PhD, I found it valuable to communicate our knowledge and knowledge gaps on RPL to the patients, as I consider the patients as my partners and my research as our common mission. I did this by collaborating with the National Patient's Association for Involuntarily Childless Patients and by giving interviews to prominent magazines and podcasts focusing on the struggle to conceive and accomplish a pregnancy. I find this meaningful and motivating as we can then share our hopes and optimism for a brighter future. It may ease the more tough situations that RPL and research also brings. The feedback from patients on the interviews and podcasts has only confirmed that they also appreciate the insight into ongoing research and results from studies where they participated. I believe it may increase their willingness to participate in scientific trials despite sometimes having some costs.

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