

The Effect of Immunomodulators in Recurrent Pregnancy Loss

Gia-Han Le

Supervised by: Dr Jen Southcombe, Prof Ingrid Granne, Dr Pedro Melo

St. Catherine's College

A thesis submitted in partial fulfilment of the requirements

For the degree of MSc by Research in Women's and Reproductive Health

I would like to express my deepest gratitude to Dr Jen Southcombe, Prof. Ingrid Granne, Dr Pedro Melo, Dr Rebecca Dragovic, and Dr Christian Becker for the invaluable guidance and expertise throughout this journey.

Thank you so much to Dr Ana Kisovar, Dr Zhixing Jin, Dr Yifan Wang, and Abbe Blatchford for the warm welcome, daily chats, and friendship.

Thank you to Holly, Liv, Bryony, and Michelle for their support and encouragement in the Thames office.

And thank you to Phoebe, Sacha, and Lal for making Oxford home.

Abstract

Recurrent pregnancy loss (RPL) is a complex, multifaceted condition affecting 1-5% of couples attempting to conceive, imposing significant psychological, clinical, and economic burdens on healthcare systems globally. Despite advancements in reproductive medicine, significant gaps remain in the ongoing research to develop effective diagnosis tools and interventions, leaving many RPL cases unexplained and difficult to manage. Research has consistently suggested that disruptions in the decidualisation of human endometrial stromal cells (hESCs) and overactivated immune responses contribute to implantation and pregnancy failure. With this inflammatory signature being common in RPL patients, drugs that modulate or suppress the immune system have been proposed as potential therapies to decrease the likelihood of a miscarriage. This thesis aims to explore the current evidence on immunomodulators used in the treatment of RPL and assess their efficacies through a systematic review. With a varying number of RCTs available for each intervention, the results of the meta-analysis showed that only steroids appear to have a significant benefit on live birth rate, whereas for intravenous immunoglobulin (IVIG), granulocyte colony stimulating factor (G-CSF), intralipid, and hydroxychloroquine (HCQ) there was significant uncertainty in the findings.

This thesis also includes further exploration of intralipid and hydroxychloroquine (HCQ), two drugs for which there is the least amount of evidence as concluded in the systematic review. This study explores how these two immunomodulators may affect decidualisation and inflammatory cytokine expression by hESCs. Stromal cell lines derived from control and RPL patients were cultured, treated, and assessed for morphological changes and decidual marker secretion. Key findings include increased expression of MCP-1 in response to intralipid and significant monocyte migration when cultured with treated-stromal cells. Although there is a lack of high-quality evidence for the use of intralipid in RPL, these results highlight the potential benefit of intralipid treatment by increasing

endometrial receptivity, by way of early recruitment of monocytes and immune tolerance, in subgroups of immunology-mediated RPL cases.

Table of Contents

Abstract 3
Table of Contents 5
Abbreviations 8
List of Figures 9
List of Tables 10
Chapter 1: Introduction to Pregnancy & Recurrent Pregnancy Loss 11
1.1. Recurrent Pregnancy Loss (RPL) 11
1.1.1. Definition and Epidemiology 11
1.1.2. Overview on the Aetiology of RPL 12
1.1.3. Clinical Significance 15
1.2.1. Structure & Function of the Endometrium 17
1.2.1.1. Cell Types in Endometrium 17
1.2.1.2. Menstrual Cycle: Endometrial & Ovarian 18
1.2.1.3. Implantation 19
1.2.2. Decidua 20
1.2.3. Decidualisation: Process and Importance 21
1.2.3.1. Effects of Impaired Decidualisation 24
1.2.4. Placenta 25
1.3. Pregnancy Immunology 26
1.2.1. Maternal-Foetal Interface: Tolerance & Immunity 26
1.2.2. Aberrations in Immune Cells in RPL 27
1.2.3. Aberrant Immune Environment 29
1.4. Treatment of RPL 32
1.4.1. Current Clinical Recommendations 32
1.4.2. Immunomodulators 33
1.4.3. Focus of Research Project 34
Chapter 2: Systematic Review of Intralipid Treatment in Recurrent Pregnancy Loss Studies. 35
2.1 | Introduction 35

2.1.1. Immunomodulators 35
2.1.1.1. Intralipid 36
2.1.1.2. Hydroxychloroquine 37
2.1.1.3. Intravenous Immunoglobulin (IVIG) 38
2.1.1.4. Granulocyte Colony Stimulating Factor (G-CSF) 39
2.1.1.5. Steroids (Glucocorticoids) 40
2.3. Methods 44
2.3.1. Search Design & Strategy 44
2.3.2. Study Selection Process 47
2.3.3. Quality Assessment 49
2.3.4. Data Extraction 50
2.3.5. Data Analysis & Synthesis 51
2.4. Results 52
2.4.1. Study Selection 52
2.4.2. Trustworthiness Assessment of Included Studies 61
2.4.3. Data Extraction 64
2.4.4. Meta-Analysis of Immunomodulators in RPL 67
2.4.4. Quality Assessment of Evidence from Included Studies 74
2.5. Discussion 82
2.5.1. Summary of Key Findings 82
2.5.2. Interpretation of Results with Existing Literature 83
2.5.3. Clinical Implications of treating RPL with Immunomodulators 87
2.5.4. Ongoing Work & Future Directions 87
2.5.5. Strengths & Limitations 88
2.6. Conclusions 90
Chapter 3: Immunomodulation and Decidualisation: The Impact of Intralipid on MCP-1 Expression in RPL 91
3. 1 | Introduction 91
3.1.1. Inducing Decidualisation 91

3. 2 | Chapter Objectives 92
3.3 | Methods 94
3.3.1. Cell Culture 94
3.3.3. Confirmation of Decidualisation 98
3.3.4. LEGENDPlex (BioLegend) Human Inflammation Immunoassay 100
3.3.5. Co-Culture of THP-1 monocytes & stromal cells 102
3.4 | Results 106
3.4.1. Decidualisation of Cell Lines 106
3.4.2. Expression of Inflammatory Cytokines 109
3.4.3. MCP-1 increases in intralipid-treated stromal cells 113
3.4.4. Migration Rates of THP-1 Monocytes in Stromal Cell Co-Culture 116
3.5. Discussion 119
3.5.1. Summary 119
3.5.2. Overview of Decidualisation in hESCs 119
3.5.3. Inflammatory Cytokine Expression 121
3.5.4. Monocyte Migration in Stromal Cell Co-Culture 125
3.5.5. Limitations 126
3.5.6. Future Directions 128
3.6. Conclusions 131
Final Conclusions 132
References 134
Appendix 151
ELISA Standard Curves 152
IGFBP-1 levels in cell line RPL5 show successful decidualisation 153

Abbreviations

HESCs	Human endometrial stromal cells
IVIG	Intravenous immunoglobulin
HCQ	Hydroxychloroquine
RPL	Recurrent pregnancy loss
G-CSF	Granulocyte colony stimulating factor
ASRM	American Society for Reproductive Medicine
RCOG	Royal College of Obstetricians and Gynaecologists
ESHRE	European Society for Human Reproduction and Embryology
APS	Antiphospholipid syndrome
ACOG	American College of Obstetricians and Gynecologists
uNK cells	Uterine natural killer cells
P4	Progesterone
E2	Estradiol/oestrogen
RIF	Recurrent implantation failure
DSC	Decidual stromal cells
IL	Interleukin
cAMP	cyclic adenosine monophosphate
HFEA	Human Fertilisation and Embryology Authority

List of Figures

Figure 1.1: Breakdown of underlying causes of RPL including idiopathic cases

Figure 1.2: Depiction of the menstrual cycle and immune cell populations

Figure 1.3: Representative drawing of decidualisation in endometrium during the window of implantation

Figure 1.4: Decidualisation relative to the immunological shift at implantation site in the endometrium

Figure 2.1: PRISMA Flow Diagram

Figure 2.2A: Forest plot of the efficacy of IVIG for recurrent pregnancy loss

Figure 2.2B: Forest plot comparing published miscarriage rates between IVIG-treated and placebo control groups

Figure 2.3A: Forest plot of the efficacy of G-CSF for recurrent pregnancy loss

Figure 2.3B: Forest plot comparing published miscarriage rates between G-CSF-treated and placebo control groups

Figure 2.4A: Forest plot of the efficacy of steroids for recurrent pregnancy loss

Figure 2.4B: Forest plot comparing published miscarriage rates between steroid-treated and placebo control groups

Figure 2.5: Forest plot of the efficacy of HCQ for recurrent pregnancy loss

Figure 2.6A: Forest plot of the efficacy of intralipids for recurrent pregnancy loss

Figure 2.6B: Forest plot comparing published miscarriage rates between intralipid-treated and placebo control groups

Figure 2.7: Risk of Bias summary of all included studies B. Risk of Bias graph of all included studies in meta-analysis

Figure 3.1: Schematic of Isolation & Decidualisation Protocol

Figure 3.2: Stromal cells morphological changes through decidualisation treatment A. Representative control cell line B. Representation RPL cell line

Figure 3.3: Prolactin secretion levels in cell lines during decidualisation

Figure 3.4: Cytokines released in response to HCQ treatment during decidualisation on Day 8

Figure 3.5A: Heatmap of inflammatory cytokines released by stromal decidualised stromal cells treated with intralipid on Day 4

Figure 3.5B: Heatmap of inflammatory cytokines released by stromal decidualised stromal cells treated with intralipid on Day 8

Figure 3.6: Change in MCP-1 levels in response to Intralipid treatment on Day 4 of decidualisation

Figure 3.7: MCP-1 secreted from stromal cells in response to intralipid treatment across an 8-day decidualisation period

Figure 3.8: A. Transwell Setup of Upper and Lower Chambers. B. Migrated THP-1 Monocytes.

Figure 3.9: Percentage of Migrated THP-1 Monocytes co-cultured with control and intralipid-treated stromal cells.

List of Tables

Table 1: Overview of the Aetiology of Recurrent Pregnancy Loss

Table 2: Search Design for Cochrane Trials database

Table 3: Completed PICOS Framework

Table 4: Breakdown of the studies not included in meta-analysis

Table 5: Summary table of randomised controlled trials pertaining to treatment of RPL with intralipid

Table 6: Summary table of randomised controlled trials pertaining to treatment of RPL with HCQ

Table 7: Summary table of randomised controlled trials pertaining to treatment of RPL with G-CSF

Table 8: Summary table of randomised controlled trials pertaining to treatment of RPL with IVIG

Table 9: Summary table of randomised controlled trials pertaining to treatment of RPL with steroids

Table 10: Compiled TRACT Checklists for all included studies

Table 11: Primary outcomes of systematic review extracted from included studies

Table 12: Secondary outcomes of systematic review extracted from included studies

Table 13: GRADE assessment of all included studies for live birth rate outcome in meta-analysis

Table 14: GRADE assessment of all included studies for miscarriage rate outcome in meta-analysis

Table 15: PRISMA Checklist

Table 16: Immunoassay Analytes

Chapter 1: Introduction to Pregnancy & Recurrent Pregnancy Loss

1.1. Recurrent Pregnancy Loss (RPL)

1.1.1. Definition and Epidemiology

Early pregnancy loss is estimated to be the most prevalent pregnancy disorder, occurring in 15-25% of pregnancies. It is defined as the loss of a clinical pregnancy before the 20th week of gestation (Wilcox et al., 1988). Recurrent Pregnancy Loss (RPL), a subset of this condition, is characterised by multiple and consecutive miscarriages. However, the exact definition and diagnostic criteria for RPL vary across international institutions. According to the European Society for Human Reproduction and Embryology (ESHRE), RPL is defined as the loss of two or more pregnancies. The American Society for Reproductive Medicine (ASRM) defines RPL as two or more pregnancy losses that are confirmed by either histology or ultrasound, although—definingly—not necessarily consecutive. In the United Kingdom, the Royal College of Obstetricians and Gynaecologists (RCOG) defines RPL as three or more consecutive pregnancy losses. RPL affects 1-5% of couples trying to conceive (Practice Committee of ASRM, 2012), with the differences in reported prevalence likely reflecting the variation in diagnostic criteria across organisations and regions.

RPL can be classified as primary, secondary, or tertiary. Primary RPL includes RPL patients who have never had a live birth, secondary RPL refers to RPL in women who have previously delivered a live baby, and tertiary RPL describes patients who experience multiple pregnancy losses between successful pregnancies (ESHRE Guidelines, 2022). With approximately 50% of RPL cases considered idiopathic, the lack of identifiable causes presents significant challenges to diagnosis and treatment (Stephenson et al., 2002). Idiopathic or unexplained RPL underscores the complexity of this fertility condition, and is likely a combination of genetic, anatomical, immunological, and environmental factors that have been historically under-researched and poorly understood (Ford & Schust, 2009). The discrepancies in the definitions of RPL, combined with its complex aetiology and

pathophysiology, further emphasize the need for further investigation and integrative approach to understand and address RPL.

1.1.2. Overview on the Aetiology of RPL

For approximately half of RPL cases the most common underlying causes are known and include: antiphospholipid syndrome (APS), uterine anatomic anomalies, chromosomal abnormalities, autoimmune disorders (i.e., thyroid-related), and hormonal disorders (i.e., thyroid disease and diabetes). Maternal age is the most significant risk factor for miscarriage in general, with a study citing a risk of 10% in women aged 25-29, rapidly increasing after 30 years, and eventually to a 53% risk in women aged 45 and over (Magnus et al., 2019). While maternal age and other factors—such as uterine malformations and aneuploidy—may contribute to both sporadic miscarriage and RPL, the latter is more often linked to chronic or systemic issues related to genetics, endocrine, immunological, and anatomical risk factors. Table 1 outlines the possible underlying causes and the conditions found to be associated with RPL.

Risk Factor	Associated Conditions/Phenotypes	Suggested Diagnostic Test
Genetic	Chromosomal abnormalities (ie. embryonic aneuploidy, translocations)	Karyotyping Parental Chromosome Analysis
Anatomical	Congenital uterine malformations (ie. uterine septum, intrauterine adhesions)	MRI 3D Pelvic Ultrasound Sonohysterography
Endocrine	Hypothyroidism Polycystic Ovary Syndrome (PCOS) Insulin Resistance (IR) Luteal phase defect (LPD)	TSH Thyroid Antibodies Insulin resistance assessment
Immunological	Thrombophilia (ie. Factor V Leiden mutation, prothrombin gene mutation, protein S deficiency) *Autoimmune conditions:* Antiphospholipid syndrome (APS) Systemic lupus erythematosus (SLE)	Blood tests Antiphospholipid Antibody Panel

Infectious Factors	Rubella Cytomegalovirus (CMV) Toxoplasma	Antibody blood tests
Lifestyle Factors	Excessive drinking Smoking	
Other	Vitamin D deficiency Male factor Immunological factors**	Blood tests

Table 1: Overview of the Aetiology of Recurrent Pregnancy Loss. The information in this table is compiled from various sources such as the American College of Obstetricians and Gynecologists (ACOG), ESHRE, the NICE guidelines, and the ASRM, in addition to reviews from Quenby et al., *Lancet* (2021), Melo et al., *Fertility & Sterility* (2023), and Turesheva et al., *Journal of Clinical Medicine* (2023), which provide an updated overview of the various risk factors and conditions associated with RPL.

For the remaining 50% of all RPL cases, the cause is unknown and is classified as unexplained, or idiopathic (Li & Marren, 2018). There is emerging evidence that changes in the immune system both during pre- and early post-conception can lead to RPL. For a pregnancy to result in a live birth, the immune system must maintain a delicate balance between tolerance and immunity. Dysregulation of the immune system, especially during inflammatory processes such as implantation and decidualisation, likely contributes to the 80% of miscarriage that occur before the 12th week of gestation (Wang et al., 2003).

Studies have shown that the immune cell profiles of RPL patients differ compared to healthy controls in populations of immune cells. It is established that women with RPL have a higher number of natural killer (NK) cells in the endometrium and peripheral blood (Quenby et al., 1999, 2005). Flow cytometry experiments have also shown that abnormal T cell ratios were found in the peripheral blood of RPL patients (Vahid et al., 2019), such as a significant increase in the Th17/Treg ratio compared to women without a history of RPL (Qian et al., 2018). More recently, single-cell transcriptomes profiling the decidual immune system in patients with RPL further demonstrate a substantial decrease in the number of dNK1 cells (a subset of decidual NK cells), and a increase in dNK3 cells, a subset that

produces pro-inflammatory cytokines (Guo et al., 2021). Furthermore, studies have demonstrated different levels of cytokines, both pro- and anti-inflammatory, in patients with RPL (Kniotek et al., 2021). With all this evidence and the understanding that pregnancy requires a carefully regulated immune system, it is likely that many of these idiopathic cases result from underlying immunological aberrations. Unfortunately, it is difficult to diagnose a patient as having immune-mediated pregnancy loss, owing to an absence of validated diagnostic tests.

Another challenge in diagnosing RPL is the increased risk of future miscarriages after previous subsequent miscarriages. In addition, there is no consensus on when to investigate for RPL, either after two, three, or more pregnancy losses (Quenby et al., 2020). Due to the heterogeneity of underlying causes and many risk factors, a thorough evaluation must include a range of tests from genetic testing to imaging and antibody screening, to accurately assess an individual's cause of RPL. Additionally, as seen in Figure 1.1, only a subset of these complete investigations succeeds in identifying a definitive cause for RPL, adding further uncertainty to management and patient counselling.

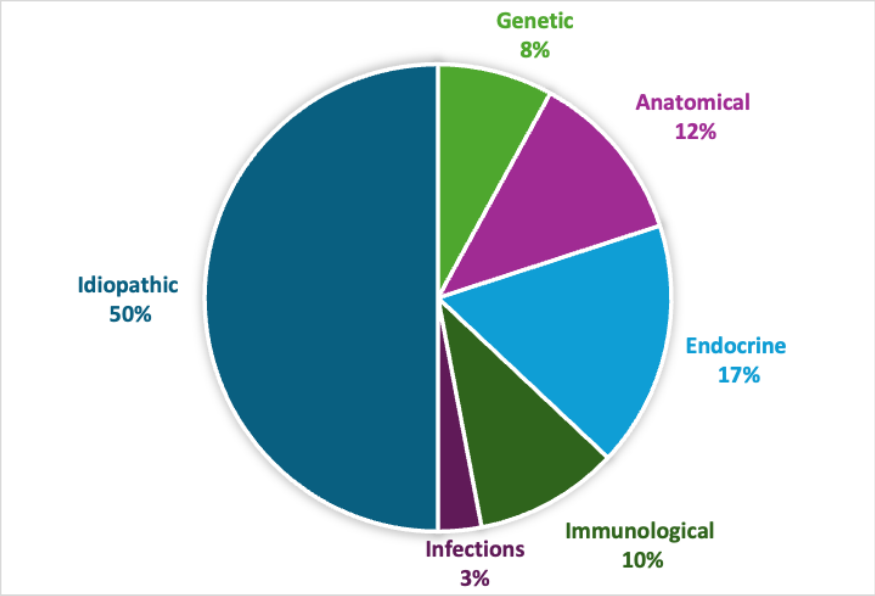


Figure 1.1: Breakdown of underlying causes of RPL including idiopathic cases. This pie chart illustrates the estimated distribution of the known and unknown causes of RPL, based on the following current data: genetic factors (2-8%), anatomical anomalies (10-15%), endocrine-related disorders (17-20%), immunological conditions (1-20%), infections (0.5-5%), and idiopathic cases (~50%). The percentages displayed on the chart reflect the ranges reported in the literature, and include the idiopathic cases to emphasize the substantial number of RPL cases with no identified causes (ASRM, *Fertility & Sterility* (2013), ESHRE, *Human Reproduction* (2018), and RCOG, *Green-top Guideline No. 17* (2011))

1.1.3. Clinical Significance

Recurrent pregnancy loss is not only a complex medical condition, but it also causes profound psychological impact and emotional distress. Studies have shown that women with RPL are more likely to experience major depression compared to women without RPL (Kolte et al., 2015). Women and their partners experiencing RPL often cite feelings of guilt, grief, and self-blame (Friedman & Gath, 1989), with the effect intensifying after each pregnancy loss (Brier, 2008). Psychological symptoms frequently persist in patients for six months to a year after a loss (Lok & Neugebauer, 2007), yet accessible mental health support for fertility patients remains inadequate (Martens et al., 2023). This underestimation of the psychological burden is compounded by the frustration experienced by both patients and clinicians, particularly in managing idiopathic cases of RPL. Beyond the emotional consequences, there are substantial clinical challenges in RPL that leave patients with unrecognised and unmet needs.

Adding to the complexity of RPL is the increased risk of future miscarriage following multiple pregnancy losses. Most couples with RPL can be reassured by the fact that the chance of having a live birth in a subsequent pregnancy is higher than the likelihood of another pregnancy loss. However, this is highly dependent on maternal age and the number of pregnancy losses. More specifically, a follow-up cohort study by De Jong et al. (2014) demonstrated that 69.7% of the participants with unexplained RPL had at least one live birth, with a lower probability of live birth in women with ≥3 miscarriages compared to women with 2 miscarriages.

While some causes of RPL, such as antiphospholipid syndrome (APS), have evidence-based treatments like heparin and low-dose aspirin that can lessen the risk of blood clots (Kutteh, 1996,

Empson et al., 2002), other aetiologies of RPL lack established therapies. For example, for RPL patients that test positive for antinuclear antibodies (ANA), ESHRE does not have specific treatment recommendations. These discrepancies in treatment options highlight the need for continued innovation and research into effective therapeutic interventions, in addition to more expansive and accurate diagnostics tools.

Furthermore, miscarriage is not only a population-specific issue but also a condition with profound societal and economic consequences. It is estimated that the short-term national economic cost of miscarriage is £471 million annually in the United Kingdom, a figure likely underestimated due to unaccounted long-term health effects (Quenby et al., 2021). Between diagnostic investigations, treatment costs, and ongoing monitoring, the financial burden is imposed not only on individuals but also on healthcare systems and society. Additionally, the lack of equitable access to specialised care for RPL exacerbates health disparities, leaving underserved populations facing greater barriers in obtaining quality patient care (Grandi et al., 2022). To address RPL holistically, a multifaceted approach is necessary, including greater research investment to develop evidence-based treatments and improvement of mental health and support services.

1.2. Endometrium and Decidualisation

1.2.1. Structure & Function of the Endometrium

The endometrium is a glandular tissue that is the innermost lining of the uterine cavity. It is composed of a layer of epithelium that sits on top of the stroma, a connective tissue containing many tubular glands in contact with the spiral arteries and luminal surface (Noyes et al., 1950). There are two distinct layers within the endometrium: the dynamic functional layer that sheds during menstruation, and the permanent basal layer that regenerates the new functional layer after each menstrual cycle. The functions of the uterine endometrium are to prepare for implantation, to maintain the pregnancy if implantation is successful, and to shed the uterine lining every month in the absence of pregnancy (Critchley et al., 2020).

1.2.1.1. Cell Types in Endometrium

The endometrium contains a variety of cell types, each contributing to its functions. Th inner lining is composed of two epithelial cell types: luminal and glandular. The luminal epithelial (LE) cells are the first maternal cells to directly contact the developing embryo after the blastocyst attaches to the uterine wall (Cha et al., 2012), whereas the glandular cells produce and secrete proteins and other essential nutrients to support embryo development (Choi et al., 2015). The stroma tissue is composed of fibroblast-like stromal cells that not only provide structural support but eventually transform into the decidual cells that allow for successful implantation and placental development (Gellersen & Brosens, 2014). Since the endometrium is a dynamic tissue that undergoes significant changes during the menstrual cycle, there are also primary vascular cells that are important for vascularisation and regeneration (Li & Ding, 2021). Finally, there are a host of immune cells that are recruited and differentiated within the endometrium to modulate and protect the endometrial microenvironment. Uterine natural killer (uNK) cells are the most abundant immune cells in the endometrium, with

macrophage, T cell (CD4+, CDC8+), and dendritic cell (DC) populations being in the greatest abundance. Additionally, B cells, regulatory T cells, mast cells, and neutrophils can be found in the endometrial tissue (Robertson et al., 2015, Chen et al., 2024, Agostinis et al., 2019). Together, these diverse cell types contribute to the structure, regenerative and immune function of the endometrium for successful implantation and pregnancy. Moreover, several studies have examined the intricate interplay between stromal, epithelial, and immune cells in the endometrium. This will be further discussed in the following sections on implantation and decidualisation.

1.2.1.2. Menstrual Cycle: Endometrial & Ovarian

The menstrual cycle is composed of the menstrual and ovarian cycles, each occurring within the endometrium and ovary, respectively. During the endometrial menstrual cycle, the endometrium undergoes changes divided across three distinct phases: proliferative phase (follicular), secretory phase (luteal), and menstrual phase. These sequential phases are regulated by circulating ovarian steroids, primarily oestradiol (E2) and progesterone (P4). Oestradiol is the dominant hormone during the proliferative phase, where the uterus lining thickens and proliferates in preparation for pregnancy (Ugwumadu, 2014). After ovulation, the corpus luteum is temporarily formed to secrete progesterone, which renders the endometrium receptive to implantation if an oocyte is fertilised. If fertilisation does not occur, the hormone levels drop, the released oocyte disintegrates, and the endometrium is shedded during the menstrual phase. After menstrual bleeding occurs, a new cycle begins starting with the follicular phase (Rosner, Samardzic, & Sarao, 2023).

While these phases are occurring, the corresponding ovarian cycle functions to produce and mature an oocyte that is released from the ovary every month during ovulation for potential fertilisation. Composed of the follicular phase, ovulation, and luteal phase, the ovarian cycle provides

the ovum and the corpus luteum structure (Rosner, Samardzic, & Sarao, 2023). The average menstrual cycle lasts for 28 days and repeats until menopause, unless a pregnancy is initiated.

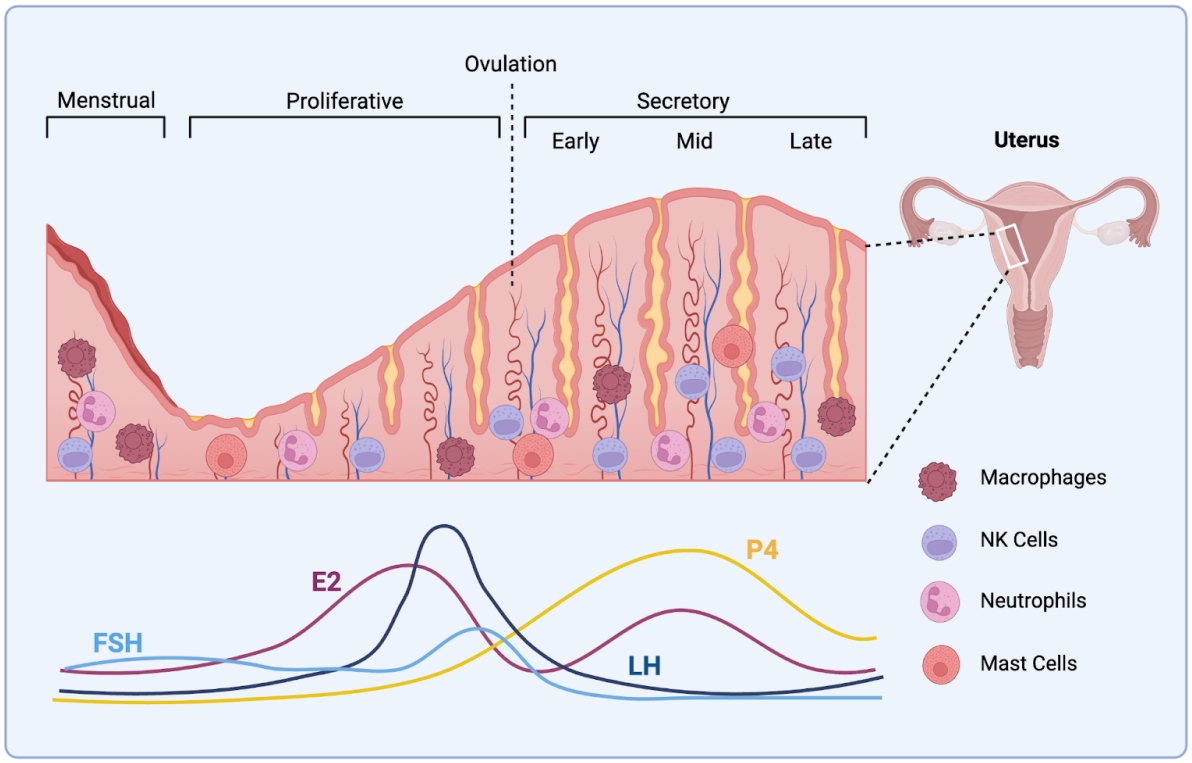


Figure 1.2: Depiction of the menstrual cycle and immune cell populations. This figure is a schematic drawing of the endometrium (located in the inner lining of the uterus) as it thickens and sheds throughout the menstrual cycle. The sequential phases are labelled from left to right: (1) menstrual, (2) proliferative, and (3) secretory (early, mid, late). The relative levels of hormones that regulate the menstrual cycle are drawn below the endometrium: estradiol (E2), progesterone (P4), luteinizing hormone (LH), and follicle-stimulating hormone (FSH). The immune cells shown are the primary host cells in the endometrium along with its relative abundance throughout the cycle. Descriptively, uterine natural killer (NK) cells are always present, but most prominent in the mid to late secretory phase. Macrophages are most abundant during the menstrual cycle, neutrophils increase significantly before menstruation, and mast cells remain relatively constant. The smaller populations of T and B cells are not shown, as well as dendritic cells. This figure was drawn on BioRender and references Starkey et al., *European Journal of Obstetrics & Gynecology and Reproductive Biology* (1991), Reed et al., *Endotext* (2000), and Mareckova, *Nature* (2024).

1.2.1.3. Implantation

After the oocyte is fertilised by the sperm, the formed blastocyst must attach to the endometrium through the process of implantation. Implantation is the first step to a successful pregnancy and is identified by the visualisation of the gestational sac through ultrasonography. The outer layer of a blastocyst is composed of trophoblast cells that eventually encounter the uterine

lining. Abnormal endometrial receptivity and immune system dysregulation within the uterus can severely affect implantation in RPL patients. This is particularly relevant for women who are diagnosed with both RPL and Recurrent Implantation Failure (RIF) and are undergoing in vitro fertilisation-embryo transfers to conceive; an estimated 75% of RPL cases are likely attributed to RIF. The RIF criteria specifically refer to the inability to achieve a pregnancy after at least four good-quality embryo transfers–meaning that the embryos fail to implant in the endometrium (Ma et al., 2023) However, there is still no consensus on the definition of RIF.

Furthermore, after implantation, it is proposed that there is a checkpoint in which the endometrium ensures that the implanted embryo is healthy enough to continue developing (Brosens et al., 2014). It is hypothesized that failure of this implantation checkpoint leads to the endometrium being unable to select against aneuploid embryos. The incidence of aneuploidy pregnancy losses due could be partially attributed to a faulty implantation checkpoint (Stephenson et al., 2002). Alternatively, if it is correct that this checkpoint allows for an endometrium to develop in response to individual embryos, it could also mean that competent embryos will still result in a miscarriage because the endometrium is insufficiently developed to support the embryo (Brosens et al., 2022). This could mean a thin uterus lining that lacks the proper thickness and vasculature, and other irregular changes to the inner cell lining that makes the uterus less receptive (Critchley et al., 2020). Overall, improper embryo selection and defects in implantation of an embryo, and subsequent decidualisation, can lead to both euploid and aneuploid miscarriage in RPL patients.

1.2.2. Decidua

The decidua is the specialized lining of the endometrium that is a transitory maternally derived uterine epithelium where implantation eventually occurs. It is formed during the secretory phase of the menstrual cycle in response to elevated levels of progesterone through the process of decidualisation. During pregnancy, this thickened and highly vascular tissue is crucial to the proper

development of the embryo and serves as the foundation of the placental bed (Mori et al., 2016). Anatomically, the decidua will eventually surround the fetus, umbilical cord, and developed placenta. The decidua is composed of decidual stromal cells (DSCs), as well as maternal vascular and blood cells. This structure continues to undergo significant tissue remodelling after the attachment of the blastocyst and is vital for proper implantation and a successful pregnancy.

1.2.3. Decidualisation: Process and Importance

For successful implantation of a competent embryo, the uterus undergoes extensive structural, immunological and hormonal changes, primarily driven by endometrial decidualisation. Decidualisation is a critical process whereby the endometrial stromal cells (ESCs) differentiate into secretory decidual stromal cells (DSCs), transforming the endometrium into the decidua (Ramathal et al., 2010). Unlike in most mammals, decidualisation is not dependent on embryo implantation, but is initiated spontaneously during the secretory phase of the menstrual cycle in response to elevated levels of the ovarian steroid hormones, oestrogen (E2) and progesterone, as well as the secondary messenger, cyclic adenosine monophosphate (cAMP) (Wetendorf et al., 2012, Jing et al., 2022). Developing on the concept of “embryo selection” mentioned above, it is the decidualising stromal cells that can respond to individual embryos to either promote implantation or initiate early rejection (Gellersen & Brosens, 2014).

Decidualisation can be distinguished in the endometrium during the different phases of the menstrual cycle, as observed in an integrated single-cell references atlas that used spatial transcriptomes to quantify and illustrate changes in the human endometrium (Mareckova et al., 2024). This study further demonstrates that stromal cell differentiation into decidual cells occurs in the late secretory phase of the menstrual cycle over approximately 10 days (de Ziegler et al., 1998), with the window of endometrial receptivity to implantation spanning around 4 days (Psychoyos, 1986). This period is marked by significant tissue remodelling and immune cell activation, particularly of

macrophages, T cells, and mast cells, which are critical for creating an immune-tolerant environment capable of supporting the allogeneic fetal. For this complex process to occur, there are key interactions between the stromal, epithelial, and immune cells in the endometrium.

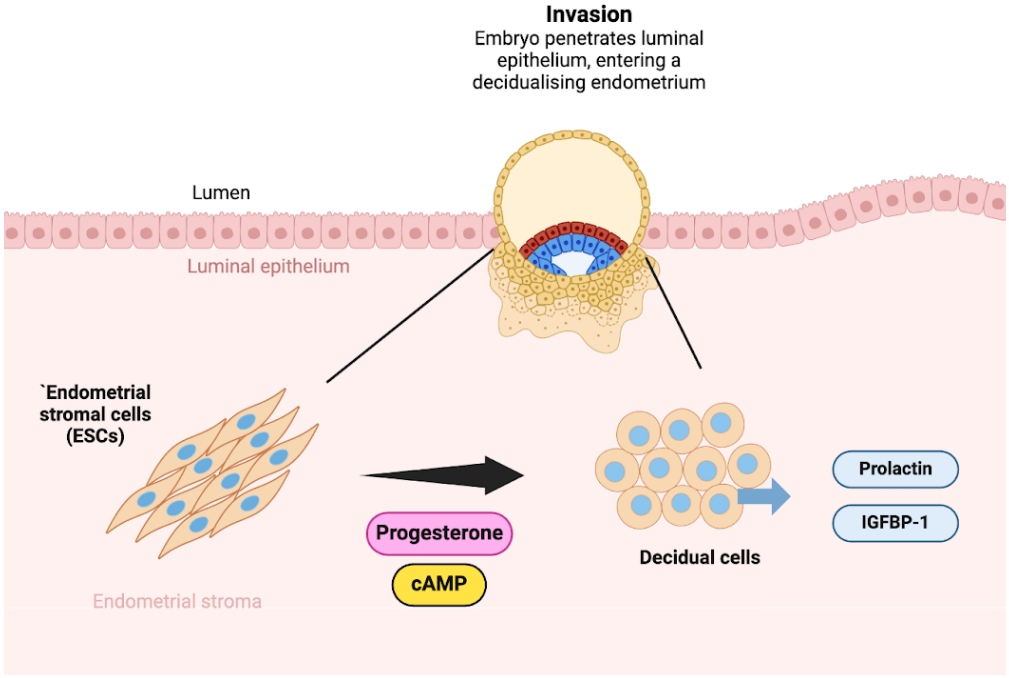


Figure 1.3: Representative drawing of decidualisation in endometrium during the window of implantation. This image depicts decidualisation as a spontaneous process that aligns with the window of implantation, not necessarily initiation by the embedding of an embryo. This process is driven by progesterone and an increase in cAMP concentration. Decidual cells release prolactin and insulin-like growth factor binding protein-1 (IGFBP-1) and are known as decidualisation markers. This figure was made on BioRender and adapted from the Human Embryo Implantation BioRender image by Nick Delrose.

Firstly, under the influence of ovarian steroid hormone signalling, stromal and epithelial cells are shown to synchronize their differentiation (Mareckova et al., 2024). This crosstalk continues across the menstrual cycle to facilitate paracrine signalling. Decidual stromal cells release key cytokines such as transforming growth factor-beta (TGF-β) (Jones et al, 2006) and the hepatocyte growth factor (HGF) to modulate receptivity and promote epithelial proliferation into the uterine lining–both crucial for the embryonic implantation (Chan et al., 2004). Another required cytokine for

implantation is the release of leukaemia inhibitory factor (LIF) by the ESCs, which guides epithelial cells to support embryo attachment (Brinsden et al., 2009). There are also epithelial-derived cytokines such as interleukin-6 (IL-6), secreted during the window of implantation that can act as a autocrine or paracrine factor on stromal cells. IL-6 has a variety of direct and indirect effects, including its ability to influence the expression of matrix metalloproteinases (MMPs), crucial in the remodelling of the extracellular matrix (ECM) (Champion et al., 2012).

Beyond the endocrine cues from ovarian steroid hormones and important factors such as cyclic adenosine monophosphate (cAMP), differentiating ESCs secrete various cytokines and chemokines to drive the decidual process. For example, uNK cells–the most abundant cells in early decidua–proliferate and mature in response to the cytokines produced by the stromal cells, such as IL-11 and IL-15 (Ashkar et al., 2003, Ain et al., 2004). Stromal cells not only reduce the cytotoxicity of uNK cells (Hanna et al. & Lash et al., 2006) but can produce gradients of chemoattractants to control the distribution and density of uNK cells throughout the endometrium (Guo et al., 2012, Quenby et al., 2005). In RPL patients, improper signalling can lead to abnormal uNK cell distribution, specifically more uNK cells in the stroma, leading to a less immune-tolerant environment and increasing the risk of implantation failure or miscarriage (Clifford et al., 1999).

Decidual stromal cells also recruit and polarise macrophages towards an anti-inflammatory phenotype by releasing cytokines such as TGF-β–a multifunctional morphogen. These decidual macrophages cooperate with uNK cells to induce immunosuppressive regulatory T cells (Vacca et al., 2010), further promoting immune tolerance. This crosstalk between the DSCs and immune cells highlights complexity of the decidual microenvironment to allow for implantation.

On the other hand, blastocyst signalling is seen as just as important in creating a receptive uterine environment. For example, the embryo secretes IL-1β, which upregulates key adhesion

molecules to signal for the completion of implantation and decidualisation. IL-1β also plays a role in the transformation of stromal cells by promoting the intense remodelling of ECM, a key characteristic of decidualisation (Gellersen & Brosens, 2014).

Decidualisation is a finely tuned and evolved process that requires the intricate interplay of various endocrine, paracrine, and autocrine pathways to achieve proper stromal cell differentiation and eventually, a receptive and supportive environment for the embryo to develop. Any disruptions or impairments transformation can lead to various pregnancy complications, including miscarriages.

1.2.3.1. Effects of Impaired Decidualisation

As researchers discover new regulatory pathways and key signalling molecules to more accurately model implantation and decidualisation in humans, it has become clear that any molecular defects in these complex processes can lead to RPL. As established in Section 1.1, embryonic chromosomal abnormalities account for 50% of all pregnancy losses, however it is known that with each additional miscarriage, the likelihood of a euploid miscarriage also increases (Hyde & Schust, 2015). A key component of the embryo selection theory at implantation is the decidualising endometrium that can either promote or reject the embedding of viable embryos. If decidualisation is impaired, it leads to the endometrium unable to select or support embryos, increasing the risk of miscarriage. Aberrant decidualisation can result in downstream effects due to inadequate production and secretion of cytokines, growth factors, and coregulators to modulate the immune system (Gellersen & Brosens, 2014).

In RPL patients, it is likely that decidualisation occurs either too early or too late and as a result, is misaligned with the window of implantation. As RPL is often associated with an out-of-phase or even prolonged window of implantation, this mistiming disrupts the coordination between the embryo and maternal tissues (Teklenburg et al., 2010). Any disturbances in steroid signalling or

intracellular signalling, such as cAMP, can lead to impaired decidualisation. Since decidualisation is an immune-regulated process, immune system dysfunction is hypothesized to contribute to unexplained cases of RPL that may stem from underlying immune system dysfunction. The proinflammatory decidual response in RPL cases and its role in decreasing the receptivity of the endometrium is further discussed in section 1.3 (Stalker et al., 2012).

1.2.4. Placenta

The placenta is a fetal-derived transient organ that develops in the uterus to link the mother and fetus during pregnancy. This highly specialised tissue provides both nourishment and immunological protection to the fetus (Balasundaram, 2023). Placental development is marked by trophoblast invasion, the process in which trophoblast cells from the embryo invade the uterine lining, leading to increased blood flow and transformation of the endometrium. This process along with decidualisation establishes the foundation of the placenta. Next, extravillous trophoblast (EVTs) further penetrate the decidua to increase blood flow to the placenta through remodelling of the spiral arteries (Burton et al., 2009). Finally, the placenta matures throughout the pregnancy, including the development of villous trophoblasts to maximise gas and nutrient exchange (Evain-Brion & Malassine, 2003). Besides providing nutrients to the developing fetus, the role of the placenta is to mediate immune tolerance, specifically to suppress the maternal immune response to prevent fetal rejection (Mor et al., 2005). Recent studies suggest that up to 90% of miscarriages can be due to complications in the placenta (Thompson et al., 2024), however further investigation is needed to determine the implicit link between the placenta and RPL cases.

1.3. Pregnancy Immunology

1.2.1. Maternal-Fetal Interface: Tolerance & Immunity

The maternal-fetal interface is a delicate point of contact between fetal cells and the maternal immune system where tolerance of the semi-allogeneic fetus is balanced with the immune system of the mother to adequately protect against systemic infections during gestation (Mor & Cardenas, 2011). Throughout implantation, decidualisation, and placentation, key cell-to-cell interactions (e.g., between decidual stromal cells and embryonic vesicles) allow for the adaptation and maintenance of the maternal-fetal immune tolerance (Wu et al., 2022). During implantation and placentation, until the early second trimester, a strong inflammatory environment is required to repair the uterine lining after all its changes to adapt to and support embryonic attachment (Dekel et al., 2010). This pro-inflammatory state shifts to be anti-inflammatory as the fetus rapidly grows and the immune response is lessened compared to the early stages of pregnancy. Finally, once the fetus is completely developed, inflammation is renewed through an influx of immune cells. Once the uterus returns to a pro-inflammatory environment, contraction of the uterus is initiated (Romero et al., 2006). The immunological state of pregnancy is constantly shifting and investigations into pregnancy as an anti-inflammatory condition continue to build our understanding of the complex paradox of pregnancy (Wegmann, 1984). Dysregulation at any stage of these immunological shifts can lead to complications, such as recurrent pregnancy loss.

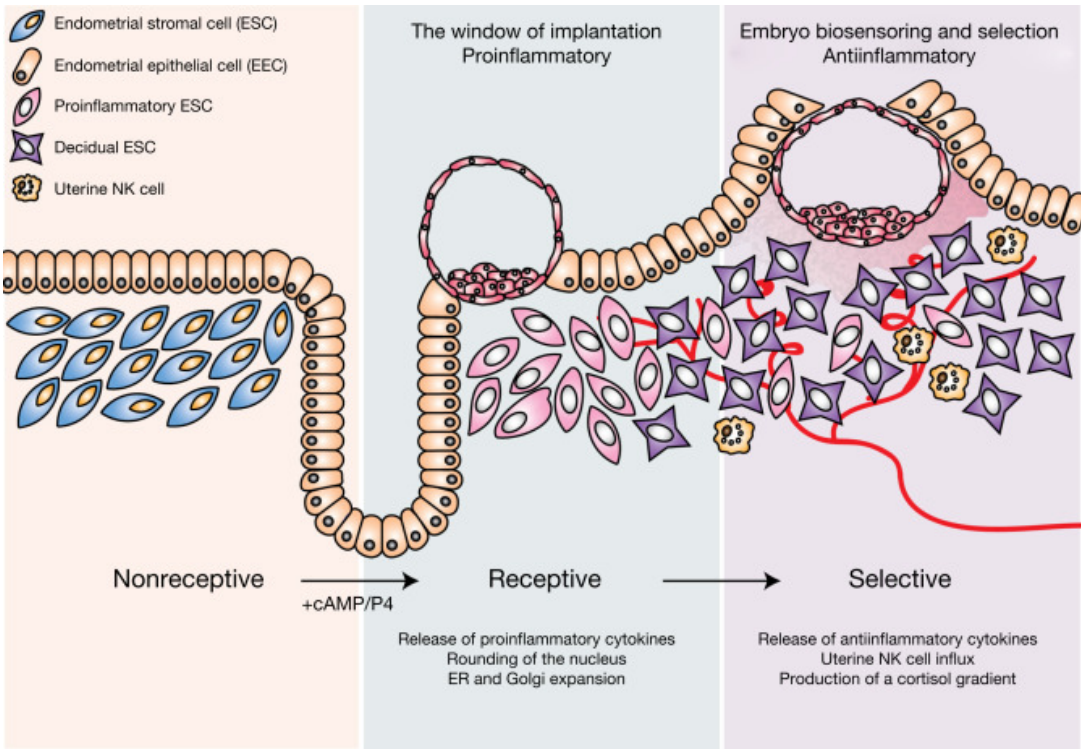


Figure 1.4: Decidualisation relative to the immunological shift at implantation site in the endometrium. This image is from the Encyclopaedia of Reproduction *Second Edition* (2018), specifically from the Female Reproduction and "Decidualisation: A Multistep Process" section by Joanne Muter, Jan J. Brosen. And although this image is primarily illustrating the process of decidualisation as having multiple steps, I have included this image to further emphasise the importance of decidualisation in shifting the endometrium from a proinflammatory to anti-inflammatory state.

1.2.2. Aberrations in Immune Cells in RPL

As discussed above, healthy pregnancies require a tightly regulated immune system, which can be attenuated through different mechanisms. In this section, immune cell populations as a driver of pro- and anti-inflammatory states and how they contribute to maternal-fetal tolerance during pregnancy will be discussed. Firstly, single-cell studies have shown significant differences in different immune cell types and subsets between biopsies from women with and without RPL (Vento-Tormo et al., 2018, Wang et al., 2022, Guo et al., 2021). Earlier, the dysregulation of NK cells has been discussed as playing a pivotal role in RPL, however higher NK cells and activity at the implantation site might not be the only accurate predictive marker of miscarriage (Dosiou & Giudice, 2005, Salazar et al., 2022).

Investigations have long shown that Th1/Th2 ratios are altered in RPL patients, with there being a higher proportion of Th1 cells compared to Th2 cells (Makhseed et al., 2001). Th1 cells primarily release cytokines like interferon gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α)–both considered to be pro-inflammatory, contributing to the immune response. Whereas Th2 cells contribute to dampening the endometrial immune response by producing known anti-inflammatory cytokines, such as interleukin-4 and 10 (IL-4 and IL-10) (Ali et al., 2020). This shift to a dominantly Th2-mediated immunity occurs after implantation to protect the embryo by balancing out the inflammatory Th1 immune response. Another subset of CD4+ T cells that have been found to be elevated in RPL patients is the Inflammatory Th17 cell and could likely contribute to unexplained RPL cases (Wang et al., 2010). Dysregulation of the different subsets of T cells can contribute to an aberrant immune environment, increasing the likelihood of miscarriage.

Previously, macrophages have been established as an integral immune cell that polarises in response to signalling from decidual stromal cells to express the anti-inflammatory M2 phenotype. In some unexplained RPL cases, decidual macrophages tend to polarize toward the M1 phenotype, which promotes pro-inflammatory cytokines resulting in recurrent pregnancy loss (Wang et al., 2022). This pro-inflammatory state in RPL patients is further indicated by a study showing high intermediate monocytes in the RPL subgroup (Comins-Boo et al., 2022). There have been further investigations into the differences in surface and secretory phenotypes between decidual macrophages and monocyte-derived macrophages. Compared to decidual macrophages, it was shown that monocytes and monocyte-derived macrophages did not secrete a detectable level of IL-10, and it is likely that the programming of the monocytes into secreting the same cytokines as decidual macrophages cannot be achieved through activation or differentiation. It is suggested that the endometrial and uteroplacental environment plays a role in programming decidual macrophages to release IL-10 (McIntire et al., 2009). Since IL-10 is an anti-inflammatory cytokine, earlier recruitment of monocytes

to the implantation site could allow for more accurate programming of these monocyte-derived macrophages to promote more of an anti-inflammatory signature in RPL patients.

Finally, in RPL patients, there is even a lack of sufficient maternal endometrial stromal cells– the structural and immunological foundation of the uterus. Within this cell population are the progenitor cells, such as the mesenchymal stem-like cells (MSCs) that drive the constant renewal and proliferation of the tissue lining. RPL is associated with a relative depletion of these cells, resulting in a loss of endometrial plasticity (Lucas et al., 2015, 2016). As a result, decidualisation is compromised and excessive senescence of decidual stromal is observed (Lucas et al., 2015, 2016, Zeng et al., 2023). Given how important functional stromal cells are in many immunological pathways in early pregnancy, RPL face detrimental downstream effect stemming from this deficiency.

These disruptions to the various cell populations and their signalling pathways contribute to an aberrant immune environment, which is further compounded by dysregulated cytokine production and inflammation, as discussed below.

1.2.3. Aberrant Immune Environment

The disruptions in immune cell populations are further proven by studies showing cytokine profiling in RPL. As mentioned in the decidualisation section, endometrium stromal cells must successfully transform to support embryonic implantation and to eventually abate from the initial proinflammatory differentiation phase. For example, IL-33, a proinflammatory cytokine, is expressed in significantly higher levels at the beginning of decidualisation. However, the reverse is seen in RPL cases, showing that the anti-inflammatory pathway that should limit the IL-33 response is dysfunctional during decidualisation (Salker et al., 2012). This prolonged inflammatory response in RPL cells is further intensified through the expression of the inflammatory cytokines listed in the section above, as well as more cytotoxic immune cells. These include IFN-gamma and tumor necrosis factor-alpha, which are seen to be secreted at greater levels in RPL patients. As the Th1 cytokine

profile is associated with pregnancy loss, it is unsurprising that these inflammatory cytokines are more prevalent (Daher at al., 2004).

Decidualisation is important not just for its transformation of the endometrial stroma, but for its triggering of the shift to an anti-inflammatory microenvironment during early pregnancy. This anti-inflammatory profile includes IL-4, IL-6, and IL-10 and are measured to be increased in healthy pregnancies and decreased in RPL patients. Improper regulation of inflammatory mediators through dysregulated immune cells, as well as a potential autoimmune response, can lead to changes in cytokine production.

With these cytokines stemming from immune cells, there are “immunological checkpoints” in the form of regulatory molecules that are necessary to control the immune response during pregnancy (Miko et al., 2019). These tend to be inhibitory, such as cytotoxic T-lymphocyte-associated antigen-4 (CTL-4), which suppresses Th1 cytokine production (Jin et al., 2011); or the lymphocyte-activation gene-3 (LAG), which suppresses Treg and CD4+ activity. Finally, the programmed cell death protein 1 (PD-1), is another inhibitory checkpoint that is expressed on many immune cells (T/B cells, NK cells). More specifically, decidual CD8+, CD4+, and Treg cells have enhanced PD-1 expression (Taglauer et al., 2008) and interact with the PD-L1 and PD-L2 ligands on stromal cells and other immune cells to establish the Th2-favored immune balance at the maternal-fetal interface. As demonstrated, immune checkpoint regulators actively participate in dampening immune activation (Dai et al., 2014, Marozio et al., 2023).

Concerning potential autoimmune responses, it is usually due to the presence of antibodies like antiphospholipid antibodies that can initiate attacks against the developing fetus or placenta. RPL patients who test positive for antinuclear antibodies (ANA), a marker of autoimmunity, are diagnosed as having autoimmuneRPL (Chen et al., 2020).

Overall, a deeper understanding of these immune regulatory mechanisms and their disruption in RPL highlights the delicate balance required for successful pregnancy. With RPL patients more likely to have a pro-inflammatory state and an impaired stress response, there is an opportunity to tailor therapeutic strategies to restore this immune balance at the maternal-fetal interface.

1.4. Treatment of RPL

1.4.1. Current Clinical Recommendations

The management of RPL is uniquely challenging–from early diagnostic investigations to ongoing fertility treatment to potential monitoring of high-risk pregnancies. Given the multifactorial nature of this condition, the treatment of RPL is dependent on the specific cause(s) in each individual patient. With approximately 50% of RPL cases labelled as unexplained, and possibly due to immunological causes, treatment options are even more limited.

Current clinical guidelines from recognised institutions, namely ASRM, RCOG and ESHRE, differ slightly in their recommendations. Firstly, for RPL due to chromosomal factors, ASRM recommends genetic counselling, whereas ESHRE guidelines identify preimplantation genetic testing (PGT) as the main treatment method. For anatomical risk factors, various surgeries to manage anomalies, such as intrauterine adhesions and a septate uterus, are tentative possible treatments within the available clinical evidence (ESHRE Guideline Group on RPL). Unfortunately, for patients with endocrine- and immunological-related factors, the treatment guidelines are less clear (Tomkiewics & Darmochwal-Kolarz, 2023).

RCOG guidelines state that there is not enough evidence to show that treatment of endocrine disorders will result in higher live birth rates. Opposingly, ASRM states that maternal endocrine disorders should be treated, specifically due to the influence of prolactin levels on ovulatory dysfunction. For different disorders, such as hypothyroidism, PCOS, and insulin resistance, there are varying recommendations based on the latest trials and meta-analysis on the current evidence.

Currently, progesterone is routinely administered to support early pregnancy, in addition to low-dose aspirin and heparin to help prevent potential blood clots during implantation. The anticoagulants are especially successful in RPL patients with antiphospholipid syndrome (APS). These treatments have quality clinical trials and substantial evidence to support their safety and

efficacy (Laskin et al., 2009, Hamulyak et al., 2020, Lai et al., 2024). However, clinical trials administering these drugs to participants with unexplained RPL, have been met with variably success rates (Cooomarasamy et al., 2016, 2020). This shows that these drugs are not universally effective for RPL, reflecting limitations in our current understanding of RPL and require further testing of other methods of treatment.

1.4.2. Immunomodulators

Based on current research and our knowledge of RPL, it is theorised that most unexplained RPL cases are most likely of immunological origin. As discussed above, it is known that endometrial immune dysfunction, impaired decidualisation, and overactivated immune responses heightens the risk of miscarriage. Therefore, therapies that can modulate or suppress this inflammatory state– referred to as immunomodulators–during implantation and pregnancy have been proposed as a potential treatment in RPL.

Historically, one of the earliest immunomodulatory treatments developed for RPL has been lymphocyte transfusions, either with paternal lymphocytes or third-party immunizations. In the late twentieth century, clinicians and researchers hypothesised that some RPL cases stem from immune intolerance of the semi allogeneic fetus (Mowbray et al., 1985, Christiansen et al., 1994). After many clinical trials yielding inconsistent outcomes and the associated risk, this treatment has been replaced with more recent advancements (Cavalcante et al., 2021, Wong et al., 2014).

Although neither novel nor new, there are more modern immunomodulatory treatments that are considered targeted approaches and safer for treating fertility. These drugs include intralipid infusions, intravenous immunoglobulin (IVIG), corticosteroids, hydroxychloroquine, granulocyte colony-stimulating factor (G-CSF), and tumour necrosis factor-alpha (TNF-alpha). These drugs have various functions, such as modulating NK cell activity, down regulating inflammatory cytokine production, suppressing autoantibodies, and overall dampening of the immune response (Kumar,

2014, Genest et al., 2022). However, there is limited evidence in the form of large-scale randomised controlled trials to show that these drugs can significantly increase the chances of a successful pregnancy. These immunomodulators will be further discussed in Chapter 2.

1.4.3. Focus of Research Project

This project aims to further our understanding of immunomodulators and to synthesize the published evidence surrounding their effectiveness in treating RPL. The next chapter is focused on a systematic review and meta-analysis of the available randomised control trials (RCTs) investigating an immunomodulatory treatment in the RPL population. Based on the results of the systematic review, the following chapter will then be focused on formulating a hypothesis on how an understudied immunomodulatory drug affects endometrial stromal cells grown in vitro. Overall, with the number of emerging immunotherapies and lack of research to prove their efficacies, this thesis aims to synthesis the presently available research and inform future research.

Chapter 2: Systematic Review of Immunomodulatory Treatments in Recurrent Pregnancy Loss Studies

2.1 | Introduction

2.1.1. Immunomodulators

Immunomodulators are a class of treatments that are used to help with fertility conditions by modulating the immune system. As RPL is often linked to immunological imbalances, these therapies aim to reduce an inflammatory response or rectify immunological abnormalities. Beyond RPL, immunomodulatory treatments have been shown to improve IVF success rates (Melo et al., 2022) and recurrent implantation failure (RIF). However, there is a lack of evidence supporting the effectiveness of these drugs in RPL due to inconsistent findings and limited high-quality trials to prove that these treatments increase the chances of achieving a live birth. Immunomodulators may be offered when clinicians believe the underlying cause of pregnancy loss is related to immune function, as there are no validated diagnostic tests to confirm immune-mediated miscarriages (Genest et al., 2022). Overall, as most immunomodulatory treatments are considered experimental, these drugs are primarily administered to patients within clinical trials. The term “immunomodulator” is used broadly to describe the different types: immunosuppressants, immunostimulants, and others that directly or indirectly influence immune function. In the ongoing systematic review, a comprehensive list of treatment will be included: progesterone, aspirin, heparin, intravenous immunoglobulin (IVIg), paternal leukocyte immunizations, lymphocyte immunotherapy, corticosteroids (prednisolone, glucocorticoids), granulocyte colony-stimulating factor (G-CSF), intralipid, tumor necrosis factor (TNF) blockers, hydroxychloroquine (HCQ), and other treatments that appear in the searches under “immune-modulating.” Evidently, not all these interventions are classically considered immunomodulators, but they are historically used to treat RPL patients—both with unexplained and known causes of miscarriage.

The review in this thesis is specifically focused on treatments with known immunomodulatory effects and are therefore “purely” immunomodulators. The first screen was conducted with the broader aim of including all drugs historically in the treatment of RPL that could be considered as an immune therapy. This thesis will focus on the RCTs evaluating “pure” immunomodulators that passed the full-text screening. By narrowing the scope of treatment, there will be more cohesive integration of the literature with the experimental findings to allow a deeper understanding of the effect of intralipid in RPL patients. In this introduction, I have compiled a summary of for each immune therapy included in this chapter’s systematic review.

2.1.1.1. Intralipid

The emergence of lipid emulsion therapy (LET) as a treatment for RPL originated from its clinical success in parenteral nutrition therapy, treating diseases associated with nutrient deficiencies (Waitzberg et al., 2006, Singh et al., 2020). Due to its anti-inflammatory properties, LET has emerged as a potential treatment of RPL and RIF. The most used lipid emulsion is Intralipid®, a lipid emulsion composed primarily of purified soya bean oil (10% or 20%), along with oocyte yolk phospholipids, glycerine, and water (FDA). Other commercially available lipid emulsions include Structolipid, MCT/LCT and Lipofundin (Canella et al., 2021). The Intralipid dosage and time of dosage(s) varies amongst the clinical trials, ranging from 100 mL to 4 mL, and is usually administered through intravenous infusion before conception.

Although the cellular mechanisms of the Intralipid drug are not clear, researchers argue that the lipid emulsion can restore abnormal NK cell activity (Dakhly et al., 2016). As many studies report increased activity of NK cells in RPL patients, Intralipid is reasoned to help dampen this inflammatory response as an immune-modulating therapy.

According to the Human Fertilisation and Embryology Authority (HFEA), there are currently no ratings to guide the usage of intralipid as an immunological treatment in RPL patients. However, the rating for using intralipids as a fertility treatment, specifically as an add-on treatment to in vitro fertilisation (IVF)/intracytoplasmic sperm injection (ICSI), is grey, meaning that there is "insufficient moderate/high quality evidence" (HFEA). Common minor to severe side effects in patients receiving intralipid treatment can include headache, nausea, and dizziness to signs of infection, bone pain, muscle weakness, and difficulty breathing. Due to the lack of knowledge about the cellular mechanism of intralipid and its conflicting data across clinical trials, intravenous intralipid is not recommended as a routine treatment for RPL patients. According to the ESHRE guidelines, there is "insufficient evidence" to recommend intralipid for improving LBR in women with unexplained RPL (ESHRE, 2022).

2.1.1.2. Hydroxychloroquine

Known as an antimalaria and anti-inflammatory drug, HCQ is an immunosuppressant drug that has extended indications to treat other dysfunctional immune-related conditions, such as rheumatoid arthritis, lupus erythematosus, and other autoimmune conditions (Bansal et al., 2020). In RPL, HCQ is thought to modulate the immune system by altering T-cell activation and inhibiting platelet aggregation (Moreuil et al., 2019). HCQ is an aminoquinoline compound and its chemical structure is similar to chloroquine, but with a hydroxyethyl group, to form $C_{18}H_{26}ClN_3O$. It has a long half-life of 30 to 60 days and can be detected in plasma and tissue even after a patient has stopped treatment. HCQ is typically orally ingested across two dosages, starting before conception and stopping at 10 weeks of gestation (National Institute of Health).

Currently, there are no published clinical data supporting that HCQ can increase live birth rates in patients with unexplained RPL. As opposed to HFEA's guidance to intralipid usage in IVF/ICSI,

HCQ is not included in the ratings, however it may help improve outcomes in IVF-ET and frozen embryo transfer (FET). With HCQ's direct and indirect immunomodulatory effects, it may be able to effectively modulate mechanisms underlying unexplained RPL.

2.1.1.3. Intravenous Immunoglobulin (IVIG)

Intravenous and subcutaneous immunoglobulin, also known as IVIG and SCIG, depending on its mode of administration and intended rate of absorption (Epland et al., 2022), is a concentrated blood product made from pooled antibodies (immunoglobulins) from the plasma from thousands of healthy blood donors (American College of Rheumatology). IVIG is primarily made of immunoglobulin G (IgG) and is known to have an anti-inflammatory effect through the interactions between its Fc receptors and immune cells, leading to tolerogenic dendritic cells and T regulatory cell expansion (Hsieh et al., 2022). IVIg was first used as a treatment for immunodeficiency disorders (Kaveri et al., 2011) and its success led to its application in other autoimmune diseases and eventually as a fertility treatment. To better understand IVIg's immune-modulating effects in RPL, researchers have studied its effect on immune cell populations. As RPL patients tend to have Th1/Th2 ratio abnormalities, it has been shown that IVIg can decrease Th1 cells and subsequently shift toward Th2 cells in RPL patients. (Graphou et al., 2003). Furthermore, IVIg seems to be beneficial to RPL patients through its ability to enhance the suppressive activity of Treg cells (Kessel et al., 2007). Among immune therapies, IVIg is one of the most extensively investigated treatments for RPL, primarily in the context of clinical trials.

Common IVIG treatments include a dosage of 0.2-0.5 g/kg per infusion, which can range in frequency depending on the trial or individual pregnancy. Treatment regimens can be initiated before conception or immediately following a positive pregnancy test and continued through at least the first trimester. Although there are a range of IVIg brands, such as Privigen and Gamunex-C, IVIG is considered the most expensive immune therapy of those commonly used in RPL (NIH). Due to this

high cost and known functionality, IVIg is typically limited to treating RPL patients where immune-mediated causes of miscarriages are established (Saab et al., 2021). Patients treated with IVIG have experienced side effects including: headache, fever, chills, fatigue, and muscle aches (NIH).

Systematic reviews analysing the efficacy of immunoglobulins in the treatment of subfertility, including RPL and recurrent implantation failure (RIF), have been regularly published since 2006 reporting varying results and effect sizes (Clark et al., 2006; Wong et al., 2014; Saab et al., 2021). According to the HFEA, the use of IVIg as part of fertility treatment is rated red for improving the chances of having a baby and for reducing the chances of miscarriages for most patients with or without immunological testing. Amongst the clinical trials on IVIg, RPL patients are sometimes screened for elevated NK cells or abnormal Th1/2 ratios or other markers of immune dysregulation. This systematic review aims to review the contradictory results of the landmark IVIg RCTs and more recent clinical trials on its effectiveness in treating RPL.

2.1.1.4. Granulocyte Colony Stimulating Factor (G-CSF)

Granulocyte colony stimulating factor, abbreviated as G-CSF, is a cytokine that is synthesised by human cells and is known to have great importance in implantation and pregnancy (Ding et al., 2022). It is most often prescribed after chemotherapy to increase white blood cell levels to protect against risk of infection (Cleveland Clinic). Researchers have shown that G-CSF treatment appears to be associated with an increase in Treg cells and dendritic cells and seemingly affects the expression of genes involved in the implantation process and local endometrial immune modulation (Cavalcante et al., 2015). G-CSF is usually administered by subcutaneous injection or by intravenous drip and given before conception to increase the likelihood of successful implantation and a positive pregnancy (He et al., 2024).

Although the HFEA does not have specific guidelines for the use of G-CSF, ESHRE and RCOG do not recommend its routine use in the treatment of RPL due to insufficient evidence (Bender Atik et al., 2018). It is important to note that the National Institute for Health and Care Excellence (NICE) does not specifically mention G-CSF in treating fertility patients, and therefore its usage should be considered within the context of current evidence and clinical judgment. Although there are two retrospective studies that report G-CSF as having a significant positive on the success rate in patients with RPL, there is a noticeable lack of large-scale high-quality RCTs to support this conclusion (Santjohanser et al., 2013, Wurfel, 2015). As a known immunomodulators, G-CSF is still an emerging immune therapy for RPL patients, whether primarily for unexplained, immune-mediated RPL or universally across this population group.

2.1.1.5. Steroids (Glucocorticoids)

Steroids, specifically corticosteroids and glucocorticoids, are drugs similar to naturally occurring steroid hormones. Generally, steroids are known as an immunosuppressant and involved in anti-inflammatory activities. In the context of RPL, steroids can suppress uNK activity and increase chances of implantation, especially in conjunction with other drugs, such as aspirin (Bequet et al., 2023). One of the most administered steroids, especially in the treatment of RPL, is prednisone. It is usually given orally in small doses at around 8 to 10 mg/kg/day with a maximum of approximately 60 mg/day, beginning immediately after the confirmation of a pregnancy until birth or miscarriage (Li et al., 2022).

Amongst the published reviews examining the efficacy of steroids in recurrent pregnancy loss, the consensus is that the intervention improves live birth rates (Li et al., 2022, Ma et al. 2022). However, according to the HFEA, the use of steroids is rated red in most fertility patients, both for improving the chances of having a baby and reducing the chances of miscarriages, including patients

undergoing immunological testing. This red rating indicates that there is sufficient evidence for this authority to recommend against clinical use of steroids due to safety hazards (HFEA). Some of the side effects observed in patients taking steroids are increased risk of infection, high blood sugar, mood swings, and even pregnancy complications, such as preeclampsia or gestational diabetes (National Institute of Health).

2.2 | Chapter Objectives

This systematic review included in this thesis is part of a larger ongoing systematic review on all immunomodulatory drugs used in the treatment of RPL. The protocol and methods used in this chapter are also going to be used in the broader review looking at safety and effectiveness across a range of drugs that is registered on PROSPERO. Due to limitation on time, this thesis chapter will focus on examining the literature on "pure immunomodulators" in the treatment of RPL in answering the question of, "Are [is] immunological-modulating therapies [intralipid] safe and effective in the women with recurrent pregnancy loss (RPL) undergoing assisted reproductive technology?" The emphasis on "pure immunomodulators" is to be intentional in the scope of the search and focus on therapies that are not routinely administered and well established as a fertility treatment, such as progesterone, aspiring, and heparin. Currently, there are systematic reviews looking at specific treatments and narrative reviews appraising the available immune therapies in RPL. However, there is no comprehensive systematic review and meta-analysis involving all immunomodulators in the treatment of RPL. To condense the substantial number of relevant studies, yield over 10,000 searches, two screens will be conducted to filter out the articles that do not meet the criteria of this review. After screening, the data with be extracted from each full-text and analysed using the Cochrane Review Manager tool. This chapter will be an excerpt of the broader systematic review to capture the current evidence on the usage of intralipid in RPL.

Chapter Objectives:

1. Design a systematic review protocol examining the effectiveness and safety of using immunomodulators in RPL and get approved by PROSPERO.
2. In collaboration, design a search strategy to perform a broad and specific search design to include all relevant articles.

3. Screen searches through a double blind, two-step screening process to narrow down to the included searches of this systematic review.
4. Extract data from included full-text and analyse for specified outcomes and across different subgroups of population.
5. Assess included studies for risk of bias, trustworthiness, and certainty using established frameworks and tools.
6. Extract data from studies and perform a meta-analysis of the published evidence.
7. Formulate a plan and timeline for the ongoing systematic review focusing on all immunomodulators in Recurrent Pregnancy Loss.

2.3. Methods

In this section, I will be documenting how I identified, selected, evaluated, and analysed the literature for this review. The protocol was registered on PROSPERO with a registration number of CRD42024512317 in February 2024. The search was designed and conducted in collaboration with Librarian Carolyn Smith from the Oxford Bodleian Library. This review was conducted according to the PRISMA guidelines for systematic reviews and the PRISMA checklist was completed.

2.3.1. Search Design & Strategy

The databases that were searched using the search terms and keywords below are Medline (Ovid), Embase, Cochrane Central Register of Controlled Trials, SCOPUS, and Web of Science Core collection. The table below displays how the exact MeSH search terms, Boolean operators, and truncations that were coded into the search platform to refine the results. Table 2 shows the search terms used for the Cochrane Trials database and the search design for the other databases are included in the Appendix. All databases were searched separately, with similar but adapted search strategies.

	Search Terms	No. Of Searches
1	(immunomod* OR immunoglob* OR Immunologi* OR glucocorti* OR steroid* OR prednisolone OR prednisone OR intralipid OR granulocyte OR lymphocyte)	142510
2	[mh "Immunologic Factors"] OR [mh Immunoglobulins] OR [mh Granulocytes] OR [mh Immunomodulation] OR [mh hydroxychloroquine] OR [mh Lymphocytes] OR [mh Steroids]	142766
3	#1 OR #2	227531
4	((recur* OR habitual OR refractory) NEAR/3 (miscarr* OR pregn* OR abort* OR gestat* OR obstet*))	1331
5	[mh "Abortion, Habitual"]	423
6	#4 or #5	1429
7	#3 and #6	616

Table 2: Search Design for Cochrane Trials database. These are the search terms used for the Cochrane trials database as well as the number of results for each search level. This is a preliminary search strategy that the following searches for other interventions and databases were built from (Appendix).

This review's inclusion and exclusion criteria were determined before the final search was run. There was also a scoping search conducted to ensure that the search design was specific, yet broad enough to include all possible relevant searches. The results of the preliminary search were cross-checked with the landmark high-quality studies listed in Table 1 investigating immune therapies that meet the criteria of this systematic review. All studies published from inception until the data of the search will be included. There will also be no restrictions on language. However, the only publications included in this search are articles published in scholarly journals. Before the final analyses, searches will be re-run, and any further relevant studies will be retrieved and included in the analysis. Additional relevant studies in the reference lists of relevant publications, such as literature reviews and systematic reviews, will also be included in the search results.

Eligibility Criteria: PICOS

This review aims to assess how safe and effective immune therapies are in the treatment of women with recurrent pregnancy loss. To ensure that the review includes articles that are specific and best to address our research question, the PICOS search tool provides the protocol framework. Endorsed by the Cochrane Collaboration, this tool—primarily used for quantitative systematic reviews—focuses on the Population, Intervention, Comparison, Outcomes, and Sites (Cochrane Tools). It is commonly used to outline and identify components of studies to provide clinical evidence for systematic reviews (Cochrane Collaboration). This review follows the completed PICOS framework below:

Population	Women with a history of RPL
Intervention	Immunological therapies**
Comparators/Control	Other immune therapies, no intervention/placebo
Outcomes	*Primary:* Live birth rate or ongoing pregnancy rate, miscarriage rate; *Secondary:* Adverse events from drugs, embryo number (IVF only), implantation rate (IVF only), clinical pregnancy rate
Sites	Hospitals, RPL clinics, fertility clinics

Table 3: Completed PICOS Framework. To properly answer the research question, the PICOS technique is used to outline every step review from defining the population to data extraction and synthesis. This table was made in Microsoft PowerPoint.

This completed PICOS outlines the inclusion criteria that the articles will be screened for. The other criteria are the type of studies to be included: only randomised control trials (RCTs) in this thesis and additionally cohort studies in the ongoing review. The following types of studies will be excluded: non-peer reviewed studies, conference abstracts, case reports, observational studies, systematic reviews, narrative reviews, quasi-randomised and crossover trials. Other articles, such as animal studies, will also be excluded.

To properly answer our research question on the safety and effectiveness of each immunomodulator, the primary outcome for "safety" is miscarriage rate and for "effectiveness" is live birth rate (LBR). With the primary goal of these therapies being to treat RPL, the effectiveness of each immunomodulator is dependent on LBR and ongoing pregnancy rate as a secondary outcome, if included. Miscarriage rate was selected as the primary safety outcome in this review because it represents a severe and clinically consequential adverse event associated with fertility treatments and would significantly negate the support evidence for a drug. While adverse outcomes such as nausea, fatigue, or immune-related side effect are important, they are inconsistently reported and often not the focus of RCTs. In contrast, miscarriage is well-documented and allows for quantitative synthesis of the clinically relevant and available data. Studies with incomplete or missing data will be excluded from the included texts. The PICOS framework, along with the other points of eligibility criteria, will guide what searches with get screened out and what texts will be included in the final

analysis. How PICOS is specifically and actively used throughout the screening process will be further explored in this Methods section. This search will focus on the following interventions: intravenous immunoglobulin (IVIg), paternal leukocyte immunizations, lymphocyte immunotherapy, corticosteroids (prednisolone, glucocorticoids), granulocyte colony-stimulating factor (G-CSF), intralipid, tumor necrosis factor (TNF) blockers, and hydroxychloroquine (HCQ).

2.3.2. Study Selection Process

The systematic review software, Rayyan, was used to facilitate screening and to remove all duplicate searches. Once all the searches from all the databases were compiled into Rayyan, which flagged possible duplicates, they were manually reviewed and identified. To easily identify any remaining duplicates not automatically flagged, entries were sorted by title and removed if in duplicate. The number of duplicates is documented in the PRISMA flow diagram.

Preliminary Screening

After the "pilot" screening phase, I refined the inclusion criteria to optimize the screening accuracy of this review. This systematic review focuses on women experiencing RPL; however, definitions of RPL vary across guidelines, such as those from ESHRE, ASRM, and RCOG. To ensure inclusivity of all high-quality studies regardless of regional or institutional definitions, I adhered to the specified definition of RPL provided by each study. Additionally, as terminology for RPL can differ, I accounted for variations in nomenclature. RPL is often referred to as "Recurrent pregnancy loss (RM)," "Recurrent Spontaneous Abortion (RSA)," "Habitual Abortion (HA)," and may include other combinations of terms such as "recurrent," "abortion," "loss," "miscarriage," and "spontaneous." Articles using any of these terms were included, provided they met all other inclusion criteria. This approach allowed for this analysis to overcome diverse terminologies and definitions. Studies that specifically include patients with RPL due to antiphospholipid (APS), thrombophilia, or primary/secondary RPL, will meet

the “Population” criteria of this review. These different subgroups will be further investigated in the analysis.

Furthermore, studies specifying only APS patients as their population of interest were highlighted and selected for because the obstetrical outcomes of APS are directly related to RPL. For other conditions, such as systemic lupus erythematosus (SLE), which is correlative with RPL (Valeff et al., 2023), is not considered to be the same obstetrically or pathologically and therefore excluded. For other conditions, decisions were made on a case-by-case basis.

First Screen: Titles & Abstracts

The first screen is a blinded, double screening of all the searches based on their titles and abstracts. For searches without abstracts, the decision to include or exclude was based solely on the title. If the search met the inclusion criteria stated above, the search was included to proceed to the second screen. For searches, where the title and abstract provided limited information, the primary screening criteria involved the type of study (RCT), the population, the intervention (intralipid), and if there was any mention of the specific outcome(s). The “outcome” criteria was loosely followed during this first screen on the basis that the authors might not include certain outcomes and results in the abstract that is included in the full text. On the Rayyan software, there are three possible labels the screener can assign to an article: Include, Exclude, or Maybe. For articles designated “Maybe,” the final decision was made after all the articles were sorted into the three different categories.

For the ongoing systematic review, I am the first screener and working with three second screeners to ensure every title and abstract is double screened. Any disagreements about whether an article should be included will be discussed and resolved.

Second Screen: Full-Text

Following the initial title and abstract screening, articles that were selected as likely to meet the inclusion criteria underwent a second, blinded, double-screening of the full-texts to decide which studies should be included in this review. PDF files of all articles categorized as “Include” were uploaded into Rayyan for evaluation. Each text was carefully assessed to confirm adherence to the established inclusion criteria.

2.3.3. Quality Assessment

I assessed all the RCTs for trustworthiness using the Trustworthiness in RAndomised Controlled Trials (TRACT) checklist. This checklist is made up of 19 items organized into the 7 categories that are applicable to every RCT: (1) governance, (2) author group, (3) plausibility of intervention usage, (4) timeframe, (5) drop-out rates, (6) baseline characteristics, and (7) outcomes. Each item was rated either having ‘No concerns,’ ‘Some concerns’/’Not applicable,’ or ‘Major concerns.’ Based on the totality of these ratings, I decided if the study required further investigation or if the study should be excluded entirely (Mol et al., 2023). The TRACT checklist is the first tool to be developed specifically to detect trustworthiness issues in RCTs and is used in this review to ensure all the identified studies in this review are reliable and of standard to evaluate the effects of intralipid in RPL.

The included RCTs will also be assessed for quality and risk of bias using the risk of bias (ROB-2) tool from Cochrane. The assessment process for each study is outlined and documented on the risk bias assessment tables from the Cochrane RevMan software of the Cochrane Center. Each outcome of a study is assessed for its risk of bias across five standard domains plus overall risk of bias: (1) bias arising from the randomization process, (2) bias due to deviations from intended interventions, (3) bias due to missing outcome data, (4) bias in measurement of the outcome, (5) bias in selection of the reported result, and "Overall risk of bias” (Cochrane Methods). To determine the level of risk, multiple signalling questions for each domain were designated one of the five options:

"Yes," "Probably yes," "Probably no," "No," or "No information." Based on all these answers and the supporting evidence, three possible judgement options were designated to each of the five domains (Low risk, Unclear, High risk). I utilized Excel tool provided by Cochrane Methods to implement this RoB 2 process and streamline the assessment of each outcome of the included studies. The guidelines and signalling questions used are listed in the Appendix section. Additionally, the certainty of all the RCTs were assessed using the Grading of Recommendations Assessment, Development and Evaluation (GRADE) framework from Cochrane. Since RCTs are all considered high-quality evidence, each trial will start with a high rating and five factors will decide if that rating will be downgraded; the five factors are risk of bias, inconsistency, imprecision, indirectness, or publication bias. An upgrade in quality can also be reflected based on three factors: large effect size, dose-response gradient, or effect of plausible residual confounding. Each outcome in a study receives an individual rating (Cochrane Training). This is a systematic approach to rating the certainty of evidence in systematic reviews.

For the ongoing review, two reviewers will respectively evaluate all RCTs for trustworthiness using the TRACT checklist, the risk of bias using the ROB-2 tool from Cochrane, and quality with the GRADE framework. All these assessments are important measures to include high-quality and reliable studies and data in this review.

2.3.4. Data Extraction

To synthesise the data from the included studies, I extracted the following information from the included study: first author, year of publication, country of origin, intervention regimen specifics, study design, inclusion criteria and number of participants, type of conception, and data concerning the primary outcomes of miscarriage rate and live birth rate. For the ongoing review, data from the studies that have the following the secondary outcomes: adverse events from drugs, clinical

pregnancy, embryo number (IVF only), implantation rate (IVF only) and clinical pregnancy rate, will also be extracted.

2.3.5. Data Analysis & Synthesis

Once the included studies were established, the PICOS characteristics of every study were first summarised. For the quantitative data, the analysis was conducted using the Cochrane RevMan software. The effect size will be demonstrated using mean difference/standardized mean difference with a 95% confidence interval (CI). For the qualitative or categorical variables, effect size was reported as risk ratio with 95% confidence internals. There was a primary analysis of all the RCTs for trustworthiness using the TRACT tool, followed by a sensitivity analysis. The main results of the primary and secondary outcomes, when applicable, were synthesized in a Summary of Findings Table. For the meta-analysis, forest plots were created to represent the extracted statistical data graphically. The heterogeneity of the meta-analysis was calculated with the chi-squared method and an the $I^2 < 50\%$ was considered as representative of low heterogeneity. The random-effect model was used in cases of significant heterogeneity, $p > 0.05$, $I^2 > 50\%$. The results are represented in forest plots that are graphical representations of significance.

2.4. Results

2.4.1. Study Selection

The database retrieval found 16,541 relevant records, including 5108 from Medline, 3410 from Embase, 558 from Cochrane, 3710 from Scopus, and 3755 from Web of Science. Before screening, 6037 duplicate records between all the databases were removed. After the first screen based on titles and abstracts, 798 studies underwent the full-text screen with 8776 excluded. In this second screen, the full texts being assessed for eligibility were assigned to the following specific immune-modulating interventions: intralipid, hydroxychloroquine, IVIG, G-CSF, and steroids, for a total of 240 reports on these drugs with known immune-modulatory roles. The remaining 558 searches were reports about the following RPL interventions: progesterone, heparin, aspiring, vitamins, and other drugs labelled as "potential immunomodulators." As shown in Figure 2.1, out of the 21 intralipid-, 17 HCQ-, 26 G-CSF-, 151 IVIG-, and 25 steroid-focused articles that were included in the 2nd screen, 1, 2, 3, 16, and 4 articles were selected for detailed assessment—respectively. I was aiming to also include other known immunomodulators, such as TNF-alpha, however there were no searches that made it past the 1st screen on this intervention.

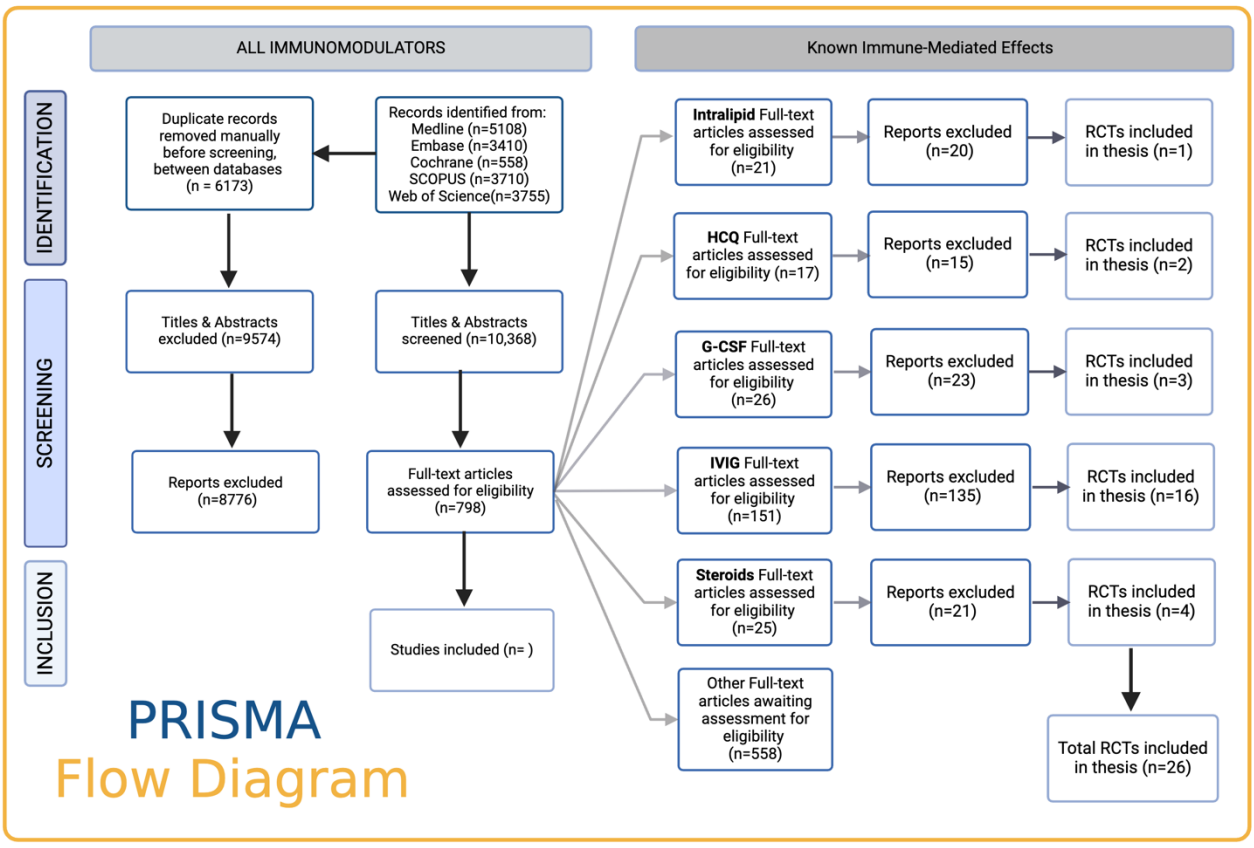


Figure 2.1: PRISMA Flow Diagram. PRISMA stands for Preferred Reporting Items for Systematic Reviews and Meta-Analyses. This flow diagram is a visual representation of the process used in selecting the studies for this review. The figure is a slow diagram that includes two branches of the process, the broader review process and the immunomodulator-specific studies used in this thesis. Each box represents the results of each step from compiling searches to screening to the number of studies included. Some of the boxes in the second "all Immunomodulators" column are not completed due to ongoing work. N = number of searches/articles.

Of the excluded studies, Table 5 further details the reasons for exclusion for each intervention. All interventions, except for steroids, had duplicates upon further investigation, such as one search being a trial's prospective registration and then the actual published study. All interventions except for intralipids, also had ongoing trials that have yet to be concluded or published; these were primarily published study protocols. Some search results are awaiting classification due to inaccessible full-text or waiting on author responses. The only intervention to have retracted or withdrawn studies is intralipid. Mostly commonly, articles that did not meet the selection criteria were due to the following reasons: (1) retrospective studies, (2) not randomised, (3) did not include the correct outcomes, or (4)

conference abstracts with no corresponding paper. The total excluded articles in Table 4 match up with the "reports excluded" in Figure 2.1.

	Manual Duplicates	Ongoing	Awaiting Classification	Withdrawn/ Retracted	Excluded	Total
Intralipid	5	0	0	3	12	**20**
HCQ	1	5	0	0	9	**15**
G-CSF	7	4	0	0	12	**23**
Steroids	0	1	1	0	19	**21**
IVIG	27	2	7	0	99	**135**

Table 4: Breakdown of the studies not included in meta-analysis. This table further details on why the excluded reports for each intervention did not pass the full-text screening. This included "Manual Duplicates" that were not just identically formatted searches detected by Rayyan reference manager, "Ongoing" studies that have yet to be concluded or published, studies "Awaiting Classification" of inclusion or exclusion, "Withdrawn/Retracted" studies that retracted by the original authors, and the remaining "Excluded" articles. The articles under the "Excluded" column refers to all the full texts that do not meet the inclusion criteria of this systematic review.

Overall, 214 of the 240 full text articles were screened out as according to Figure 2.1 and Table 5 across all five interventions and 26 randomised controlled trials that met this review's inclusion criteria were selected to be further investigated for trustworthiness for inclusion in the overall meta-analyses.

2.4.1.1. Summary of the Included Studies for Intralipid

The singular intralipid-focused study has a total participant sample size of 192 and was published in 2015. Meng and their team conducted prospective, randomised clinical trial from December 2010 to December 2012 and recruited participants with unexplained RPL.

Authorship	Drug Regimen	Inclusion Criteria	Sample size (enrolment)	Mean age (years)	Types of miscarriage (stratification)
Meng et al, 2015	250 mL of 20% Intralipid on Day 3 of menstrual cycle; injection time no less than 2 h. 25g of IVIG on Day 8, 9, or 10 of menstrual cycle; injection time no less than 8 h.	Patients with 3 or more miscarriages before 12th week of gestation	192	31.6 in intralipid group; 31.2 in IVIG group	Unexplained RPL

Table 5: Summary table of randomised controlled trials pertaining to treatment of RPL with intralipid. This table summarises the characteristics of the singular RCT included in this systematic review that evaluated intralipid therapy for RPL. The columns detail the study's first authorship, drug regimen (including dosage, timing, and method of administration), inclusion criteria for participants, sample size of enrolment, mean age in both the intervention and control groups, and any potential stratification of participants based on the types of miscarriage and RPL detailed in this study. GW = gestational week.

In this study, the participants must have CD56+CD16+ levels greater than 20% to enrol and be randomly allocated into the intralipid (treatment) and IVIG (comparator) groups. The study concluded that the live birth rates between the two groups were not significantly different, 92.1 vs. 88.2 ($p = 0.415$) for the intralipid and IVIG groups, respectively. The miscarriage rate in Table 12 only included the early pregnancy losses and not the losses after the 20th week of gestation. The mean age of both groups is not statistically significant, according to the article (Meng et al., 2015) Both groups had 30 and 18 patients not follow-up after the 12th gestational week and therefore the outcomes of these patients were not included. This singular RCT will be further assessed for trustworthiness.

2.4.1.2. Summary of the Included Studies for HCQ

For the HCQ meta-analysis, 2 RCTs with 149 patients were chosen for further assessment. Within these two studies, both the treatment and control group did not differ significantly by the included baseline characteristics—as seen with the mean age in Table 7. The studies included participants with either autoimmune-related or unexplained RPL. The timing of the HCQ regimen is different between the two studies, with one given before conception and during pregnancy (Elsenity et al., 2022) and the other only after conception (Moini et al., 2022). Next, these 2 studies will be investigated for trustworthiness. One study reported that the HCQ group was significantly more effective at increasing the LBR (Elsenity et al., 2022). The other study only reported miscarriage rates and observed no significant difference between the two groups. These two conflicted studies on the efficacy and safety of using HCQ will next be assessed for trustworthiness.

Authors	Drug Regimen	Inclusion Criteria	Sample size (enrolment)	Mean age (years)	Types of miscarriage (stratification)
Elsenity et al., 2022	During preconception & gestation, group received HCQ, as well as LDA and LMWH. Placebo given to control group.	History of autoimmune RPL	120	*Not available*	Autoimmune-related RPL
Moini et al., 2022	Participants received 200 mg HCQ twice a day from conception until GW 20 or abortion. Placebo given to control group.	History of ≥ 2 unexplained spontaneous abortions	29	31.57 in HCQ group; 30.13 in placebo group (p = 0.325)	Unexplained RPL

Table 6: Summary table of randomised controlled trials pertaining to treatment of RPL with HCQ. This table summarises the characteristics of the 2 RCTs included in this systematic review that evaluated hydroxychloroquine therapy for RPL. The columns detail the study's first authorship, drug regimen (including dosage, timing, and method of administration), inclusion criteria for participants, sample size of enrolment, mean age in both the intervention and control groups, and any potential stratification of participants based on the types of miscarriage and RPL detailed in the study. This information allows for a clear comparison of the study population, breath of protocol for the intervention, and the scale of the studies overall. GW = gestational week.

2.4.1.3. Summary of the Included Studies for G-CSF

For the G-CSF branch of this review's meta-analysis, 3 RCTS with 286 patients were chosen for further assessment. Across the baseline characteristics available in two of the studies, there was no significant difference between the G-CSF-treatment and control group (Zafardoust et al., 2015, Eapen et al., 2018), with mean age shown in Table 8. All three studies only included RPL patients with unexplained aetiology, with two only enrolling primary RPL (Scarpellini & Sbracia, 2009, Eapen et al., 2018). Most notably, the method of administration is distinct across all three RCTs. One study reported the G-CSF could be effective in treating unexplained RPL, the other two studies reported no significant differences between the two groups (Zafardoust et al., 2015, Eapen et al, 2018).

Authors	Drug Regimen	Inclusion Criteria	Sample size (enrolment)	Mean age (years)	Types of miscarriage (stratification)
Zafardoust et al., 2015	300 µg of G-CSF (Filgrastim), transvaginally an insemination catheter on 12-13 day of menstrual cycle	Under 40 YO; ≥2 sequential unexplained abortions	68	30.2 in G-CSF group; 31.6 in control group (p = 0.082)	Unexplained RPL
Scarpellini & Sbracia, 2009	G-CSF (1 µg/kg/day) starting on 6th day after ovulation	Unexplained primary RM (≥4 consecutive miscarriages)	68	*Not available*	Primary RPL
Eapen et al., 2018	subcutaneous injections of recombinant human G-CSF (130 µg) & placebo that appears identical	Unexplained primary recurrent miscarriage	150	32 in G-CSF group; 31 in placebo group	Primary RPL

Table 7: Summary table of randomised controlled trials pertaining to treatment of RPL with G-CSF. This table summarises the characteristics of the 3 RCTs included in this systematic review that evaluated G-CSF therapy for RPL. The columns detail the study's first authorship, drug regimen (including dosage, timing, and method of administration), inclusion criteria for participants, sample size of enrolment, mean age in both the intervention and control groups, and any potential stratification of participants based on the types of miscarriage and RPL detailed in the study. This information allows for a clear comparison of the study population, breath of protocol for the intervention, and the scale of the studies overall. GW = gestational week.

2.4.1.4. Summary of the Included Studies for IVIG

In this review, the intervention with the most RCTs chosen for further assessment is IVIG with 15 and a total of 866 patients. Overall, there are 7 studies that cannot recommend IVIG due to no reported treatment benefit (Stephenson et al., 2010, Triolo et al., 2003, Jablonowska et al., 1999, Christiansen et al., 2015, German/IVIG, Perino et al., 1997, Stephenson et al., 1998). Contradictorily, 5 trials show promising IVIG treatment benefit (Christiansen et al., 1995, Christiansen et al., 2002, Yamada et al., 2022, Coulam et al., 1995), with one citing reporting a 55% therapeutic gain (Christiansen et al., 1992). For 3 studies, the comparator or control group was treated with the routine heparin and aspirin without IVIG, and they report a higher or comparable efficacy to the IVIG treated group (Dendrinos et al., 2009, Nazari et al., 2015, Triolo et al., 2003). The Mahmoud et al. study published in 2004 did not have the scale size to recommend or report a significant difference. For the

studies reporting benefit, all studies included only RPL patients that had an unexplained aetiology. These conflicting results across these 15 studies will be further investigated after the data extraction process.

Authors	Drug Regimen	Inclusion Criteria	Sample size (enrolment)	Mean age (years)	Types of miscarriage (stratification)
Stephenson et al., 1998	5% IVIG (Gamimune N) in normal saline at 500 mg/kg starting in follocular phase for max of 6 cycles	≥2 consecutive spontaneous pregnancy losses under 20 GW	62	35.2 in IVIG group; 34.6 in control group	Primary, secondary, and unexplained RPL
Stephenson et al., 2010	IVIG (Gamimune or Gamunex) at a dose of 500 mg/kg starting preconception & every 4 weeks until GW 18-20	Idiopathic secondary RPL (at least one prior ongoing pregnancy followed by ≥3 consecutive miscarriages)	82	36 in IVIG group; 35 in control group	Secondary RPL
Christiansen et al., 1995	25-40g of IVIG (Nordimmun)/placebo in GW 5-7 (weekly, 8-34 (biweekly)	Secondary recurrent spontaneous abortion (≥3 consecutive pregnancy loss before 28th GW)	34	31.0 in IVIG group; 32.0 in control group	Secondary RPL
Christiansen et al., 2002	1st infusion of IVIG (Nordimmun) after randomisation; subsequent infusions until GW 20; 0.8-1 g/kg/infusion	History of ≥4 unexplained miscarriages (before end of GW26)	56	31 in IVIG group & control group	Primary, secondary RPL
Christiansen et al., 2014	8 total infusions until GW14-15; 25-35g of IVIG (Privigen)	History of ≥4 unexplained miscarriages (before end of GW14) & ≥1 birth after GW28	82	34.5 in IVIG group; 34.2 in control group	Secondary RPL
German RSA/IVIG Group, 1994	IVIG (verum) or 5% human albumin (placebo) infusions	History of unexplained primary RPL	64	*Not available*	Primary RPL
Coulam et al., 1995	IVIg infusions of 500 mg/kg/month for 4 months; after conception, infusion every 28 days until delivery or GW28-32	≥2 consecutive unexplained spontaneous abortions	95	35 in IVIG group & control group	Primary, secondary RPL

Perino et al., 1997	2 consecutive, initial doses of 25 g/day Ig; 3rd dose of 25 g administered 3 weeks after confirmed pregnancy	≥3 recurrent pregnancy losses in 1st trimester & no prior live births; all conceived spontaneously	48	30 in IVIG group; 29.3 in control group	Primary RPL
Jablonowska et al., 1999	IVIG (20 g Gammonativ®, 400 ml) or placebo (saline, 400 ml) every 3 weeks X5 if viable pregnancy	Recurrent spontaneous abortion (RSA) (≥3 consecutive fetal losses before GW20)	41	30.0 in IVIG group; 32.0 in control group	Primary, secondary RPL
Triolo et al., 2003	400 mg/kg/day dosage of IVIG (IgVENA) for 2 days, then every month until GW31 or miscarriages	≥3 recurrent abortions before GW10 & ≥2 positive results for anticardiolipin	42	32.1 IVIG group; 30.3 in control group	APS associated RPL
Mahmoud et al., 2004	500 mg IVIg/kg/month or multivitamin (placebo)	History 3 previous RPL, positive antiphospholipid syndrome (APS)	15	*Not available*	Elevated NK cell levels
Dendrinos et al., 2009	start IVIG after positive pregnancy test; 400 mg/kg every 28 days until GW32	≥3 consecutive spontaneous abortions before GW10 with APS	78	32 in IVIG group; 31.1 in control group	APS associated RPL
Nazari et al., 2015	200 mg/kg monthly up to 24 weeks of gestation with 40 mg enoxaparinn/80 mg aspirin daily	≥3 consecutive spontaneous abortions before GW24 with APS	68	29.7 in IVIG group; 31.2 in control group (p = 0.72)	Unexplained RPL
Yamada et al., 2022	400 mg/kg administered by intravenous drip infusion for 5 days	Primary RPL with unexplained aetiology (≥4 consecutive pregnancy losses)	99	35.2 in IVIG group; 35.0 in control group	Primary RPL

***GW = gestation week**

Table 8: Summary table of randomised controlled trials pertaining to treatment of RPL with IVIG. Using the language directly as written by the study, this table summarises the characteristics of the 15 RCTs included in this systematic review that evaluated IVIG therapy for RPL. The columns detail the study's first authorship, drug regimen (including dosage, timing, and method of administration), inclusion criteria for participants, sample size of enrolment, mean age in both the intervention and control groups, and any potential stratification of participants based on the types of miscarriage and RPL detailed in the study. This information allows for a clear comparison of the study population, breath of protocol for the intervention, and the scale of the studies overall.

2.4.1.5. Summary of the Included Studies for Steroids

For the steroids branch of this review's meta-analysis, 4 RCTS with 610 patients were chosen for further assessment. As seen in Table 10, the steroids used in all 4 RCTs is either prednisone or prednisolone, and they were all taken orally. There is no significant difference in any of the given baseline characteristics in all studies, except one, where no characteristics were given (Tang et al., 2011). Overall, these 4 studies are split in their recommendation of IVIG as a beneficial intervention for RPL, with 2 reporting no signficant difference (Fawzy et al., 2008, Laskin et al., 1997) and 2 reporting a potential benefit report (Gomaa et al., 2014, Tang et al., 2011).

Authors	Drug Regimen	Inclusion Criteria	Sample size (enrolment)	Mean age (years)	Types of miscarriage (stratification)
Tang et al., 2011	prednisolone 20mg for 6 weeks, 10mg for a week, 5 mg for a week	≥3 consecutive miscarriages RM (3 or more consecutive); raised uNK cell density	68	*Not available*	Elevated uNK cell density
Fawzy et al., 2008	oral prednisone 20 mg/day, progesterone 20 mg/day for first12 weeks of gestation, aspirin 75 mg/day until GW32	Idiopathic recurrent miscarriage (≥3 fetal losses)	160	29.1 in steroid group; 29.4 in control group	Primary, secondary RPL
Gomaa et al., 2014	prednisolone tablets (Hostacortin®) taken daily orally (20 mg/day)	Viable early pregnancy; ≥2 miscarriage before GW20	180	26.13 in steroids group; 27.09 in control group	Unexplained RPL
Laskin et al., 1997	prednisone (Deltasone) at 0.8 mg/kg/day for four weeks (maximum, 60 mg); followed by 0.5 mg/kg/day (maximum, 40 mg) until delivery or fetal loss	≥2 consecutive fetal losses before GW32	202	33 in steroids group; 32 in control group	Unexplained RPL

Table 10: Summary table of randomised controlled trials pertaining to treatment of RPL with steroids. Using the language directly as written by the study, this table summarises the characteristics of the 4 RCTs included in this systematic review that evaluated steroid (prednisone) therapy for RPL. The columns detail the study's first authorship, drug regimen (including dosage, timing, and method of administration), inclusion criteria for participants, sample size of enrolment, mean age in both the intervention and control groups, and any

potential stratification of participants based on the types of miscarriage and RPL detailed in the study. This information allows for a clear comparison of the study population, breath of protocol for the intervention, and the scale of the studies overall. GW = gestational week.

2.4.2. Trustworthiness Assessment of Included Studies

The first screening tool the study included will be evaluated against is the TRACT framework. This screening tool requires information found in the full text of the trial report and trial registration, if accessible. For each item, I chose a rating and wrote a rationale (with concerns and issues if there were any) based on examples and common domain items to look for listed in the TRACT guideline. However, to better summarise the results of the trustworthiness assessment, I have only included the rationales for studies and domain ratings that are of "major concern," this rating warrants possible exclusion of the study in this review.

Study	Governance	Author Group	Plausibility of Intervention Usage	Timeframe	Drop-Out Rates	Baseline Characteristics	Outcomes
Stephenson et al., 2010	**Some concerns**	**No concerns**	**No concerns**	**No concerns**	**Some concerns**	**No concerns**	**No concerns**
Christiansen et al., 2014	**No concerns**	**No concerns**	**No concerns**	**No concerns**	**No concerns**	**No concerns**	**No concerns**
Meng et al., 2016	**No concerns**	**Some concerns**	**Some concerns**	**No concerns**	**Some concerns**	**No concerns**	**Some concerns**
Nazari et al., 2015	**No concerns**	**Some concerns**	**No concerns**	**No concerns**	**Some concerns**	**No concerns**	**No concerns**
Perino et al., 1997	**Some concerns**	**No concerns**	**No concerns**	**No concerns**	**No concerns**	**Major concerns** Only mentioned of baseline characteristics of entire patient selected pool; no comparison between study groups.	**No concerns**
Jablonowska et al., 1999	**Some concerns**	**No concerns**	**No concerns**	**No concerns**	**No concerns**	**Some concerns**	**No concerns**
Christiansen et al., 2002	**Some concerns**	**No concerns**	**No concerns**	**No concerns**	**No concerns**	**No concerns**	**No concerns**
Kim et al., 2012	**Major concerns** No description of ethics or found registration.	**No concerns**	**Some concerns**	**Major concerns** Timeframe not mentioned.	**No concerns**	**Major concerns** No mention of baseline characteristics.	**No concerns**

Coulam et al., 1995	**Some concerns**	**No concerns**	**No concerns**	**No concerns**	**No concerns**	**Major concerns** No mention of baseline characteristics.	**No concerns**
Triolo et al., 2003	**Some concerns**	**No concerns**	**No concerns**	**No concerns**	**No concerns**	**No concerns**	**No concerns**
Yamada et al., 2022	**No concerns**	**No concerns**	**No concerns**	**No concerns**	**Some concerns**	**No concerns**	**No concerns**
Mahmoud et al., 2004	**Some concerns**	**No concerns**	**No concerns**	**Major concerns** Timeframe not mentioned.	**No concerns**	**Some concerns**	**Some concerns**
German RSA/IVIG Group, 1994	**Some concerns**	**No concerns**	**No concerns**	**No concerns**	**Some concerns**	**No concerns**	**No concerns**
Dendrinos et al., 2009	**Some concerns**	**No concerns**	**No concerns**	**No concerns**	**No concerns**	**No concerns**	**No concerns**
Christiansen et al., 1995	**Some concerns**	**No concerns**	**No concerns**	**Major concerns** Timeframe not mentioned.	**No concerns**	**No concerns**	**No concerns**
Stephenson et al., 1998	**Some concerns**	**No concerns**	**No concerns**	**No concerns**	**No concerns**	**Some concerns**	**No concerns**
Meng et al., 2016	**No concerns**	**Some concerns**	**Some concerns**	**No concerns**	**Some concerns**	**No concerns**	**Some concerns**
Elsenity et al., 2022	**No concerns**	**No concerns**	**No concerns**	**No concerns**	**No concerns**	**Some concerns**	**No concerns**
Moini et al., 2022	**No concerns**	**No concerns**	**No concerns**	**No concerns**	**Some concerns**	**No concerns**	**No concerns**
Tang et al., 2011	**Some concerns**	**No concerns**	**Some concerns**	**No concerns**	**No concerns**	**Major concerns** No baseline characteristics listed in any of results.	**No concerns**
Fawzy et al., 2008	**Some concerns**	**No concerns**	**No concerns**	**No concerns**	**Some concerns**	**No concerns**	**No concerns**
Gomaa et al., 2014	**No concerns**	**Some concerns**	**Some concerns**	**No concerns**	**Some concerns**	**Some concerns**	**No concerns**
Laskin et al., 1997	**Some concerns**	**No concerns**	**No concerns**	**No concerns**	**No concerns**	**No concerns**	**No concerns**
Zafardoust et al., 2015	**No concerns**	**No concerns**	**No concerns**	**Some concerns**	**No concerns**	**Some concerns**	**No concerns**
Scarpellini & Sbracia, 2009	**Some concerns**	**No concerns**	**No concerns**	**No concerns**	**No concerns**	**No concerns**	**No concerns**
Eapen et al., 2018	**No concerns**	**No concerns**	**Some concerns**	**No concerns**	**No concerns**	**No concerns**	**No concerns**

Table 10: Compiled TRACT Checklists for all included studies. The leftmost column lists the studies that meet the criteria of this review and classified as fitting to include after the two screens. The seven domains that the TRACT tool assesses

trustworthiness by are labelled at the top of each subsequent column. The red cells represent the study and the specific domain(s) that it received a “major concerns” rating for. The different colours that the studies are divided into are based on the immunomodulator of focus: green is IVIG, blue is intralipid, pink is HCQ, orange is steroids, and yellow is G-CSF. An N of 26 studies was included in this tract analysis.

From the trustworthiness analysis using the TRACT guidelines, one IVIG full text (Kim at al., 2012) will be excluded from the data extraction step and eventual meta-analysis. This full text received three “major concerns” ratings for the domains of governance, timeframe, and baseline characteristics. As a result, in reference to the TRACT guidelines, this many “major concerns” rating warrant exclusion and I decided to exclude the results of this study from my analysis due to untrustworthiness study design and incomplete patient data. Furthermore, although most of the ratings were designated of “no concern,” there were a substantial number of “some concerns” ratings across the seven different domains. Some of the most common reasons for each domain include: (governance) study commenced after 2010 and have no accessible registration, (author group) the ratio of the number of authors to scale of study is relatively low, (plausibility) the intervention protocol would not be easily replicable, (timeframe) no timeframe explicitly stated, (drop-out rate) minimal explanation or even mention of withdrawn participants or those lost in follow-up, (baseline) limited number of given characteristics, and (outcomes) final outcomes did not match up completely with prospective registration. Overall, the included studies did not indicate evidence of low-quality or quasi-experimental studies. Most studies met the exemplar standards for each of the domains, such as including (1) well-descript allocation/intervention protocols and reasons for dropouts, (2) comprehensive list of baseline characteristics that were not significantly different between the two study groups, (3) reputable author group, (4) prospective trial registration in public domains, and (5) reasonable timeframe from recruitment to trial conclusion. The result of this initial quality assessment is 25 studies being included in the next step—data extraction.

2.4.3. Data Extraction

For all the included studies, I manually extracted all the results that correspond to the primary and secondary outcomes as written in the PICOS criteria. The primary outcomes are live birth rate (or ongoing pregnancy rate) (LBR) and miscarriage rate. For studies that include both arm-level and contrast-level results, I recorded both the event numbers (if available) per group size, along with the odds ratio or risk ratio, confidence interval (95%), and p-values—if statistical analysis was included.

	Intervention Group			Control/Comparator Group	
Study	**Intervention**	**Live Birth Rate**	**Miscarriage**	**Live Birth Rate**	**Miscarriage**
Meng et al., 2015	Intralipid	39/76	9/70	48/78	14/67
Elsenity et al., 2022	HCQ	40/40 (66%)	N/A	25/60 (42.3%)	N/A
Moini et al., 2022	HCQ	N/A	5/14 (OR 2.36; 95% CI 1.07-8.93)	N/A	4/15
Zafardoust et al., 2015	G-CSF	N/A	33%	N/A	37.5%
Scarpellini & Sbracia, 2009	G-CSF	29/35 (p=.0061, OR 5.1, 95% 1.5-18.4)	N/A	16/33	N/A
Eapen et al., 2018	GCSF	45/76	N/A	48/74	N/A
Tang et al., 2011	Steroids	12/20 (RR 1.5; 95% CI 0.8-2.9)	7/20	8/20	9/20
Fawzy et al., 2008	Steroids	45/53	8/53	24/50	26/50
Gomaa et al., 2014	Steroids	52/74 (RR 7.63; 95% CI 3.7-15.7)	22/74	7/76	69/76
Laskin et al., 1997	Steroids	66/101	35/101	57/101	44/101
Stephenson et al., 1998	IVIG	[total] 12/21 [primary] 7/10 OR 1.94; 95%-CI .32-11.76)	9 events	[total] 11/22 [primary] 6/11	11 events
Stephenson et al., 2010	IVIG	LBR (16/23) (P = 0.760) (OR 1.37) (85% CI 0.41-4.61)	7/23	15/24	9/24
Christiansen et al., 1995	IVIG	9/17	N/A	5/17	N/A
Christiansen et al., 2002	IVIG	13/29	16 events	13/29	16 events
Christiansen et al., 2014	IVIG	23/44	20/44	20/43	23/43

German RSA/IVIG Group, 1994	IVIG	20/33	N/A	21/31	N/A
Coulam et al., 1995	IVIG	18/29 (P = 0.04; OR 3.1)	11/29	11/32	21/32
Perino et al., 1997	IVIG	16/22	6/22	20/24	4/24
Jablonowska et al., 1999	IVIG	[primary] 8/11 OR 1.14 95% CI .17-7.60 [total] 17/22	5/22	[primary] 7/10 [total] 15/19	4/19
Triolo et al., 2003	IVIG	12/21 (P = 0.06)	9/21	16/19	3/19
Mahmoud et al., 2004	IVIG	6/8 (p > 0.05)	2/8 (p > 0.05)	5/7	2/7
Dendrinos et al., 2009	IVIG	15/38 (P = 0.003)	21/28 (P = 0.01)	29/40	11/40
Nazari et al., 2015	IVIG	25/28	3 events	28/32	4 events
Yamada et al., 2022	IVIG	29/50 (OR 2.60; 95% CI 1.15-5.86; p = 0.03)	19/50	17/49	31/49

Table 11: Primary outcomes of systematic review extracted from included studies. This table presents the primary outcomes included in this systematic review. These outcomes focus on the efficacy and safety of these immunomodulatory interventions for RPL. The data includes live birth rates (LBR), miscarriage rate, and any associated effect measures (relative risk or odds ratio with confidence intervals).

Study	Intervention	Adverse Events	Ongoing Pregnancies	Clinical Pregnancy Rate	Embryo Number	Implantation Rate
Meng et al., 2015	Intralipid	No side effects seen in intralipid group. 5 IVIG group patients had fever, headache, and no other side effects.	[IVIG] 67/78 [intralipid] 70/76	N/A	N/A	N/A
Elsenity et al., 2022	HCQ	No adverse events	N/A	N/A	N/A	N/A
Moini et al., 2022	HCQ	No adverse events	N/A	N/A	N/A	N/A
Zafardoust et al., 2015	G-CSF	No adverse events	[G-CSF] 6/23 (p=0.781) [control] 8/27	N/A	N/A	N/A
Scarpellini & Sbracia, 2009	G-CSF	No adverse events	N/A	N/A	N/A	N/A
Eapen et al., 2018	GCSF	Adverse events occurred in 52 in G-CSF; 43 in placebo. neonatal congenital anomalies	N/A	[G-CSF] 59.2% (45/76) [placebo] 64.9% (48/74)	N/A	N/A
Tang et al., 2011	Steroids	Insomnia in steroid group, no adverse effects otherwise.	N/A	N/A	N/A	N/A

Fawzy et al., 2008	Steroids	No side effects observed.	N/A	N/A	N/A	N/A
Gomaa et al., 2014	Steroids	No adverse events	N/A	N/A	N/A	N/A
Laskin et al., 1997	Steroids	Hypertension and gestational diabetes mellitus common with cataracts developed in 2 women in treatment group.	N/A	N/A	N/A	N/A
Stephenson et al., 1998	IVIG	1 stillbirth at GW21 after intractable abdominal pain secondary to large degenerating fibroids. 1 genetic termination after Kleinfelter Syndrome.	N/A	N/A	N/A	N/A
Stephenson et al., 2010	IVIG	1 birth in IVIG group was preterm at GW26 following HELLP syndrome.	N/A	[IVIG] 23/38 [control] 24/39	N/A	N/A
Christiansen et al., 1995	IVIG	N/A	N/A	N/A	N/A	N/A
Christiansen et al., 2002	IVIG	2 patients developed transient skin rashes.	N/A	N/A	N/A	N/A
Christiansen et al., 2014	IVIG	Rashes, headaches, 2 children from births had congenital disorders.	N/A	N/A	N/A	N/A
German RSA/IVIG Group, 1994	IVIG	N/A	N/A	N/A	N/A	N/A
Coulam et al., 1995	IVIG	No reactions to medication.	N/A	N/A	N/A	N/A
Perino et al., 1997	IVIG	1 case due to cervical dystocia, 1 to intrauterine growth retardation.	N/A	N/A	N/A	N/A
Jablonowska et al., 1999	IVIG	Mild skin rashes and itching, vaginal bleeding in two patients, elevated body temp.	N/A	N/A	N/A	N/A
Triolo et al., 2003	IVIG	1 case of premature membrane rupture in comparator group (heparin/aspirin), 1 case of preeclampsia in IVIG	N/A	N/A	N/A	N/A
Mahmoud et al., 2004	IVIG	No effects.	N/A	N/A	N/A	N/A
Dendrinos et al., 2009	IVIG	1 preterm vaginal delivery; 2 intrauterine deaths; nausea, hypotension, and tachycardia in 3 patients taking IVIG.	N/A	N/A	N/A	N/A
Nazari et al., 2015	IVIG	Fever and chills in one patient.	N/A	N/A	N/A	N/A
Yamada et al., 2022	IVIG	1 patient in control group had induced abortion (fetal anencephaly) in intention-to-treat group	31/50 (OR 3.07; 95% CI 1.35-6.97; P =.009)	N/A	N/A	N/A

Table 12: Secondary outcomes of systematic review extracted from included studies. This table presents the secondary outcomes that will not be the outcomes of focus in the meta-analyses. Outcomes such as adverse events, ongoing and clinical pregnancy rates, and embryo number and implantation rate, if the study includes IVF treatment, for all studies are summarised in this table. Not all studies report all these outcomes, and the missing data is denoted as "N/A." All the comparators and controls are a placebo group except for Meng et al., 2015, which compared IVIG and intralipid treatments.

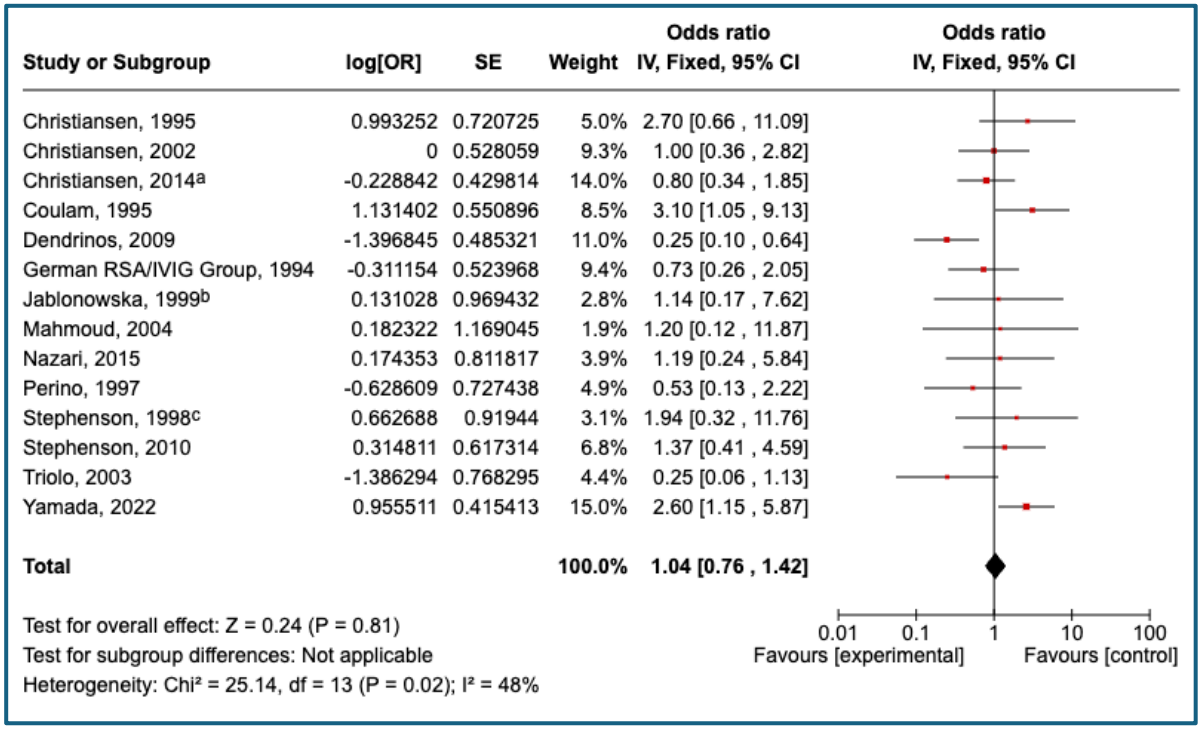
2.4.4. Meta-Analysis of Immunomodulators in RPL

After completing the data extraction and entering the results manually into RevMan, I made the following forest plots using the same software. All the data was dichotomous in nature for live birth rate (LBR), rate of miscarriage (MR), and rate of ongoing pregnancy (OPR). For studies that published contrast-level results, I included the author group's calculated odds ratio or risk ratio (RR), confidence intervals (all 95%), and p-values. However, if the study only included arm-level results, such as total group sizes and the number of live births, miscarriage, and ongoing pregnancies, I inputted the data as arm-level results. To compile the data into each intervention meta-analysis, I included both contrast- and arm-level results, with a preference of contrast-level results if both are given. This choice was made to minimise the amount of arm-level data manipulation to calculate effect measures and based on the moderate-high quality of the studies (as described below), limits any potential miscalculations by trusting the calculation of the studies' authors to be more precise and accurate. The live birth rate corresponds to this review's measure of efficacy and the miscarriage rate to the safety of the intervention.

2.4.5.1. Intravenous Immunoglobulin: Effectiveness & Safety

This section evaluates the efficacy and safety of IVIG therapy in improving live birth and miscarriage rates in women with RPL, through the assessment of 15 studies. Five RCTs involving 866 participants were included in the analysis of IVIG. Study populations varied in mean age (29.7-35) and the number of previous miscarriages, as shown in Table 9. Figure 2.2 shows the pooled effect of IVIG on live birth rates and miscarriage rates compared to the control group. Each study's effect size and weight are represented along with the calculated overall odds ratio. In Figure 2.2.A, the overall odds ratio is 1.04 [95% CI 0.76, 1.42; p-value = 0.81; I^2 = 48%], indicating that there is a slightly higher chance of a live

birth occurring in the IVIG-treatment group, compared to the control group. However, the pooled analysis reveals that this effect is not significant. Moderate to high heterogeneity was observed, which may be due to the variation in the study population or dosage of IVIG, making the results less interpretable.



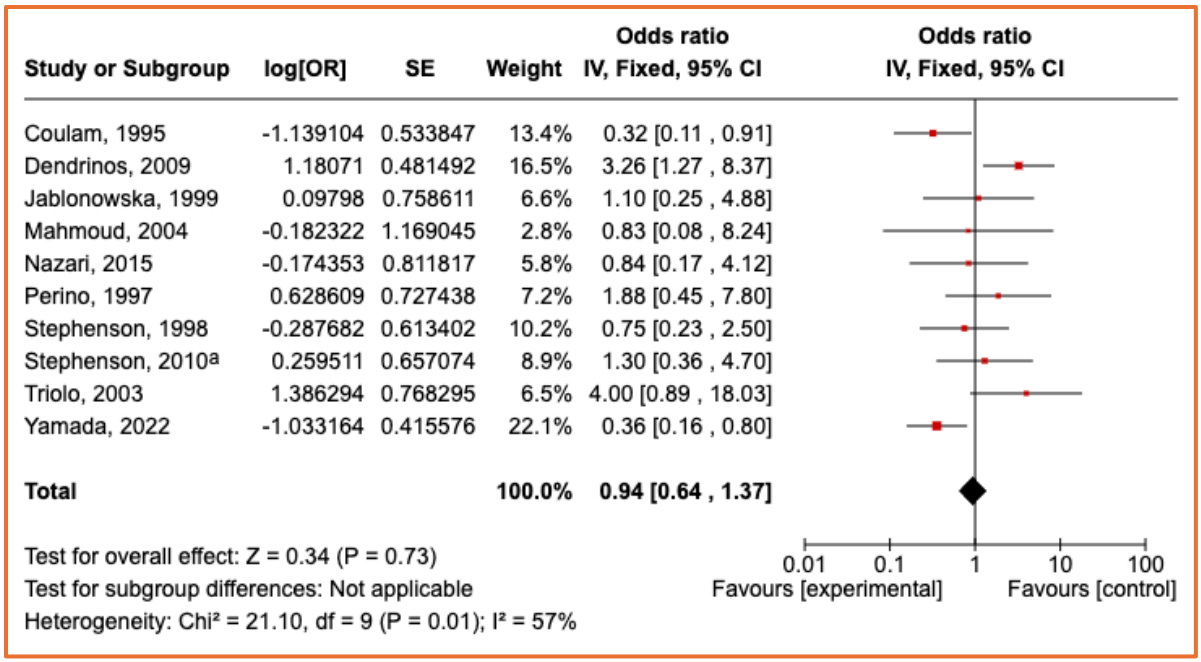


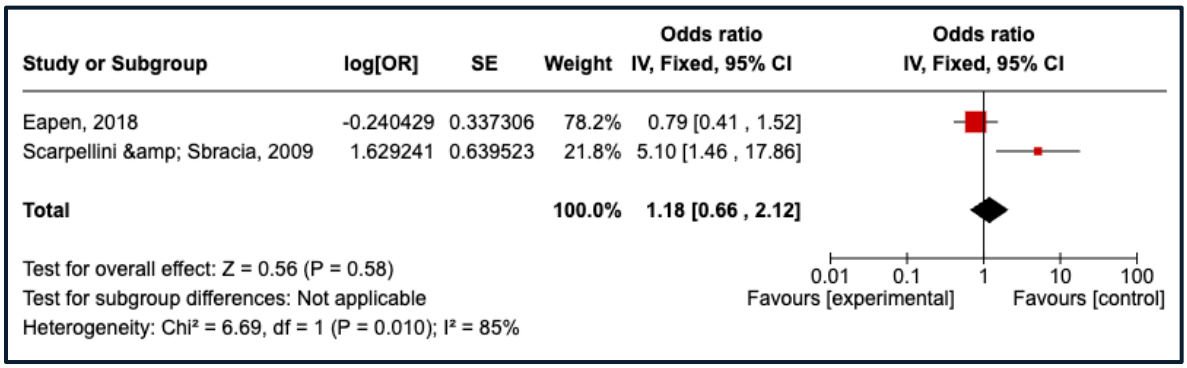
Figure 2.2: A. Forest plot of the efficacy of IVIG for recurrent pregnancy loss. This forest displays the pooled effect with odds ratio (OR) of IVIG therapy on live birth rates in RPL patients. The individual study results are displayed alongside the overall meta-analysis results. The diamond on the bottom axis indicates an overall OR of 1.04 [95% CI 0.76, 1.42]. **B. Forest plot comparing published miscarriage rates between IVIG-treated and placebo control groups.** This forest displays the pooled odds ratio (OR) for miscarriage rates comparing IVIG to placebo/control group. The individual study results are displayed alongside the overall meta-analysis results. The diamond on the bottom axis indicates an overall OR of 0.94 [95% CI 0.64, 1.37]. In both plots, each study is represented by a point estimate (the square) along the horizontal lines (95% confidence intervals). The weight is represented by the size of the square. SE = standard error.

In part B of the Figure 2.2, the odds ratio is 0.94 [95% CI 0.64, 1.37; p-value = 0.73; $I^2 = 57\%$], showing that the likelihood of having a miscarriage is higher in the control group. This result is also insignificant compared to the IVIG-treated group. Compared to live birth rates, an even higher heterogeneity was observed in the miscarriage analysis, further making the results inconclusive. These results suggest that IVIG therapy may increase both live birth rates and miscarriage rates in patients with RPL. However, these results did not reach statistical significance, and further trials are needed to explore the safety and efficacy of IVIG as a potential immune therapy.

2.4.5.2. G-CSF: Effectiveness & Safety

This next section evaluates the efficacy and safety of G-CSF therapy in improving live birth and miscarriage rates in women with RPL across 2 studies. Figure 2.3 illustrates the overall effect of G-CSF therapy on live birth and miscarriage rates. The meta-analysis in Figure 2.3.A found the G-CSF

intervention treatment to yield a greater benefit on LBR compared to the control group with an OR of 1.18 [95% CI 0.66, 2.12; p = 0.58]. There is high heterogeneity with an I^2 = 85%, likely due to the small pool of 2 studies. In Figure 2.3.B, there is only one included study with an OR of 0.91 [95% CI 0.28, 2.89, p = 0.87]. There is not measure of heterogeneity in the meta-analysis for MR with only one included study. With the lack of evidence to make a conclusion about the efficacy and safety of G-CSF in patients with RPL, further trials testing G-CSF are needed to establish G-CSF as a viable immune therapy.



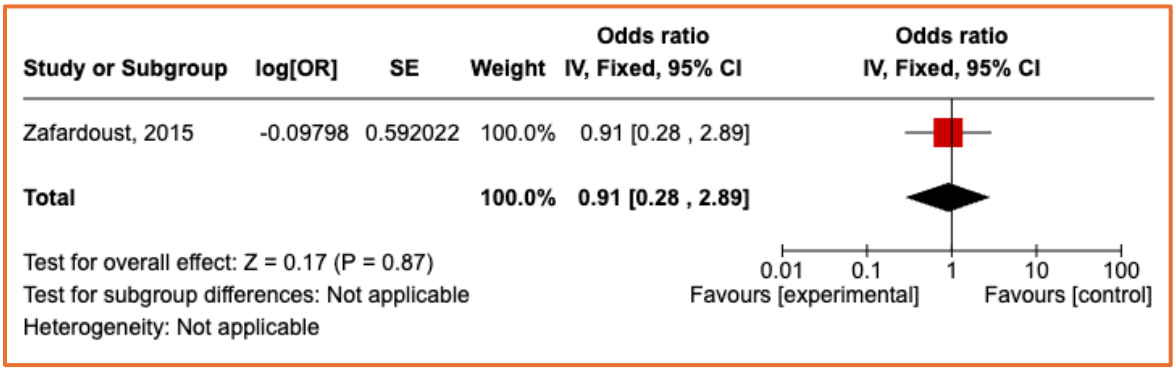
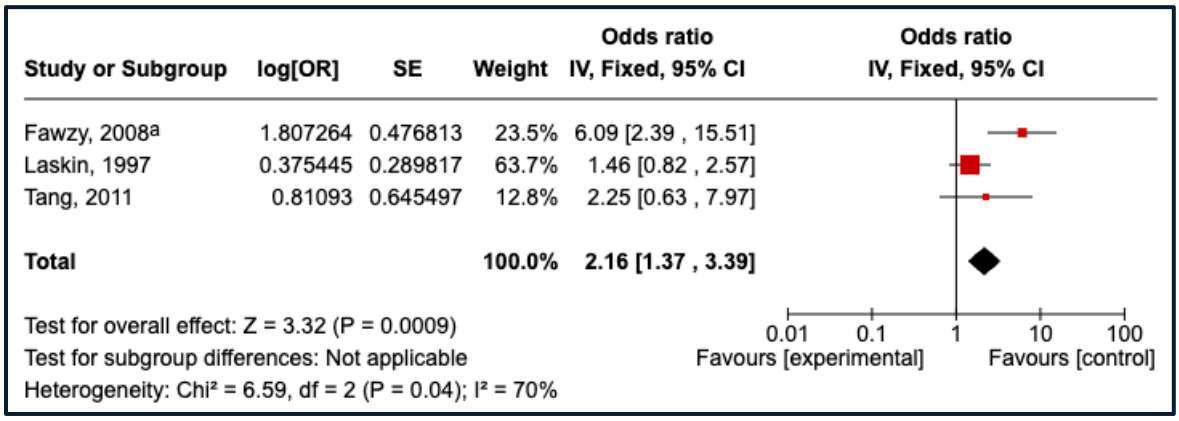


Figure 2.3: A. Forest plot of the efficacy of G-CSF for recurrent pregnancy loss. This forest displays the pooled effect with odds ratio (OR) of G-CSF therapy on live birth rates in RPL patients. The individual study results are displayed alongside the overall meta-analysis results. The diamond on the bottom axis indicates an overall OR of 1.18 [95% CI 0.66, 2.12]. **B. Forest plot comparing published miscarriage rates between G-CSF-treated and placebo control groups.** This forest displays the pooled odds ratio (OR) for miscarriage rates comparing G-CSF to placebo/control group. The individual study results are displayed alongside the overall meta-analysis results. The diamond on the bottom axis indicates an overall OR of 0.91 [95% CI 0.28, 2.89]. In both plots, each study is represented by a point estimate (the square) along the horizontal lines (95% confidence intervals). The weight is represented by the size of the square. SE = standard error.

2.4.5.3. Steroids: Effectiveness & Safety

This section evaluates the efficacy and safety of immune therapy using steroids in improving live birth and miscarriage rates in women with RPL across 4 studies. Figure 2.4 displays the results of the results of the meta-analysis done on the included studies reported live birth and miscarriage rates. The analysis revealed that steroid intervention significantly increased live birth rates compared to the control group (OR: 2.16, 95% CI 1.37, 3.39, p = 0.0009). According to Figure 2.4.B, the pooled analysis showed that in the steroids group, miscarriage rates are significantly lower than in the control group (OR: 0.29, 95% CI 0.20, 0.44, p <0.00001). The heterogeneity of the studies in both the LBR and MR analysis are high with an I^2 = 70% and 89%, respectively. This shows great variance between the studies, likely attributed to differences in intervention regimens or poor randomisation in allocation of the two groups. Although these results are significant, there is still only 3 studies in total that reported LBR and 4 studies for MR. More large-scale randomised trials are required to provide further evidence of steroids as a promising immune therapy for RPL patients.



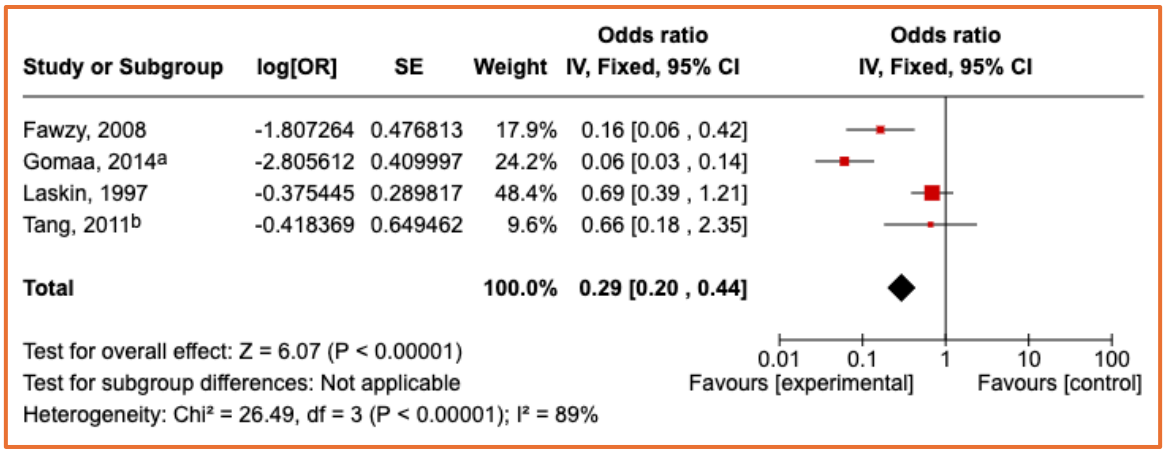


Figure 2.4: A. Forest plot of the efficacy of steroids for recurrent pregnancy loss. This forest displays the pooled effect with odds ratio (OR) of steroid-based immune therapy on live birth rates in RPL patients. The individual study results are displayed alongside the overall meta-analysis results. The diamond on the bottom axis indicates an overall OR of 2.16 [95% CI 1.37, 3.39]. **B. Forest plot comparing published miscarriage rates between steroid-treated and placebo control groups.** This forest displays the pooled odds ratio (OR) for miscarriage rates comparing steroid-based immune therapy to the placebo/control group. The individual study results are displayed alongside the overall meta-analysis results. The diamond on the bottom axis indicates an overall OR of 0.29 [95% CI 0.20, 0.44]. In both plots, each study is represented by a point estimate (the square) along the horizontal lines (95% confidence intervals). The weight is represented by the size of the square. SE = standard error.

2.4.5.4. Hydroxychloroquine: Effectiveness & Safety

This section evaluates the efficacy of hydroxychloroquine as an immune therapy in improving live birth in women with RPL across 2 studies. In either of these two studies, there was no reported miscarriage rate and therefore, I am not able to comment on the safety of HCQ using miscarriage rate due to the lack of evidence and subsequent meta-analysis. The analysis in Figure 2.5 reveals that HCQ significantly increase the LBR, compared to the control group (OR: 2.12, 95% CI 1.13, 3.95, p = 0.02). Heterogeneity of this analysis is also low (I^2 = 0%), which shows that both studies yielded similar results with low variance. Although the meta-analysis provides significant results to support the efficacy of HCQ in treating RPL patients, this is only based on two trials. With more evidence needed to establish HCQ, it is promising that there are at least 5 ongoing studies.

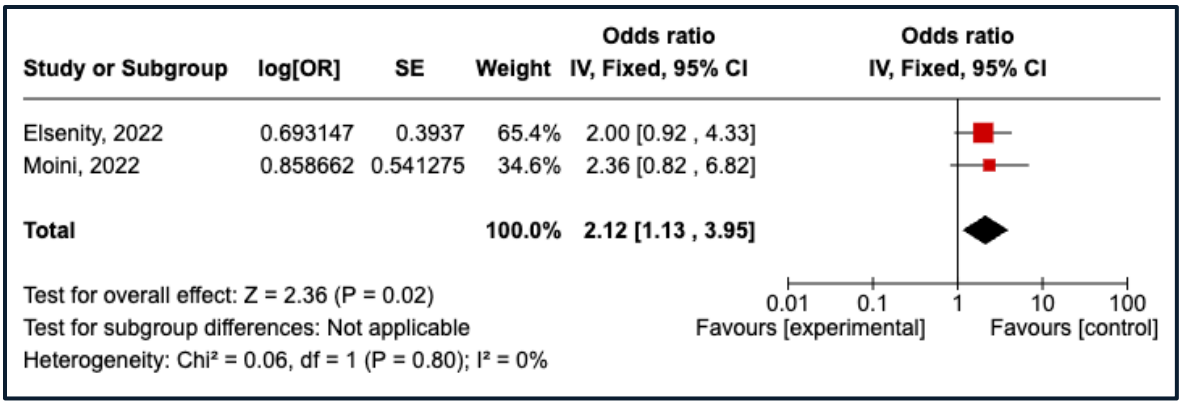
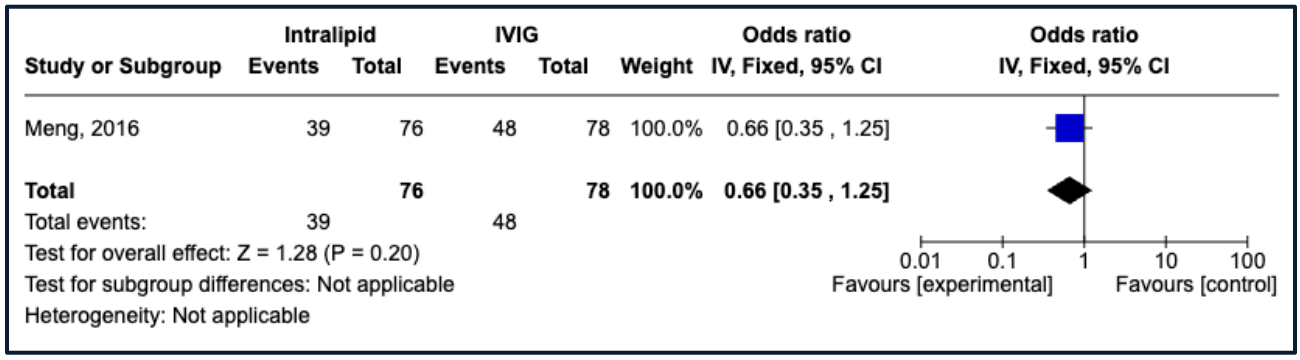


Figure 2.5: Forest plot of the efficacy of HCQ for recurrent pregnancy loss. This forest displays the pooled effect with odds ratio (OR) of HCQ therapy on live birth rates in RPL patients. Each study is represented by a point estimate (the square) along the horizontal lines (95% confidence intervals). The weight is represented by the size of the square. The individual study results are displayed alongside the overall meta-analysis results. The diamond on the bottom axis indicates an overall OR of 2.12 [95% CI 1.13, 3.95]. SE = standard error.

2.4.5.5. Intralipid: Effectiveness & Safety

This final section was meant to evaluate the efficacy and safety of intralipid as an immune therapy in improving live birth and miscarriage rates in women with RPL. However, with only one included study, no meta-analysis was able to be conducted. The forest plots in Figure 2.6 are redundant with the inclusion of only one study, but they display the total live birth and miscarriage events in both groups.



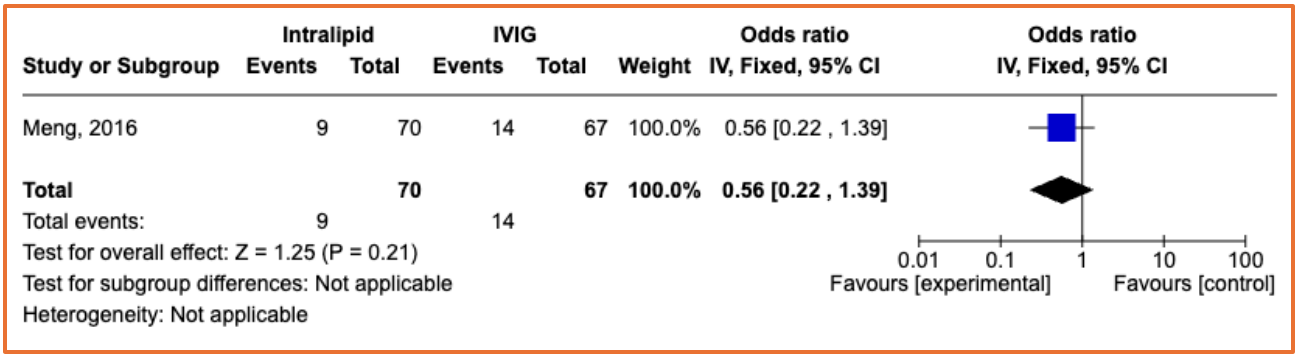


Figure 2.6: Forest plot of the efficacy of intralipids for recurrent pregnancy loss. This forest displays the results of one study measuring the potential effect on intralipid on live birth rates in RPL patients. The diamond on the bottom axis indicates an overall OR that is equal to that of the single study, 0.66 [95% CI 0.35, 1.25]. **B. Forest plot comparing published miscarriage rates between intralipid-treated and placebo control groups.** Only the miscarriage rate from one study is included in this forest plot. The diamond on the bottom axis indicates an overall OR that is equal to that of the single study, 0.56 [95% CI, 0.22, 1.39]. In both plots, each study is represented by a point estimate (the square) along the horizontal lines (95% confidence intervals). The weight is represented by the size of the square, which in both plots is 100% for the singular included study. SE = standard error.

In this one study, the live birth rates are 97.5% and 98.0% for the intralipid and IVIG groups, respectively. Amongst the 192 patients in both study groups that started the clinical trial, only 40 in the intralipid group and 49 in the IVIG group followed up post-delivery. Without almost 50% of the original patient pool missing pregnancy outcomes, these rates must be interpreted with this significant limitation in consideration. Also, rather than the comparator group being a control group receiving a placebo intervention, this trial is comparing the intralipid to another immunomodulator, IVIG. The findings are limited to conclusions about how comparable the efficacy of intralipid is to IVIG in RPL patients.

These results will be further discussed in the context of the current literature on each immunomodulator in the Discussion section below.

2.4.4. Quality Assessment of Evidence from Included Studies

2.4.4.1. Risk of Bias Assessment

The overall integrity risk level of the 25 studies across the seven bias judgments are primarily low risk to unclear risk of bias. As seen in Figure 2.7, in terms of selection bias, 100% of the studies are of low risk of bias due the usage of random sequence generation. 50% maintain a low risk of bias due to clear use of allocation concealment, however for the other 50% of studies it is unclear the risk

because concealment was not explicitly stated or included in protocol description. Blinding as a part of the study design is not part of this review's inclusion criteria, and unsurprisingly, more than 50% of the studies are unclear in risk of performance bias. The rest of the studies are primarily double-blinded studies, and therefore of low risk for performance bias. For assessment of possible detection bias, the rate of low risk and unclear risk is the similar within the included studies. In terms of attrition bias, 87.5% of the studies were of low risk with two studies being of unclear risk, and one being of major risk due to the outcome numbers not clearly adding up to the total enrolment number (Meng et al., 2016). The same distribution of potential risk across the studies for selection bias with one study considered of major risk due to no reporting of event numbers, only percentage (Zafardoust et al., 2015). For the last ratings for "other bias," 87.5% of the studies were of low risk and three studies considered unclear of risk due to concerns not easily categorised by the other specific domains, like concerns with very sparse baseline characteristics, follow-up corrections to the original publication, and very small sample sizes.

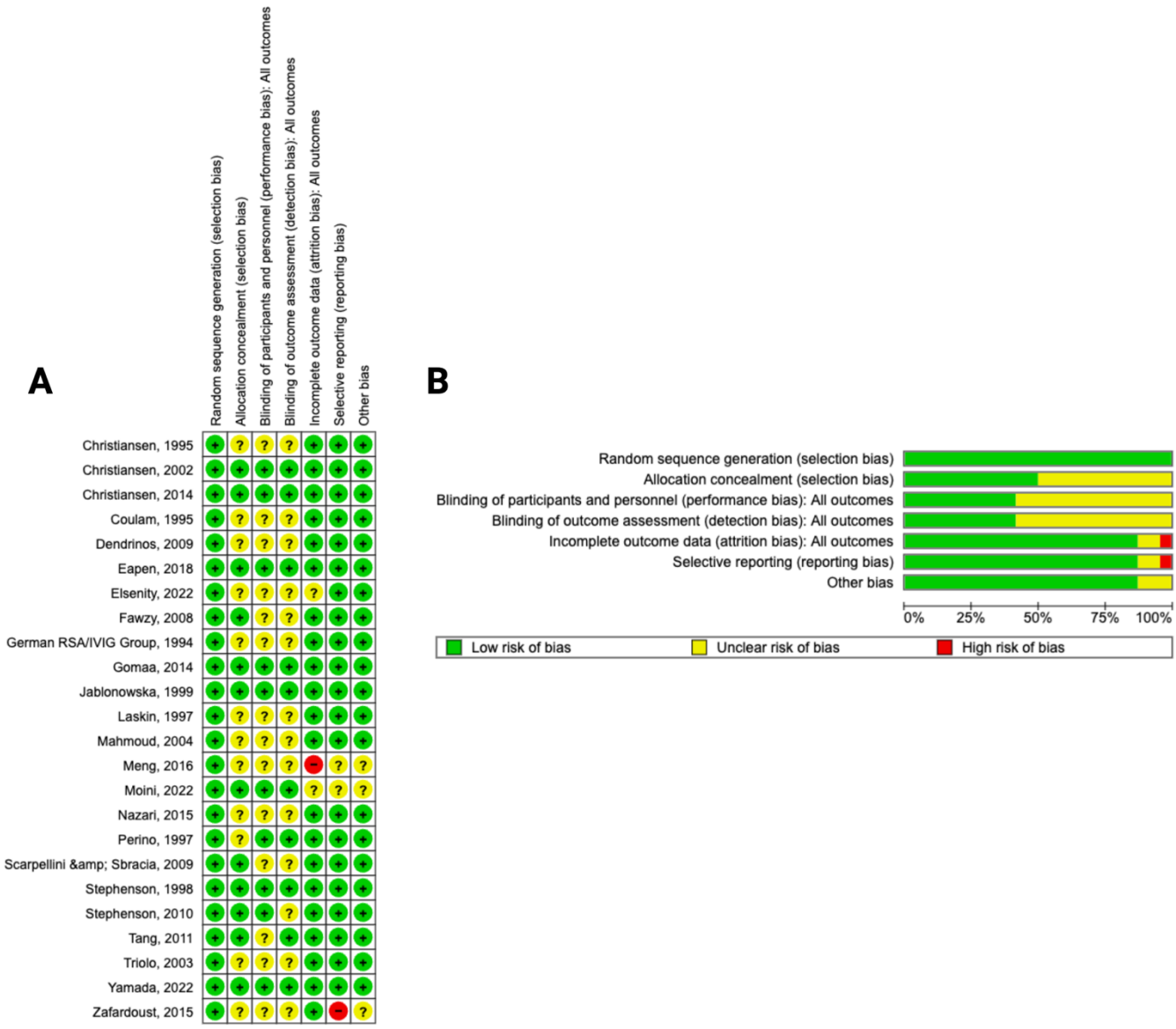


Figure 2.7: A. Risk of Bias summary of all included studies. The lattice summarises the risk ratings given to each included study across the seven risks of bias criteria listed on the top of each column. The green circles correspond to the "low risk" rating, the yellow to the "unclear risk of bias" rating, and finally the red corresponds to the "high risk" rating. **B. Risk of Bias graph of all included studies in meta-analysis.** This graph further summarises the results of the risk of bias ratings and gives a better visualisation of the potential overall risk of bias across all studies in each criterion. This figure reports the quality and limitations of the study. The graph gives the percentage of studies that fall in each of the three ratings across the seven judgments. The unique ID that each assessment is tracked by included information to identify the study. This figure was generated by RevMan.

Most studies overall are considered low risk of bias for the live birth rate and miscarriage rate (when included) outcomes. With no study receiving more than one major risk of bias judgment, all studies are considered not only relevant to this review but should be considered as moderate- to high-quality evidence.

2.4.4.2. Grade Assessment of Included Studies

For this last assessment of quality, the GRADE approach assigned an evidence quality rating to each of the two outcomes—live birth rate (LBR) and miscarriage rate (MR)—in every included study included.

No. of studies	Design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other	Certainty (Overall Score)
Outcome: Live Birth Rate							
1 Meng, 2015	RCT (+4)	Some concern (-1)	Some important inconsistency concerns due to missing outcomes (-1)	No serious indirectness (0)	No serious imprecision (0)	None	⊕⊕◯◯
2 Elsenity et al., 2022	RCT (+4)	Some concern (-1)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0	None	⊕⊕⊕◯
3 Scarpellini & Sbracia, 2009	RCT (+4)	No concerns (0)	No concerns (0)	No serious indirectness (0)	Wide confidence intervals (-1)	None	⊕⊕⊕◯
4 Eapen et al., 2018	RCT (+4)	No concerns (0)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	None	⊕⊕⊕⊕
5 Tang et al., 2011	RCT (+4)	No concerns (0)	No concerns (0)	No serious indirectness (0)	Large effect size (+1)	Small study pool (-1)	⊕⊕⊕⊕
6 Fawzy et al., 2008	RCT (+4)	No concerns (0)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	None	⊕⊕⊕⊕
7 Gomaa et al., 2014	RCT (+4)	No concerns (0)	Some concerns (-1)	No serious indirectness (0)	Wide confidence intervals (-1)	Large effect size (+1)	⊕⊕⊕◯
8 Laskin et al., 1997	RCT (+4)	No concerns (0)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	None	⊕⊕⊕⊕
9 Stephenson et al., 1998	RCT (+4)	No concerns (0)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	None	⊕⊕⊕⊕
10 Stephenson et al., 2010	RCT (+4)	No concerns (0)	No concerns (0)	No serious indirectness (0)	Wide confidence intervals (-1)	None	⊕⊕⊕◯
11 Christiansen et al., 1995	RCT (+4)	Some concerns (-1)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	Small study pool (-1)	⊕⊕◯◯

12 Christiansen et al., 2002	RCT (+4)	No concerns (0)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	None	⊕⊕⊕⊕
13 Christiansen et al., 2014	RCT (+4)	No concerns (0)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	None	⊕⊕⊕⊕
14 German RSA/IVIG Group, 1994	RCT (+4)	Some concerns (-1)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	None	⊕⊕⊕⊕
15 Coulam et al., 1995	RCT (+4)	Some concerns (-1)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	Large effect size (+1)	⊕⊕⊕⊕
16 Perino et al., 1997	RCT (+4)	No concerns (0)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	None	⊕⊕⊕⊕
17 Jablonowska et al., 1999	RCT (+4)	No concerns (0)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	Small study pool (-1)	⊕⊕⊕◯
18 Triolo et al., 2003	RCT (+4)	Some concerns (-1)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	Small study pool (-1)	⊕⊕◯◯
19 Mahmoud et al., 2004	RCT (+4)	No concerns (0)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	Small study pool (-1)	⊕⊕⊕◯
20 Dendrinos et al., 2009	RCT (+4)	Some concerns (-1)	No concerns (0)	No serious indirectness (0)	Large effect size (+1)	None	⊕⊕⊕⊕
21 Nazari et al., 2015	RCT (+4)	Some concerns (-1)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	None	⊕⊕⊕◯
22 Yamada et al., 2022	RCT (+4)	No concerns (0)	No concerns (0)	No serious indirectness (0)	Large effect size (+1)	None	⊕⊕⊕⊕

Table 13: GRADE assessment of all included studies for live birth rate outcome in meta-analysis. This GRADE framework on the included study was made using an Excel template provided by GRADE guidelines and the Cochrane working group, with some adaptation. The value in the parentheses in the "Design" column is the starting rating for the quality of evidence in the specific outcome. The numbers in the following columns represent the increase or decrease in rating based on the stated factor. The overall score is the resulting rating shown by the number of filled in circles. The highest possible rating is 4, indicating a "high" indication to "moderate," "low," and "very low," for 3, 2, and 1, respectively.

No. of studies	Design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other	Certainty (Overall Score)
Outcome: Miscarriage Rate							
1 Meng, 2015	RCT (+4)	Some concern (-1)	Some important inconsistency concerns due to missing outcomes (-1)	No serious indirectness (0)	No serious imprecision (0)	None	⊕⊕◯◯
2 Moini et al., 2022	RCT (+4)	Confounders considered (+1)	No serious inconsistencies (0)	No serious indirectness (0)	No serious imprecision (0)	Small sample size (-1)	⊕⊕⊕⊕

3 Zafardoust et al., 2015	RCT (+4)	Some concern (-1)	No serious inconsistencies (0)	No serious indirectness (0)	No serious imprecision (0)	None	⊕⊕⊕◯
4 Tang et al., 2011	RCT (+4)	No concerns (0)	No serious inconsistencies (0)	No serious indirectness (0)	No serious imprecision (0)	Small study pool size (-1)	⊕⊕⊕◯
5 Fawzy et al., 2008	RCT (+4)	No concerns (0)	No serious inconsistencies (0)	No serious indirectness (0)	Large effect size (+1)	None	⊕⊕⊕⊕
6 Gomaa et al., 2014	RCT (+4)	No concerns (0)	Some concerns (-1)	No serious indirectness (0)	No serious imprecision (0)	None	⊕⊕⊕◯
7 Laskin et al., 1997	RCT (+4)	No concerns (0)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	Large effect size (+1)	⊕⊕⊕⊕
8 Stephenson et al., 1998	RCT (+4)	No concerns (0)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	None	⊕⊕⊕⊕
9 Stephenson et al., 2010	RCT (+4)	No concerns (0)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	None	⊕⊕⊕⊕
10 Christiansen et al., 2002	RCT (+4)	Some concerns (-1)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	None	⊕⊕⊕⊕
11 Christiansen et al., 2014	RCT (+4)	No concerns (0)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	None	⊕⊕⊕⊕
12 Coulam et al., 1995	RCT (+4)	No concerns (0)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	Large effective size (+1)	⊕⊕⊕⊕
13 Perino et al., 1997	RCT (+4)	No concerns (0)	No concerns (0)	No serious indirectness (0)	Some concerns (-1)	None	⊕⊕⊕◯
14 Jablonowska et al., 1999	RCT (+4)	No concerns (0)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	Small study pool (-1)	⊕⊕⊕◯
15 Triolo et al., 2003	RCT (+4)	Some concerns (-1)	No concerns (0)	No serious indirectness (0)	Some concerns as secondary outcome (-1)	Small study pool (-1)	⊕◯◯◯
16 Mahmoud et al., 2004	RCT (+4)	No concerns (0)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	Small study pool (-1)	⊕⊕⊕◯
17 Dendrinos et al., 2009	RCT (+4)	Some concerns (-1)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	None	⊕⊕⊕◯
18 Nazari et al., 2015	RCT (+4)	Some concerns (-1)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	None	⊕⊕⊕◯
19 Yamada et al., 2022	RCT (+4)	No concerns (0)	No concerns (0)	No serious indirectness (0)	No serious imprecision (0)	None	⊕⊕⊕⊕

Table 14: GRADE assessment of all included studies for miscarriage rate outcome in meta-analysis. This GRADE framework on the included study was made using an Excel template provided by GRADE guidelines and the Cochrane working group, with some adaptation. The value in the parentheses in the "Design" column is the starting rating for the quality of evidence in the specific outcome. The numbers in the following columns represent the increase or decrease in rating based on the stated factor. The overall score is the resulting rating shown by the number of filled in circles. The highest possible rating is 4, indicating a "high" indication to "moderate," "low," and "very low," for 3, 2, and 1, respectively.

With all the studies being randomised controlled trials, each rating started at a 4 and decreased due to risk of bias, inconsistency, indirectness, imprecision, and/or other. The likelihood that the observed effect will be substantially different from the true effect is moderate to high. The 2-ratings correspond to a "low," or some indication of the likely effect, however only three studies received a rating of 2 for the LBR outcome and two studies receiving a 2 or lower rating for miscarriage rates. Therefore, the results of the evidence for the effectiveness of the IVIG, steroids, HCQ, and G-CSF in this systematic review is of moderate certainty. For intralipids, there is only one study of low quality, so it is likely that this study provides some indication of the likely effectiveness. Overall, I designated the outcomes, miscarriage rate and LBR, as moderate to high in quality in the evidence provided by the included studies. The GRADE assessments for each study and outcomes are summarised in Tables 14 and 15.

Since the purpose of this systematic review is to gather and report all evidence on the efficacy and safety of treating RPL with various immunomodulators, studies were not excluded based on their quality. However, a thorough explanation of the quality assessment results is provided to give context for the findings. Using the Cochrane RoB 2 tool, the studies were primarily of low risk of bias. This highlights potential biases, particularly in domains of study design and selective reporting of results. This is a caution to be considered when interpreting the findings. In terms of certainty using the GRADE assessment, the effect size of the meta-analyses is a very good indication of the likely effect for all immunomodulators, except for intralipid. It is evident that many of the included studies are high-quality studies with a few outliers.

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	2.1
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	N/A
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	2.1.2
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	2.2
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	2.3.1
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	2.3.1
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	2.3.2
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	2.3
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	2.3.4
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	2.4
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	2.4
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	2.3.5
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	

Table 15: PRISMA Checklist. This 27-item PRISMA checklist is provided by the GoodReports platform for usage in systematic review. This list allows for complete transparency and accuracy on the researcher's part when completing a systematic review that is up to the PRISMA standard. Under the right column, the sections in which the item is met is noted for reference. This table only includes the first half of the PRISMA checklist, the sections of the review that are most relevant to this study. The second half is included in the Appendix and will be used for the ongoing systematic review and meta-analysis.

2.5. Discussion

This systematic review provides a foundation for understanding how effective intralipids, IVIG, HCQ, G-CSF, and steroids are in the management of RPL, which is integral to understanding the clinical perspective and literature on immunomodulators. Although there were 25 studies included across all the meta-analyses for each intervention, more than half of these RCTs are investigating the effect of IVIG. With only a total of 10 RCTs focused on the other four interventions, there is an apparent lack of RCTs and insufficient data to draw conclusions about the effect size from. However, with the overall aims to summarise the current evidence and gain an understanding of the scope of literature for each immunomodulator, I can clearly show the discrepancies in the depth of research between the known immune therapies, in addition to the results of the meta-analyses. In this discussion section, I will discuss the screening process, explore this review's limitations and strengths, the results of my meta-analysis, and the clinical application of these immune therapies in context with the current literature.

2.5.1. Summary of Key Findings

Among the interventions analysed, steroids demonstrated the most significant effect in improving the live birth rates (LBR) and reducing miscarriage rates (MR) in patients with RPL. Steroids had pooled odds ratio of 2.16 for LBR and 0.29 for MR, which are both statistically significant. Although only having evidence from two studies, Hydroxychloroquine (HCQ) emerged as a potential therapy with a statistically significant improvement in LBR (OR: 2.12) and the lowest heterogeneity of all interventions analysed (I^2 = 0%). The results for the IVIG and G-CSF both favoured the intervention treatment, but did not achieve statistical significance for either primary outcome, with p-values greater than 0.05 and a 95% confidence interval includes 0. Both also demonstrated high heterogeneity, which further limited interpretability of the results. Finally, intralipid was the only

intervention to not have sufficient data for a meta-analysis, with only one study comparing intralipid to IVIG, rather than a placebo/control group—limiting conclusions about its effect on LBR or MR.

2.5.2. Interpretation of Results with Existing Literature

According to Cochrane Reviews, the minimum number of studies need to achieve a strong meta-analysis with robust conclusions is 5—depending on the topic and level of heterogeneity across the studies. For HCQ as a RPL intervention, there was not even a minimum of two included studies to conduct a small meta-analysis for this review. In prioritising quality over quantity and only including randomised, controlled trials, many studies such as retrospective studies on HCQ (and other interventions) were excluded. However, with the promising results about the effect size of HCQ on LBR and the five ongoing trials found through this review's search, the depth of evidence on HCQ will rapidly develop and allow clinicians to make more informed conclusions about this immunosuppressant in treating RPL. Unsurprisingly, there are currently no published reviews focused on examining the available evidence on HQC for RPL. There are a few limited cohort studies on HCQ registry patients that make it difficult to determine if HCQ could be beneficial for some subgroups, or using a specific drug regimen (Dernoncourt et al., 2024).

After intralipid and HCQ, studies focusing on G-CSF as an immunomodulatory intervention for RPL were most limited for both the LBR and MR outcomes (n = 2, n=1, respectively). For the LBR outcome, the minimum number of studies was just met to perform the meta-analysis. Based on these two studies, the intervention was shown to be effective, but did not achieve statistical significance. According to a systematic review published in 2023, the findings stated that G-CSF may improve LBR in patients with RPL, however this result was not significant (RR=1.35, 95%CI: 0.99–1.84, P = 0.06). This study also included conception rate as an outcome and gave evidence to show G-CSF significantly improves rate of conception in unexplained RPL (RR=1.34, 95%CI: 1.03–1.74, P = 0.028) (Mu et al.). Although the odds ratios for LBR of this previous study and this chapter's meta-analysis

does not show clear and conclusive support for G-CSF as an intervention, it does not rule out G-CSF as a potential therapy for RPL patients. With this study showing a significant increase in conception rate, future trials could investigate the efficacy of G-CSF in combination with other immune therapies from before conception to after. As more studies further elucidate and delineate each immune therapy's specific immunomodulatory role in pregnancy, these different drugs could work in conjunction to efficiently and effectively treat RPL.

With a total of 4 included studies, this systematic review revealed steroids as having the greatest effect on improving LBR (OR: 2.16) and reducing MR (OR: 0.29)—both significant findings. A previous systematic review published in 2022 by Li et al., also concluded that glucocorticoid treatment can increase LBR (RR 1.92, 95% CI 1.17–3.16) and clinical pregnancy rate. However, this study cited no effect on MR, regardless of the timing of administration. Another review published earlier in 2015 provides evidence that steroid-therapy, specifically prednisolone treatment, significantly improves LBR (RR 1.58, 95% CI 1.23-2.02) (Dan et al., 2015). The results of this chapter's review support the promising findings of these studies; however, this analysis is limited by the high heterogeneity and cautions overgeneralising this potential therapeutic effect (Tempfer et al., 2006). These limitations and the contradictory trial results may be why the HFEA has given steroids a red rating for improving the chances of a live birth in fertility patients, even though this meta-analysis result illustrates this drug as a promising option. With this being the only drug with a significant beneficial effect, I believe steroid's known anti-inflammatory properties are conducive to suppressing the overactive immune response common in the endometrial environment of RPL patients. For patients, with elevating NK cells or a higher Th1/Th2 ratio, steroids can downregulate pro-inflammatory cytokines, such as TNF and IFN, and promote a Th2-dominant environment (Luu et al., 2022, Elenkov, 2006). This mechanism could be further examined through future stratification of studies to include RPL patients with a profile of elevated NK cells and an altered Th1/Th2.

Several systematic reviews have examined the efficacy of IVIG in treating RPL, with conflicting results. One of these reviews published in 2022 reported that when compared with the control group, IVIG can increase LBR (OR = 3.06, 95% CI (1.23, 7.64), P = 0.2) with an evidence base of 15 articles (Shi et al.). This finding aligns with an earlier review by Wang et al. (2016), which also reported a significant increase in LBR in the IVIG-treated group. Conversely, a more recent study synthesizing data across 15 studies found no significant effect of IVIG on LBR (OR = 1.07, 95% CI (0.65, 1.75), P= 0.80) (Ling et al., 2024).

The discrepancy between these studies can be partially attributed to differences in study inclusion criteria. The former study included non-randomised studies excluded in this chapter's review, whereas the Ling et al., study including only RCTs also. The pooled odds ratio revealed in this chapter's meta-analysis (OR: 1.04, 95% CI (0.76, 1.42), P = 0.81) supports the conclusion that IVIG group does not provide a consistent therapeutic benefit for RPL overall. Nevertheless, IVIG remains a potential treatment, but possibly for specific subgroups of RPL patients. For example, IVIG may not only be more effective in patients with unexplained RPL, but also in cases of secondary RPL. Although, these effects are not consistent across all reviews (Shi et al., 2022, Wang et al., 2016). Another important factor is the timing of IVIG administration. Some studies suggest that administering IVIG before conception—rather than after—can increase the live birth rate significantly (Wang et al., 2016). With the extensive depth of IVIG trials and potential for future trials that focus on stratifying RPL participants by immunological markers (raised NK cells) or type of miscarriage, more robust and target evidence may emerge amongst the literature. This research could clarify IVIG's efficacy and reduce the high heterogeneity in this review and many others, allowing clinicians to make more accurate conclusions on IVIG's therapeutic value. Currently, this review cannot support the use of IVIG nor conclusively rule out its benefits in treating RPL.

Intralipid, although a commonly prescribed treatment for parenteral nutrition, is still controversial in this field. In this systematic review, only studies (in the 2nd screen) involving intralipid were retracted or withdrawn (n = 3). As a result, the findings of previous reviews examining the efficacy of intralipid in RPL that include these studies, are less reliable (Kumar et al., 2021). The relatively high number of withdrawn trials reveal how challenging it is to conduct a high-quality, low risk randomised clinical trial. Although it is not known why these trials were retracted, it has critical implications for previous systematic reviews that included the results of these trials. This review includes a trial that is now withdrawn according to the Int J Gynaecol Obstetrics journal (Dakhly et al., 2016, Martini et al., 2018). This is not the only review that includes withdrawn trials, and these situations reflect the broader issue of research misconduct and data integrity in medical research. Other reviews on intralipid in RPL or recurrent implantation failure (RIF) also state that the quality of included studies is poor (Placais et al., 2020). The inclusion of questionable studies in previous reviews raises concerns about the reliability of conclusions and clinical recommendations made on intralipids. This further underlines the importance of rigorous screening and quality assessment of included studies. Compared to the literature on intralipids, the inconclusive findings in this meta-analysis are due to lack of evidence and the conclusions are inconsistent with other reviews. The one included intralipid article, Meng et al., is a trial that measure the efficacy of intralipid against a comparator group receiving IVIG—rather than a control/placebo group. The single study included in this review shows that there is a need for clinical trials investigating the intralipid as an effective treatment in RPL that are of proper study design: randomised, double-blinded, placebo group, and single/multicentred.

Overall, the meta-analysis in this chapter only provides evidence in support of steroids in the treatment of RPL, with IVIG and G-CSF requiring more evidence with less variability. Furthermore, no conclusions could be drawn about the efficacy of HCQ and intralipid in RPL, reflecting the need for more RCTs to demonstrate these drugs as efficient immune therapies. According to the HFEA,

intralipids is considered a potential intervention, and unlike immune therapies such as IVIG and steroids that have received a negative rating, intralipid does not yet have enough evidence to warrant a conclusive rating yet.

2.5.3. Clinical Implications of treating RPL with Immunomodulators

In terms of clinical applications, the findings of this review hold relevance for all five of the included immunotherapies. However, the limited evidence base and significant heterogeneity in most of these interventions require careful interpretation of the results. Any conclusion should be understood and received within the broader context of the existing literature and tailored to the clinical needs of each individual patient. Ultimately, a personalised approach to RPL treatment remains critical in effective treatment of this complex condition. As discussed, each immunomodulator may be most beneficial in specific subgroups of patients, or in conjunction with other drugs to maximise its efficacy. Future research should focus on identifying biomarkers or clinical characteristics that further stratify RPL patients, allowing better predictions for treatment response and optimisation of drug regimens.

2.5.4. Ongoing Work & Future Directions

This chapter focuses on the trials investigating the efficacy of known immunomodulators in RPL, however the ongoing work is aimed towards a comprehensive review of all therapies with either known or unknown immunomodulatory functions. This includes common RPL therapies, such as progesterone, heparin, and aspirin. Once this review is completed, it will be the first systematic review/meta-analysis of all the relevant immunomodulators in the treatment of RPL, whether unexplained or known causes. There is a substantial number of systematic reviews focused on a singular immunomodulator (Devall et al., 2021, Scarrone et al., 2024, Ling et al., 2024) This review will scale these results and conclusion about effect size across all therapies, such as IVIG, heparin, G-CSF, and leukocyte infusions. The aim is a publication that can incorporate high-quality evidence for

clinical recommendations. For utmost relevancy and accuracy, the searches will also be re-run every six months until completion and the new studies will be updated on Rayyan, the review manager.

2.5.5. Strengths & Limitations

This review is not completed according to the initial plain from creating the research question to the meta-analysis, however some key elements such as the reproducible methods to avoid bias, the use of multiple search databases, and critical assessment of the included study are strengths of this chapter. Firstly, the broad search yielded over 10,000 articles across 5 databases. With a minimum of 3 expected in a quality systematic review (McGowan and Sampson, 2005), the searches included in this review's screening minimised the likelihood of a relevant RCT being excluded. However, this required more time and effort to accurately review all titles and abstracts. Furthermore, after finishing the full text screening, it is evident that there was low yield of relevant and eligible studies compared to the total amount in the initial screen. This could signify a shortcoming in the study design or search terms of it being too broad. Additionally, the methodology of this review is based on established tools provided by reputable organizations such as the Cochrane Guidelines for Systematic Reviews and PRISMA guidelines. By adhering to these protocols, the aim is to mitigate the inclusion of low-quality studies that are not relevant to the research question. This approach is critical, given the inconsistencies surrounding the use of intralipids in RPL treatment and a strength of this thesis.

Firstly, a strength of this review is the inclusion of different studies that have varying drug regimens, such as different dosages and modes of administration, and synthesising all the data across the different study pool. A signal that a trial should be considered high-quality evidence is if it provides a dosage gradient of the drug. This criterion of quality evidence can be extrapolated to the broader meta-analysis across multiple trials. Subgroup analyses of the intralipid patients may have included different dosages, mode of conception, preliminary screening of RPL immune profiles, and

primary or secondary RPL patients. For example, while manually screening the searches, I noticed different trials will either specifically include or exclude RPL patients due to antiphospholipid syndrome or explicitly state that the patient cases included are unexplained. These delineations could have provided stratification of RPL patients and variables to compare effect sizes across. Another relevant subgroup would have been if the patients were using assisted reproductive technologies (ART). Assisted conception would have been displayed as a weighted estimate in the forest plots. Overall, the lack of trustworthy and relevant RCTs prevented clear conclusions about the effectiveness of intralipid, HCQ, G-CSF, and IVIG in RPL from being made.

Also, this review's aim to gather and report all evidence on the efficacy and safety of treating RPL with various immunomodulators, and therefore studies were not excluded based on their quality. This could have limited the quality of evidence; however, a strength of this study is the high-quality of evidence included. Using the Cochrane RoB 2 tool, the studies were primarily of low risk of bias. In terms of certainty using the GRADE assessment, the effect size of the meta-analyses is a very good indication of the likely effect for all immunomodulators, except for intralipid. It is evident that many of the included studies are high-quality studies with a few outliers. This is likely because this review only includes RCTs and traditionally, RCTs are considered a "gold standard" for evidence due to their ability to eliminate bias through its particular methodology (Fernainy et al., 2024). However, recent reports on the prevalence of flawed or completely falsified trials have experts estimating that up to 1 in 4 clinical trials should be deemed untrustworthy (Van Noorden, 2023). These quality assessments highlights that although some individuals have potential biases, particularly in domains of study design and selective reporting of results, these findings can be interpreted with moderate to high confidence and low risk of bias.

2.6. Conclusions

As discussed in the section above, this limitation underscores the need for further randomized controlled trials with robust methodologies and larger sample sizes to confirm the benefits of intralipid, HCQ, IVIG, and G-CSF in RPL. However, the evidence supports steroids as a intervention with the biggest significant effect size on improving LBR and decreasing MR. Until such data becomes available, the current evidence, while promising, is insufficient to draw definitive conclusions or recommend widespread clinical implementation. This review utilizes quality assessments, such as risk of bias tools and trustworthiness frameworks, to provide information on the quality and certainty of included studies; these methods will be applied to the included studies for other immunomodulators as the review is continued in this upcoming year. The evident lack of literature on intralipids in RPL shows a need for more *in vitro* and translational research to further our understanding of the drug on a molecular and cellular level. This is further explored in the following chapter where intralipid and HCQ are tested in patient-derived cell line models.

Chapter 3: Immunomodulation and Decidualisation: The Impact of Intralipid on MCP-1 Expression in RPL

3. 1 | Introduction

3.1.1. Inducing Decidualisation

As established in Chapter 1, decidualisation is a tightly regulated and necessary process for successful pregnancy. This process is initiated by an increase in cAMP levels, driven by estradiol and progesterone. Key molecular markers of decidualisation include insulin growth factor binding protein-1 (IGFBP-1) and prolactin (PRL), both preferentially expression and secreted by decidual stromal cells (Tamura et al., 2012). These markers are widely used to evaluate the extent of decidualisation in human endometrial stromal cells (hESCs).

Patients with RPL exhibit reduced prolactin levels and altered expression of Prokineticin-1 (PROK1), a critical regulator of endometrium receptivity controlled by progesterone (Salker et al., 2010). This is one of the many key discoveries that have furthered our incomplete understanding of the precise signal transduction pathways mediating the effects of progesterone and other decidualisation stimuli. It is known that cAMP activates the protein kinase A (PKA) pathway, inducing the expression of progesterone isoforms PGR-B and PGR-AB (Retis-Resendiz et al., 2024).

In *in vitro* studies, it is shown that cAMP is a more effective stimulus for decidualisation and subsequently, IGFBP-1 and PRL gene transcription in ESCs than progesterone alone (Gellersen & Brosens, 2003). Accordingly, to mimic the in vivo environment of the endometrium in my experiments, I incubated primary ESCs with dibutyrl-cyclic adenosine monophosphate (dbcAMP), a cell-permeable synthetic analogue of cAMP with progesterone in my experiments to optimize decidualisation outcomes.

3. 2 | Chapter Objectives

This chapter focuses on understanding the immunomodulatory effects of immune-modulating drugs in the treatment of recurrent pregnancy loss. With endometrial stromal cells (ESCs) playing a critical role in the decidualisation and initiating endometrial receptivity to implantation, derived hESC cell lines are the choice of model in this thesis. Based on the results of the review in Chapter 2, this chapter is focused on studying the effect of intralipids and hydroxychloroquine (HCQ). As seen with the paucity of quality intralipid clinical trials (Table 6), our understanding of intralipid in the treatment of RPL is not well established. Additionally, the meta-analysis revealed HCQ as a promising intervention in RPL, however this conclusion is based on evidence from two trials (Figure 2.5). For both understudied interventions, there is also a lack of translational research examining the exact mechanisms of these drugs—this chapter aims to lessen this gap. After establishing model human endometrial stromal cells (hESCs) and confirming each cell line can decidualise, an exploratory experiment was conducted to formulate a hypothesis as to how intralipid and HCQ can modulate inflammatory cytokines. Using patient-derived cell lines, I aim to strengthen and test this hypothesis to bring clinical relevance to the findings of this thesis. The methodology outlined in this chapter included optimization steps and experiment designed concurrently as the results of the previous experiments are analysed. In this chapter, the impact of two immunomodulator drugs on stromal cells during decidualisation will be investigated, with the aim of elucidating their potential role in enhancing or disrupting this critical process. I hypothesize that both intralipid and HCQ will promote an increase in cytokines associated with an anti-inflammatory profile, based on the concept that immune therapies may counteract the excessively inflammatory endometrial environment observed in RPL patients,

Chapter Objectives:

1. To establish primary human endometrial stromal cell lines derived from both control and recurrent pregnancy loss patients.
2. To optimize a protocol for the decidualisation of stromal cells into secretory decidual cells, incorporating intralipid and hydroxychloroquine drug treatments.
3. To profile the inflammatory cytokines released from intralipid- and HCQ-treated stromal cells throughout decidualisation using an immunoassay.
4. Further examine the effect of intralipid on stromal cells at varying concentrations and timepoints.

3.3 | Methods

3.3.1. Cell Culture

All cells used in this thesis were kept in incubator conditions of 37°C, 5% CO2. Human endometrial stromal cells were cultured in DMEM/F12 media supplemented with 10% FBS (Sigma, F7524), 1% Penicillin-Streptomycin (Sigma, P4333), and 1% L-Glutamine, with media changes every two days.

3.3.1.1. Patient Samples & Establishment of Stromal Cell Lines

All cell lines were derived from donated patient endometrial sample. The RPL cells lines originated from patients attending the Oxford University Hospitals' Recurrent Pregnancy Loss (RPL) clinic over the past last 8 years. The earliest sample used in this study (RPL4) was thawed from cryostorage from 2019. While patient age data for the control samples is unavailable, the RPL patients ranged from 31 to 37, with an average age of 34.4. Other information including the number of miscarriages is unavailable, however, these patients are all diagnosed using the RCOG definition of RPL, meaning they have experienced a minimum of 3 consecutive miscarriages.

All control and recurrent pregnancy loss cell lines were thawed from cryopreserved vials of stromal cells stored in liquid nitrogen. The cryotubes were thawed in a 37°C water bath and re-suspended in pre-warm growth medium (phenol red-free DMEM/F-12 media supplemented with 10% fetal bovine serum (FBS) (Sigma, F7524), 1% penicillin-streptomycin). The cell suspension was plated in culture flasks and incubated overnight to allow stromal cells adherence. After 24 hours, non-adherent cells and media were removed, and fresh growth media was added.

Cell line RPL4 was grown from a frozen vial of human endometrial cells (HECs) after the stromal cells were left to adhere overnight in supplemented growth medium. Cell lines RPL5 and RPL6 were derived

from processed human endometrial tissues using the Methods of sample processing referenced in the Appendix.

3.3.1.2. Passaging

The stromal cell culture medium was replaced every two days, and cells were passaged when they reached approximately 80% confluence. For passage, cells were washed with phosphate-buffered saline (PBS) and detached using trypsin (Sigma, T3924). After a three-minute incubation at 37°C, the trypsin was neutralized with an equal volume of growth medium. The cell suspension was transferred to a centrifuge tube and centrifuged at 300 x g for three minutes. The supernatant was removed, and the cell pellet was washed with PBS. Cells were then re-suspended in growth medium for re-plating. Cells were counted using cell counting slides and a hemocytometer (BioRad).

3.3.1.3. Storing Cells

After cellular detachment, stromal cells were washed then cryopreserved in freezing medium (FBS with 10% DMSO) for storage at low passages (P2-P5). The cryotubes were frozen at a controlled rate using a Mr. Frosty Nalgene (Nunc) box stored in -80°C freezer. For long-term storage, the cryotubes were transferred into liquid nitrogen tanks.

3.3.2. Decidualisation of Stromal Cells to Decidual Cells

The decidualisation protocol used in this thesis project is based on protocols established in the published literature for human endometrial stromal cells and from Dr. Magda Mareckova's Dphil thesis (Brar et al., 1997, Okada et al., 2018).

The stromal cells were seeded at 1 x 10^5 cells/well in a 12-well plate and left to adhere for 3 to 6 hours. Of note cell counts were obtained with 1:1 dilution of 0.4% trypan blue staining for viability. Media was next changed to decidualisation medium (DMEM/F-12 medium with 0.05 mM N^6,2'O-

Dibutyryladenosine 3',5'-cyclic-monophosphate sodium salt (dbcAMP) (Sigma, D0627) and 1 μM progesterone (Sigma, P8783) (Kautz et al., 2015). The decidualisation medium was changed every two days for an 8-day period with the supernatant collected in Eppendorf tubes and stored at -20°C for analysis.

3.3.2.1. Optimization of Decidualisation Protocol

To optimize the decidualisation protocol, the following experiments were completed:

- Seeding cells in 6-well, 12-well, and 24-well plates
- Maximum passage of stromal cells for successful decidualisation
- Changing the initial seeding density of stromal cells in 12-well plates (50,000/well and 100,000/well) to prevent maximum confluency before the last timepoint
- Length of protocol (6, 7, 8 days)
- Composition of decidualisation medium (with/without estradiol, progesterone, and dbcAMP)
- Amount of decidualisation medium per well

All stromal cells were decidualised in the following conditions and protocol:

- seeding concentration of 1 x 10^5 cells/well to maximize the chemokine secretory signal during decidualisation
- 8-day protocol was used rather than 6- or 7-days to accommodate for the cell lines that did not decidualise until after the Day 2 or Day 4 timepoint
- As seen in Figure 3.7, for two of the recurrent pregnancy loss cell lines, there was still an upward trend in prolactin concentration, a marker of decidualisation.
- Attempts to decidualize cells after passage 6 to 8, depending on the cell line, were unsuccessful. Therefore, all cells were on lower passages, from P2 to P5.

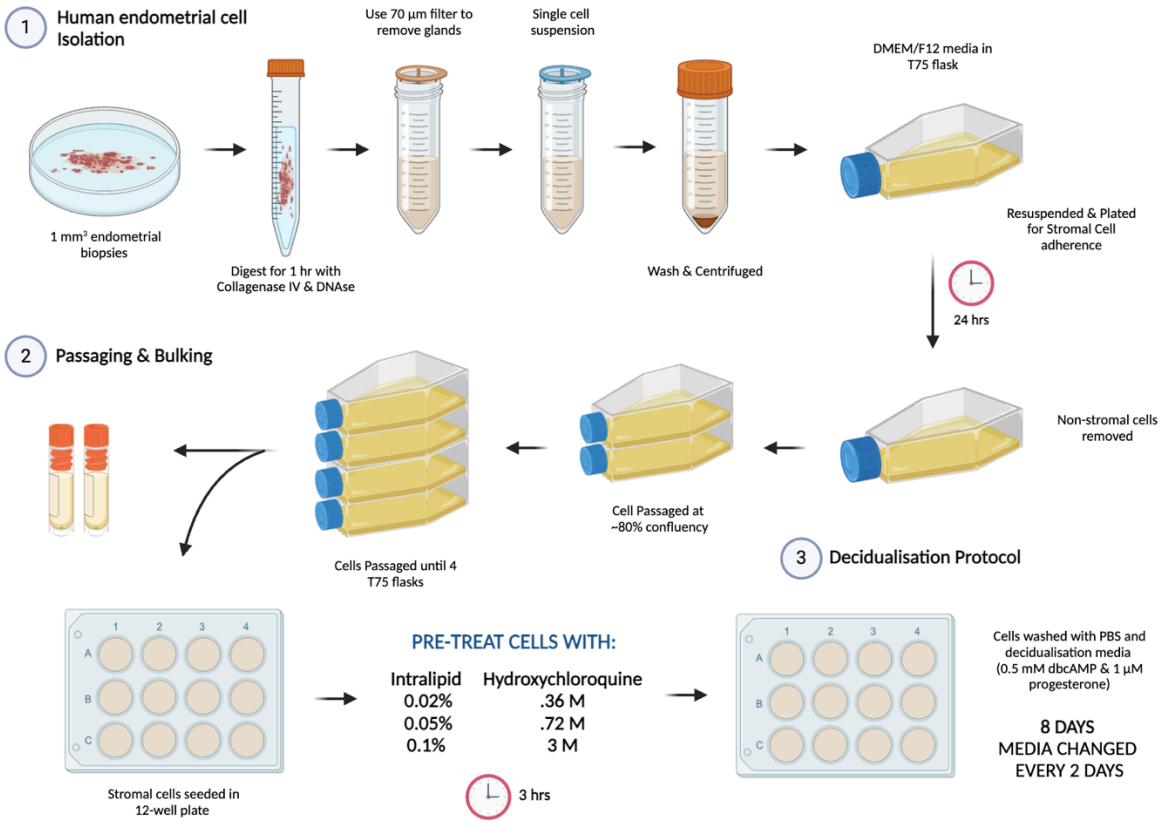


Figure 3.1: Schematic of Isolation & Decidualisation Protocol. This image was made using BioRender.

3.3.2.2. Treating Cells with HCQ & Intralipid

The concentration range of hydroxychloroquine was chosen based on studies on endometrial cells and the optimized hydroxychloroquine dosing schemes. 500-2000 ng/mL has been proposed as safe for clinical usage, and the three-molar concentrations of HCQ were calculated from this range: 0.36 M, 0.72 M, and 3 M.

For the hydroxychloroquine drug treatment experiment, the stromal cells were seeded and pre-treated with growth medium containing just hydroxychloroquine at the following concentrations: 0.36 M, 0.72 M, and 3 M on Day 0. After a 3-hour incubation period, the cells were washed with PBS and decidualisation media with 0.05 mM dbcAMP and 1 µM progesterone. On Day 2, 4, and 6, the

medium was changed with fresh decidualisation media (including the HCQ) for an 8-day decidualisation period.

The concentration range of Intralipid 20% was determined based on dosage used in clinical trials in the treatment of RPL. Using data from these trials, which utilized a 20% intralipid infusion ranging from 100 mL to 4 mL (Foyle, El-Gegawy), I calculated the corresponding percentage relative to the average blood volume of a human patient (1 mL per 5 L, or 0.02%). Given the potential impact of the intralipid's fatty acids on the stromal cell viability, including the risk of inducing cellular senescence, stromal cells in this project were treated with intralipid only prior to initiating the decidualisation protocol, consistent with typical clinical regimens involving one or two initial intralipid infusions.

A titration experiment was conducted to establish the maximum intralipid concentration that the stromal cells could tolerate. Based on microscopy images showing non-viable cells above 0.1% intralipid, three concentrations--0.02%, 0.05%, and 0.1%--were selected for further experimentation. On Day 0 of the decidualisation protocol, the seeded stromal cells were treated with growth medium containing the three intralipid concentrations for 3-hours. After this incubation period, the cells were washed with PBS and incubated in decidualisation medium.

3.3.3. Confirmation of Decidualisation

Decidualisation can be confirmed through two methods: morphological changes and the release of biomarkers.

3.3.3.1. Microscope Imaging

Each repeat of every well containing stromal cells was imaged on Day 0 and every 2 days before the decidualisation medium changes. The cells were captured at a magnification of 10X on

brightfield microscopy (Leica Microsystems). One image of each repeat represents the morphology of the cells under each treatment and timepoint.

3.3.3.2. ELISA Assays: Human Prolactin, IGFBP-1 & MCP-1

Enzyme-linked immunosorbent assays (ELISA) were used to detect and quantify Prolactin and IGFBP-1 in the supernatant collected throughout the decidualisation protocols. Each ELISA assay followed a standardized plate preparation and assay procedure from corresponding ELISA DuoSet kits (R&D), with specific antibodies and reagent diluents: prolactin (1% BSA in PBS) (R&D, DY682), CCL2/MCP-1 (1% BSA in PBS) (R&D, DY279), and IGFBP-1 (5% Tween 20 in PBS) (R&D, DY871).

For plate preparation, a 96-well flat-bottom plate was coated with 100 µL of the capture antibody, diluted to the working concentration (Table 1), per well. The plate was sealed and incubated overnight at RT. The following day, wells were aspirated and washed three times with 400 µL of wash buffer (0.05% Tween 20 in PBS), with each wash followed by inverting and blotted against clean paper towels to ensure removal of all liquid. The wells were than blocked with the addition of 300 µL of Reagent Diluent per well and incubated at RT for a minimum of 1 hour. After blocking, the plate underwent three washes to prepare for standard and sample addition.

Serial dilutions of the seven standards were made in reagent diluent, while the supernatants were used undiluted. Standards and samples were run in duplicate, with 100 µL of each added to the respective wells and incubated at RT for 2 hours. Following incubation, the plate was washed three times and 100 µL of the detection antibody, diluted to working concentration in reagent diluent, was added to each well for a 2-hour incubation at RT. The plate was washed again three times, followed by the addition of 100 µL of the HRP-conjugated streptavidin, diluted with reagent diluent, to each well for incubation in the dark for 20 minutes at RT.

For the last incubation, the plate was washed three times, and 100 µL of the substrate solution was added per well for 20 minutes. After the reaction, 50 µL of stop solution was added per well, and the absorbance was read at 540 nm using a microplate reader (OPTIMA). All incubation steps were performed promptly and with the aid of a multi-channel pipette to ensure consistency and precision. Before every incubation period, the plate was sealed with a plate sealer.

For analysis of the raw ELISA data, blank wells with fresh growth medium and the supernatant samples untreated stromal cells served as a correction-factor and the threshold of significance for the absorbances of the unknown samples. Based on 95% confidence intervals and the standard error, I calculated the different error range and set the threshold for "decidualisation" at 25 pg/mL to signify significant secretion for decidualisation markers.

3.3.4. LEGENDPlex (BioLegend) Human Inflammation Immunoassay

The LEGENDplex Human Inflammation Panel 1 with the V-bottom plate kit from BioLegend (740809) was used. The flow cytometry settings provided by BioLegend were adjusted to these samples and the cell samples were analysed on the Northern Lights Flow Cytometry System (Cytek).

The assay is a bead-based immunoassay that uses the same basic principle as sandwich assays. There are 13 beads conjugated with a specific antibody that captures the corresponding analyte. Each bead is differentiated by size and internal fluorescence intensities allowing for analyte-specific populations to be selected and quantified on a flow cytometer. The concentration of a particular analyte, or cytokine, is determined using a standard curve generated in the same assay.

3.3.4.1. Sample Preparation & Assay Protocol

Supernatant samples from the previous decidualisation experiments with the intralipid and hydroxychloroquine treatments were left at room temperature (RT) to thaw. Since the levels of analyte can vary greatly from sample to sample, the samples were left undiluted to measure the strongest

analyte signal. The standards were prepared using a lyophilized standard cocktail with assay buffer using a serial dilution method with varying starting concentrations of each analyte. Maximum standard values are shown in Table 14.

After all reagents and supernatants are warmed to RT, the assay was prepared in a 96-well well v-bottom plate. The standards and samples were added to the corresponding wells along with 25 µL of assay buffer. Then, the mixed beads were vortexed for 30 seconds and 25 µL of mixed beads was added to each well. To avoid the beads settling, the mixed beads were intermittently vortexed. The plate was sealed with a plate sealer and covered entirely in aluminium foil to protect it from light and placed on a 200 x g plate shaker for 2 hours at RT.

After the 2-hour incubation period, the plate was centrifuged using a swinging bucket rotor (G.H 3.8) with a microplate adaptor (Beckman-Coulter). Immediately after, the supernatant was removed by quickly inverting and flicking the plate and blotted on clean paper towels. The plate was then washed by added 200 µL of wash buffer into each well and a 1-minute incubation. The plate was then centrifuged and underwent a second wash step to reduce background. Next, 25 µL of the detection antibodies was added to each well and incubated for 1 hour at RT on the plate shaker, once again wrapped in aluminium foil. After incubation, 25 µL of SA-PE was added directly to each well, without a washing. The plate was then sealed and wrapped in aluminium foil and left to incubate on the plate shaker at RT. The plate was then centrifuged and washed with wash buffer. The beads were then re-suspended with 150 µL of wash buffer in each well by pipetting. The plate was then stored at 4°C until the samples were read on a flow cytometer.

Analyte	Bead ID	Top Standard Concentration (ng/mL)
IL-1B	A4	13
IFN-A2	A5	12
IFN-Y	A6	11
TNF-A	A7	12
MCP-1	A8	13
IL-6	A10	11
IL-8	B2	12
IL-10	B3	15
IL-12p70	B4	14
IL-17A	B5	3
IL-18	B6	16
IL-23	B7	18
IL-33	B8	80

Table 16: Immunoassay Analytes. The analytes and the corresponding bead ID included in the BioLegend LEGENDplex Human Inflammation Panel I assay (740809) is displayed along with the top standard concentration determined by the lot number of the kit (B408881). There are thirteen analytes included, and the beads are divided into two bead populations: smaller (A) and larger (B) beads. All beads can be detected using FL3, FL4, or APC channel.

3.3.4.2. Flow Cytometer Setup

The cytometer was programmed to automatically read samples by column from top to bottom, left to right on a 96-well v-bottom plate. The assay bead emission spectra were analysed using R4 as the Bead Classification and B4 as the Reporter channels. The Raw beads and the PE beads were run to verify that that bead populations were on scale on the B4 axis on the R4 vs. B4 plots. Subsequently, the cytometer was set to the following settings: 79 (FSC), 4 (SSC), and 28 (SSC-B) with the gains of all channels lowered to 80%. While reading the samples, the cytometer was set to a shake time of 3 and shake speed of 1200 before every well was read. Each sample was read until 5000 events were recorded.

3.3.5. Co-Culture of THP-1 monocytes & stromal cells

Through trial-and-error, I developed the following co-culture protocol with guidance from various sources about *in vitro* cell migration assays and models. The aim of this experiment was to co-culture stromal cells and a monocyte cells line, THP-1, to examine the possible effect of migration

due to the MCP-1 chemokine gradient. (ThermoFisher, Olejnik et al., Justus et al., *Transwell In Vitro Cell Migration and Invasion Assays*)

3.3.5.1. Controls

Baseline migration was assessed using a control with only THP-1 cells in the upper chamber and no stromal cells in the lower chamber, with three replicates. Since the stromal cells are adherent, no control for baseline stromal cells migration was needed.

3.3.5.2. Cell Preparation & Seeding

THP-1 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin. THP-1 cells were kept in an upright T75 flasks at a density of 0.5-1 x 10^6 cells/mL, media was changed every three days.

Stromal cells were trypsinized and re-suspended in complete DMEM/F-12 medium to achieve a final concentration of 1 x 10^5 cell/mL.

Stromal Cell Seeding in the Lower Chamber

5 x 10^4 stromal cells were added to each well of a 24-well plate, allowing for three repeats of each intralipid treatment concentration and cell line. The cells were incubated at 37°C in a 5% CO2 incubator for 3 hours to allow for adherence and establishment of a monolayer. After adherence, the cells were treated with 600 µL of 0.02%, 0.05%, or 0.1% intralipid medium for 3 hours. Complete DMEM/F-12 medium without intralipid was added to the control wells. The stromal cells were then washed with PBS and decidualisation was induced using medium consisting of 0.05 mM dbcAMP and 1 µM progesterone in complete DMEM/F-12 medium. The decidualisation medium was changed on Day 2 and Day 4.

3.3.5.3. Transwell Insert Setup & Incubation

On Day 4 of decidualisation after the medium of the lower chamber was replaced with fresh decidualisation media, THP-1 cells were prepared in serum-free RPMI-1640 medium and adjusted to a concentration of x 10^6 cells/mL. The transwell inserts were prepped by allowing each insert well and its membrane to incubate in warm serum-free RPMI-1640 medium for 10 minutes. A 100 µL aliquot (containing 1 x 10^5 cells) of the THP-1 suspension was seeded into the upper chamber of the transwell insert with an 8 µm pore size membrane. The transwell insert was placed into each well containing the decidualised stromal cells. The 24-well plate was gently agitated to ensure even distribution of cells across the insert. The wells with the inserts were incubated for 24 hours to allow for the THP-1 cells to respond to the chemokine gradient released from the stromal cells.

3.3.5.4. Assessment of Cell Migration

After the incubation period, the transwell inserts were carefully removed from the wells. The non-adherent THP-1 cells are now suspended in the lower chamber of the assay having migrated through the pores of the transwell membrane. The supernatant, containing the migrated cells, was collected and prepared for quantification. After careful pipetting to ensure an evenly distributed cell suspension of the collected THP-1 cells, 10 µL aliquots were loaded onto cell counter slides (BioRad) and counted using a hemocytometer. The total number of migrated cells was calculated using the equation:

Total Number of Migrated Cells = [__ cell/mL] X collected volume

3.3.5.5. Optimization

Based on other studies examining THP-1 in migration assays, the most common pore size is 8 µm, which is the size used in this experiment. Due to the relatively weak MCP-1 chemoattractant gradient, the incubation time was 24 hours to allow the THP-1 cells to interact with the chemoattracts and travel across the insert membrane. Stromal cell samples RPL2 and RPL6 were chosen as the

representative cell lines in this experiment due to the relatively high levels of MCP-1 secreted by the secretory decidual cells as shown in the Results section. Stromal cells RPL1 was also included since there was no trend or significant chance in MCP-1 concentrations with or without the intralipid treatment. Stromal cells from sample control 1 were used as the control and the relatively equal levels of MCP-1 concentration with or without intralipid. With the stromal cells being adherent and THP-1 cells non-adherent, the readout data will be efficiently collected using a haemocytometer (Justus et al., 2023).

3.3.5.6. Data Analysis

Migration rates of THP-1 cells under no treatment and the varying intralipid concentrations were compared to evaluate the influence of intralipid-treated stromal cells on THP-1 migration. Migrated cell numbers were corrected with the baseline migration control average.

3.4 | Results

3.4.1. Decidualisation of Cell Lines

I generated nine human primary endometrial stromal cell lines, both control (n = 3) and derived from RPL patients (n = 6). I was able to visualise the decidualisation process occurring over an 8-day time course by taking images of the cells every 2 days. Based on the morphological changes and secretory biomarkers, all 9 cell lines decidualised upon treatment with progesterone (1 μM) and dbcAMP (0.5 mM). As seen in Figure 3.2, endometrial stromal cells are fibroblast-like cells that appear elongated and spindle-shaped but become rounded and cobblestone-like when transforming into secretory decidual cells.

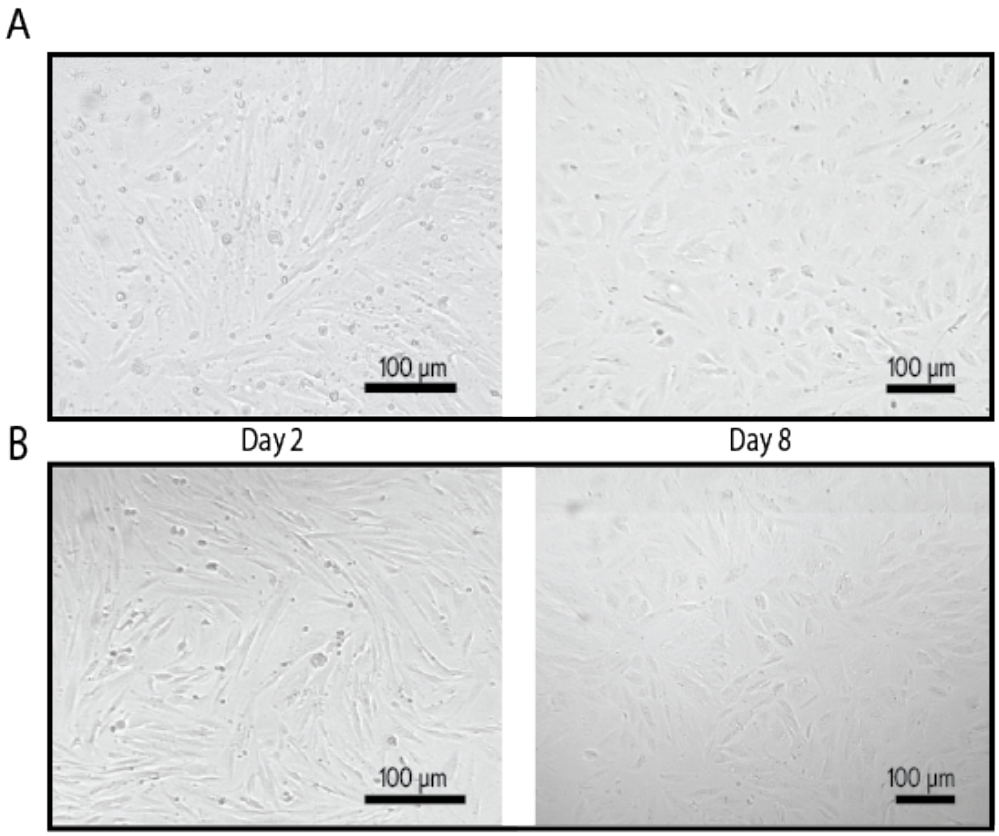


Figure 3.2: Stromal cells morphological changes through decidualisation treatment. A. Representative control cell line. The control 2 (C2) cell line is shown. **B. Representation RPL cell line.** The RPL 2 cell line is shown. This figure depicts human endometrial stromal cells (hESCs) on the 2nd and 8th day of the decidualisation protocol. The hESCs were seeded in a 12-well plate with a cell density of (1 x 10^6) and grown in decidualisation medium (1μM progesterone and 0.5 mM dbcAMP) for 8 days. Above are representative images of the morphology and size of hESCs before and after they undergo transformation from rounded stromal cells into secretory decidual cells that are fibroblast-like in shape. With three repeats

of each cell line for every 2-day timepoint, the clearest image for C2 and RPL2 on Day 2 and 8 was chosen. The images were taken on a 10X objective, in brightfield mode, and the figure was made in Adobe Illustrator. Scale bars representing 100 microns (μm) are in all four images.

All nine cell lines established showed morphological changes between days 2 and 8, with cell lines control 2 and RPL2 shown as examples in Figure 3.2. Decidualisation is also assessed using the stromal cell line supernatants and ELISA assays for decidualisation protein markers, IGFBP1 and PRL.

3.4.1.2. Prolactin & IGFBP-1 released after successful decidualisation

ELISA assays were performed analysing the supernatant samples collected every two days during the decidualisation protocol to determine the levels prolactin and IGFBP-1. As seen in Figure 3.3, eight cell lines (C1, C2, C3, RPL1, RPL2, RPL3, RPL4, RPL6) were determined to have successfully decidualised by the significant levels of prolactin released by the transformed ESCs.

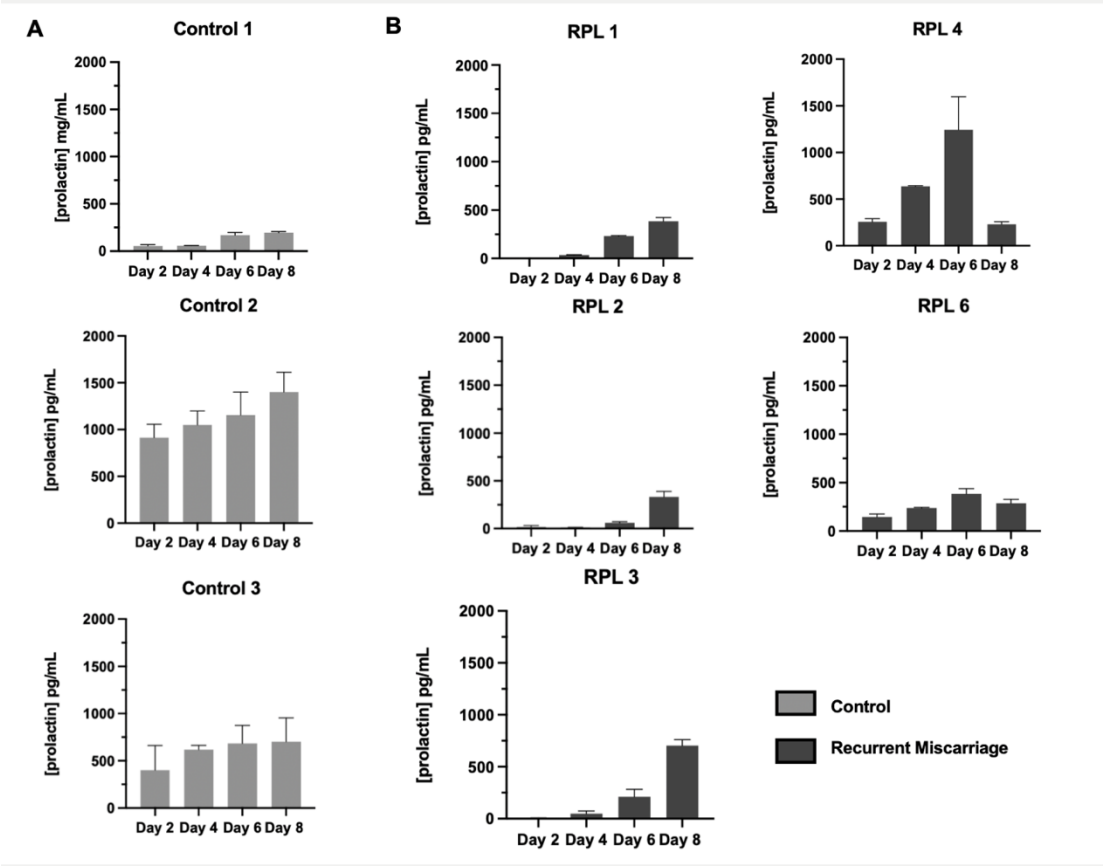


Figure 3.3: Prolactin secretion levels in cell lines during decidualisation. Human endometrial stromal cells isolated from individual patients were treated with decidualisation medium (progesterone (1μM) and dbcAMP (0.5 mM)) in 12-well plate across three identical repeats (N=3). The supernatant samples were collected every two days before replaced with fresh decidualisation medium. Prolactin was detected by ELISA (R&D), bar graphs display the mean concentration with standard error bars (SEM) across the three repeats at each 2-day timepoint across.

Based on the blank ELISA samples for background noise and wells with untreated stromal cell (negative control for no decidualisation), I corrected all the raw ELISA values of the treated cells, ensuring that any prolactin signal greater than 0 pg/mL shows decidualisation. All cell lines eventually reached prolactin concentrations greater than 0 pg/mL with all cell lines reaching 150 pg/mL or greater. The largest prolactin concentration was exhibited in cell line Control 2 on Day 8 (1,400.97 pg/mL) and the lowest range by Control 1 (142.36 pg/mL). Between the control and RPL samples, there is no evident correlation between levels of prolactin and the two groups. Samples RPL1, RPL2, and RPL3 had relatively lower levels of prolactin on Day 2 and Day 4, reaching their highest concentration level on Day 8. The level of prolactin secreted by RPL1 was initially 0 pg/mL, but from

day 4 to day 8, increased by a fold of 10.74 and is significant with a p-value of 0.0132 using a one-way ANOVA. Similarly, RPL 2 increased by a fold of 20.94 ($p = 0.05$) and RPL 3 by a fold change of 9.47 ($p = 0.0092$) from day 4 to day 8. For RPL4 and RPL6, significant levels of prolactin were secreted across all two-day timepoints, with RPL4 increasing 4.83-fold ($p = 0.1034$) from day 2 to day 6 before decreasing by 5.40-fold on day 8 ($p=0.0982$). RPL6 prolactin levels similarly followed this trend, increasing 2.63-fold ($p=0.0848$) from day 2 to 6 and decreasing by 1.33-fold ($p=0.0263$) on day 8. I analysed the supernatants of the RPL5 samples using IGFBP-1 because I had run out of the necessary antibodies from the prolactin kit. Due to time limitations and access to an IGFBP-1 ELISA kit, I pivoted to IGFBP-1 for RPL5 and significant levels of IGFBP-1 were released (Appendix) to show successful decidualisation.

3.4.2. Expression of Inflammatory Cytokines

To model the *in vivo* treatment of intralipid and hydroxychloroquine in RPL patients, I treated the hESCs with varying concentrations of intralipid or hydroxychloroquine and examined the impact on the expression of common inflammatory cytokines. The goal of this preliminary and exploratory experiment is to identify a robust cytokine that is differentially expressed in response to either intralipid or HCQ.The flow cytometry standard (FCS) files that store the quantitative flow cytometry data for light scatter and fluorescence of each single sample were analysed using the Qognit software provided by BioLegend.

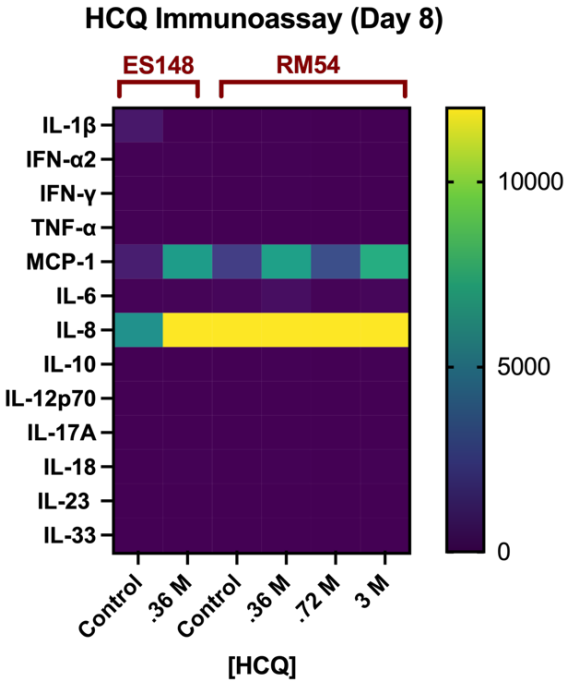


Figure 3.4: Cytokines released in response to HCQ treatment during decidualisation on Day 8. This figure is a heatmap showing the results of a 13-bead LegendPLEX immunoassay. The samples are the supernatants of the stromal cells treatment with decidualisation media (1 μM progesterone, 0.5 mM dbcAMP) with no HCQ as a control and .36 or .72 M HCQ. Samples collected on the last day of the 8-day decidualisation protocol are shown. Each square represents the raw concentration of each cytokine analyte in pg/mL. The samples tested were CONTROL1 (Control 1) and RPL2 (RPL 2) shown in succession from left to right. The values shown are averages. Each square represents the raw concentration of each cytokine analyte in pg/mL and are averages. The purple color represents the lower extreme (0-5000 pg/mL) and the yellow represents the higher extreme (>10000 pg/mL) (0-13,000 pg/mL linear gradient).

In Figure 3.4, the heatmap seems to show that when the stromal cells are treated with HCQ, there is an increase in MCP-1 expression, however this change in insignificant ($p = 0.2677$) in RPL2. MCP-1 is seen not seen to consistently trend up across all concentrations of HCQ when compared to the control in RPL2. In Control 1, the increase in MCP-1 is significant with consistent results across all three replicates ($p < .05$). Due to the limited number of samples in the immunoassay, the results of the HCQ treated stromal cells are limited to two cell lines.

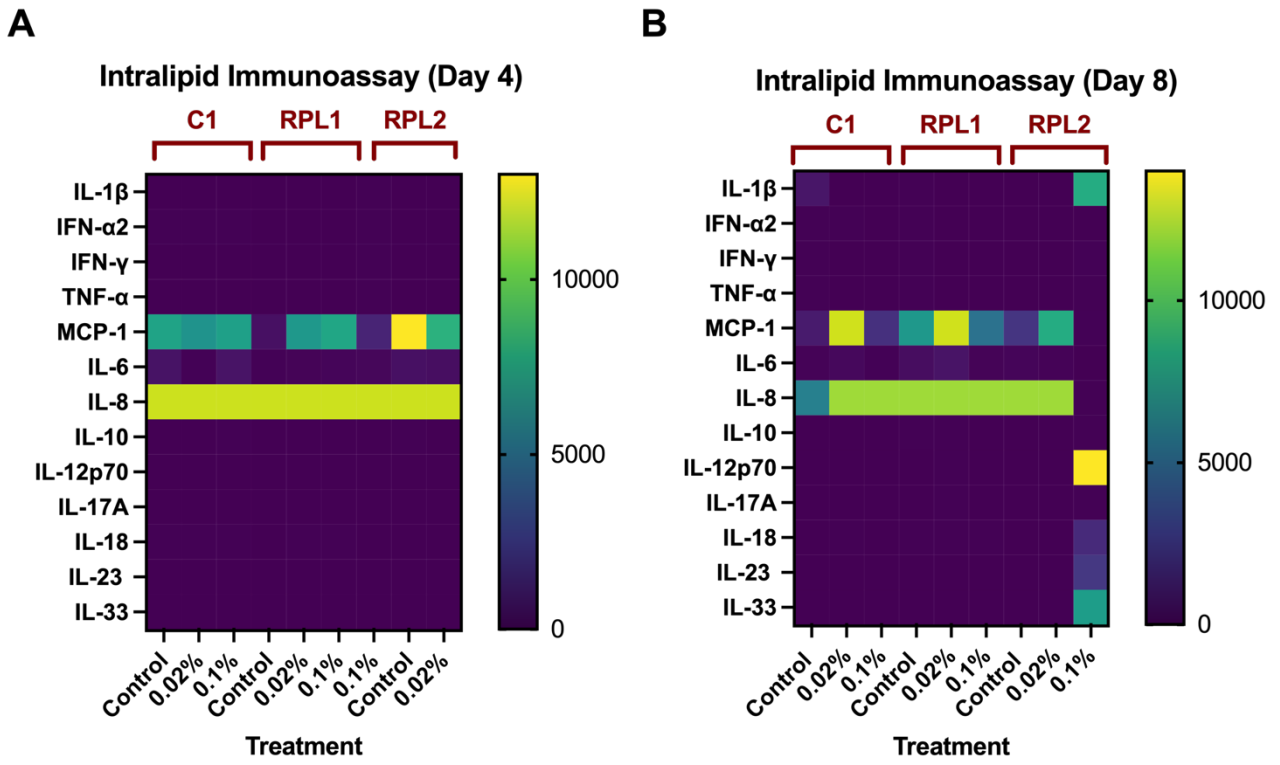


Figure 3.5: A. Heatmap of inflammatory cytokines released by stromal decidualised stromal cells treated with intralipid on Day 4. This figure a visual representation of the concentration (pg/mL) of 13 inflammatory cytokines measured from supernatant samples using a bead-based 13-analyte LegendPLEX (BioLegend) multiplex assay. The supernatant samples analysed were collected from a control (n=1) and RPL cell lines (n=2) after treatment with no intralipid as a control, 0.02%, and 0.1% intralipid in decidualisation media (1 µM progesterone, 0.5 mM dbcAMP) across 8 days. The samples displayed were collected on the last day, day 8. Each square represents the raw concentration of each cytokine analyte in pg/mL and are averages. The samples tested were Control 1, RPL 1, and RPL 2, shown in succession from left to right. **B. Day 8** This heatmap is showing the results of the same bead-based multiplex assay in part A, however the data shows the concentrations in supernatants collected on Day 8. The supernatant samples analysed were collected from a control (n=1) and a RPL cell line (n=2) after treatment with no intralipid as a control and 0.02% and 0.1% intralipid in decidualisation media (1 µM progesterone, 0.5 mM dbcAMP) across 8 days. The samples displayed were collected on the last day, day 8. Each square represents the raw concentration of each cytokine analyte in pg/mL and are averages. The purple color represents the lower extreme (0-5000 pg/mL) and the yellow represents the higher extreme (>10000 pg/mL) (0-13,000 pg/mL linear gradient).

As seen in Figure 3.5, only monocyte chemotactic protein-1 (MCP-1) and interleukin-8 (IL-8) expression is significant out of all 13 cytokines. Although IL-8 expression is significant in stromal cells treated with both intralipid and HCQ (Figure 3.4), it is unclear whether it is differentially expressed in response to these drugs. The immunoassay is limited to reporting IL-8 expression at a maximum of 12,000 pg/mL, therefore I am unable to conclude whether IL-8 increases, decreases, or is unaffected by either immunomodulatory drug. In Figure 3.5, not only is MCP-1 differentially expressed between the control and treatment groups, but also when comparing the untreated and treated cells. In both

the RPL cells lines on Day 4 and 8, MCP-1 increases when the stromal cells are treated with intralipid. However, two-way ANOVA analysis showed this increase is not significant with a p-value of 0.0705 when comparing untreated and 0.02%-intralipid treated cells on Day 8. This p-value is the closest to the alpha value of 0.05, when compared to how significant the effect of HCQ is on MCP-1 expression. Furthermore, unlike in Figure 3.4 with HCQ, all concentrations of intralipid had higher levels of MCP-1 when compared to the control in RPL1 and RPL2 on Day 4.

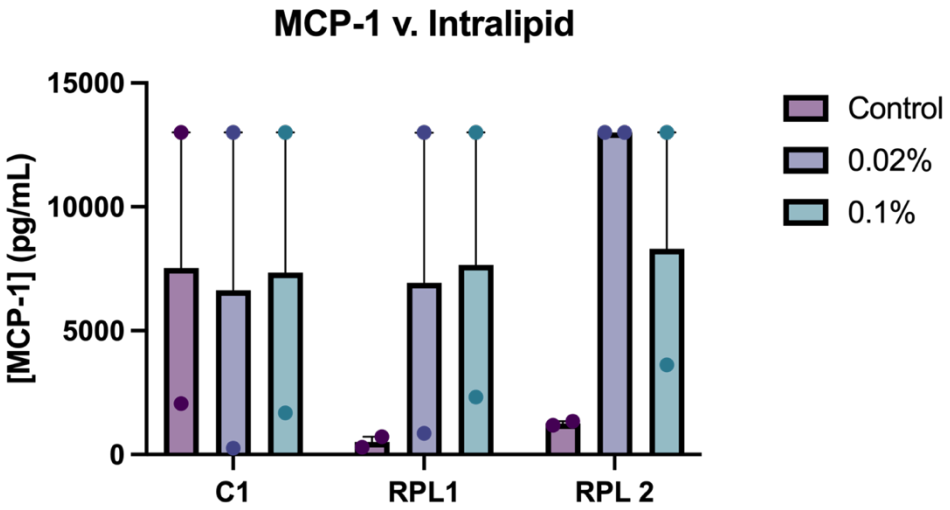


Figure 3.6: Change in MCP-1 levels in response to Intralipid treatment on Day 4 of decidualisation. This figure focuses on the MCP-1 cytokine concentration data from the BioLegend LEGENDplex immunoassay. Bar graphs represent the mean corrected concentrations (pg/mL) from three different stromal cell lines (N = 3), with three replicates per conditions (untreated, 0.02%, and 0.1% intralipid) grown in decidualisation medium (1 μM progesterone, 0.5 mM dbcAMP). Error bars indicated the standard error of the mean (SEM). Statistical significance was assessed using ANOVA. The y-axis represents MCP-1 concentrations in pg/mL, and the x-axis denotes the three cell lines. That assay detection top concentration was 13,000 pg/mL.

With MCP-1 trending up in response to intralipid being consistent across two timepoints and seen in two RPL cell lines, as seen in Figure 3.5, I have focused on only MCP-1 in Figure 3.6. MCP-1 levels do not differ much when comparing control and intralipid-treated cells. However, MCP-1 levels in the RPL cell lines are significantly lower than that of the control cell line in the untreated stromal cells ($p < .0001$). Although our statistical analysis showed that the increase in MCP-1 is insignificant,

this result could be further tested in more cell lines, specifically derived from RPL to increase statistical power. Therefore, I have formed the hypothesis below:

Compared to no treatment, expression of MCP-1 increases in response to the addition of intralipid during decidualisation, particularly in stromal cells derived from RPL patients.

For other cytokines shown in Figures 3.4 and 3.5, there was no detectable concentration, except for IL-6. However, a two-way ANOVA statistical analysis revealed no significant changes in IL-6 levels in response to intralipid or HCQ ($p = 0.0.3290$ and 0.6886, respectively). Between MCP-1, IL-6, and IL-8, MCP-1 expression seemed to be most affected by the intralipid treatment and warranted further investigation.

3.4.3. MCP-1 increases in intralipid-treated stromal cells

To test the hypothesis on MCP-1 expression, the intralipid decidualisation protocol was repeated on two more control cell lines and four recurrent pregnancy loss cell lines. The supernatants collected in these repeats were analysed using an MCP-1 ELISA assay to measure the MCP-1 concentration levels. After collecting this data, I normalized the data sets from each cell line to Z-scores that ranged from –1 to +1 to account for the differences in the scale of MCP-1 secreted by each sample. Regardless of identical seeding numbers and growth medium conditions, the cell lines seem to differ in secretion levels. This was observed with prolactin levels as seen in Figure 3.3 and the Z-score normalization was used to provide more accurate analysis of the MCP-1 levels.

In Figure 3.7, the highest levels of MCP-1 were observed in the 0.02% intralipid treatment, compared to both the control and other intralipid treatment groups. For example, in RPL5, there is a substantial increase in 0.02% intralipid treatment cells compared to the control across all 8 days of decidualisation. The average difference of MCP-1 between the control and treated group is 171.3 pg/mL, the greatest margin of increase across all cell lines. However, this increase was not

consistently shown across all cell samples. In RPL3 and control 3, the highest MCP-1 concentrations were seen in the samples without any intralipid treatment. Unexpectedly, there was not a noticeable upward trend in MCP-1 as the concentration of intralipid increase except for RPL2. In the last four rows representing RPL2 secretion of MCP-1 on Day 2, 4, 6, and 8, the highest MCP-1 levels were seen with the 0.1% intralipid-treated cells. There seems to be the greatest range in MCP-1 levels under the 0.5% intralipid treatment, with the highest number of dark purple cells and two yellow cell points.

Overall, the heatmap in Figure 3.7 provides support for my hypothesis that MCP-1 levels increase in response to intralipid in stromal cells. However, this increase is only observed in 0.02% intralipid groups. A two-way ANOVA analysis comparing the untreated to 0.02%-intralipid treated cells revealed there is a significant increase ($p < 0.0001$) with an average increase of 342.3 pg/mL in intralipid-treated cells (95% CI: 265.2 to 419.3).

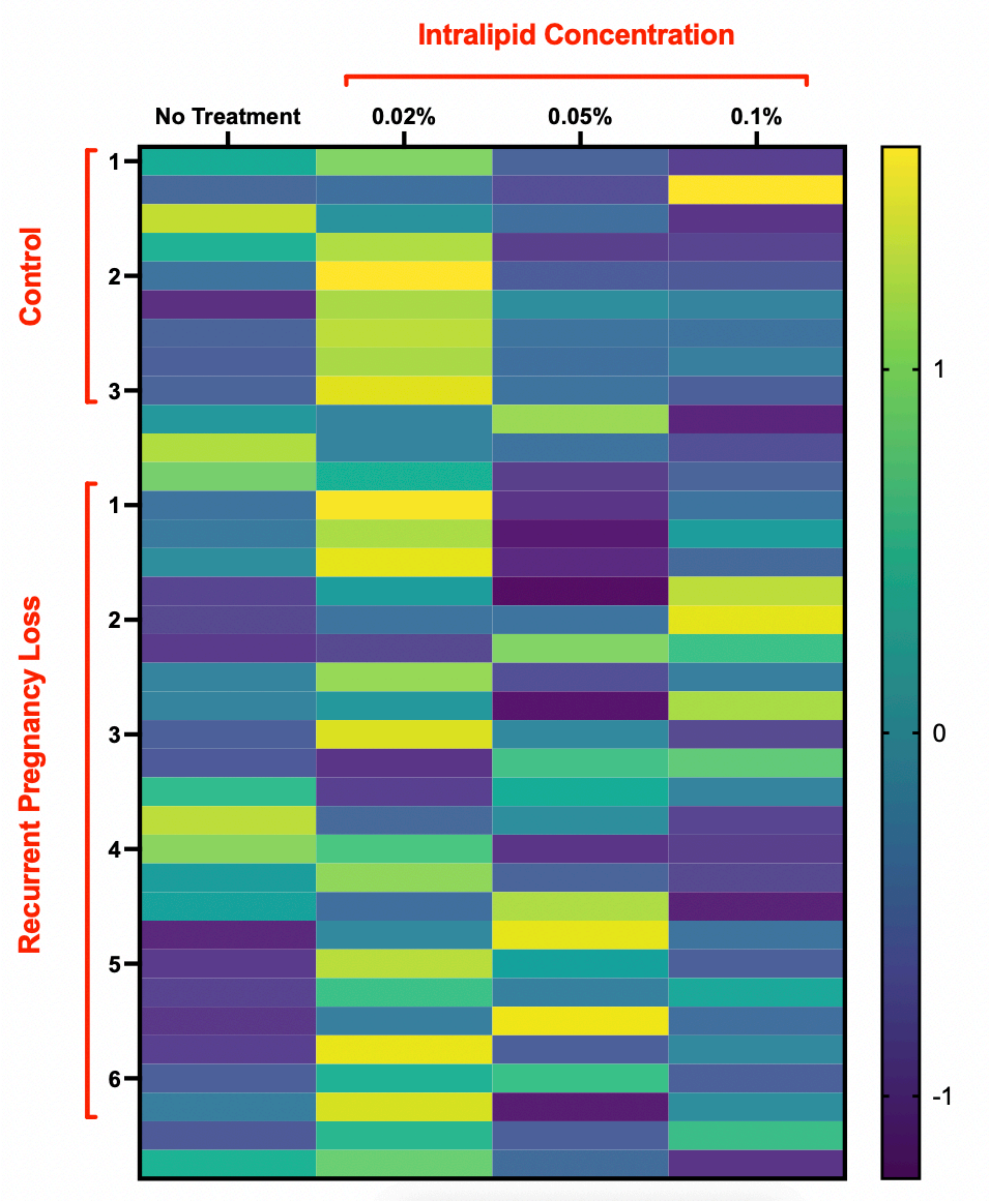


Figure 3.7: MCP-1 secreted from stromal cells in response to intralipid treatment across an 8-day decidualisation period. Sample sizes of N = 3 and N = 6 for control and recurrent pregnancy loss samples, respectively. Stromal cells were seeded in 12-well plates and treated with decidualisation medium (1 μM progesterone, 0.5 mM dbcAMP) for 8 days with the corresponding intralipid concentrations (0.02%, 0.05%, 0.1%). MCP-1 ELISAs were run using the supernatants collected every 2 days to analyse the concentration of the MCP-1 cytokine released from these stromal cells. This figure shows the average Z-score representing MCP-1 levels across the three repeats of each sample and its corresponding control treatment (no intralipid) and three varying concentrations of intralipid treatment across the x-axis. For every sample, the Z-scores across the four timepoints (Day 2, 4, 6, 8) are shown sequentially under each numbered tick mark. The Z-score is calculated using mean and standard deviation to normalize the MCP-1 levels within each cell line. Controls 1-3: ES148, ES151, ES163 and RM 1-6: RM24, RM54, RM43, RM47, OXF048, OXF049, respectively. Displayed are the mean Z-scores.

3.4.4. Migration Rates of THP-1 Monocytes in Stromal Cell Co-Culture

As MCP-1 is known as a chemokine that recruits and attracts monocytes, I wanted to investigate if the increase in MCP-1 due to 0.02% intralipid treatment during decidualisation affects the migration of monocyte cells in a migration assay. Using Transwell inserts with a pore-filled membrane initially separating THP-1 monocyte cells from the seeding stromal cells, I was able to observe the differences in the percentage of migrated monocytes in Figure 3.8 and Figure 3.9. The cell lines chosen for this experiment was based on the Results of the MCP-1 ELISAs that showed RPL1, RPL2, and RPL6 as having the most significant differences in MCP-1 expression when treated with intralipid.

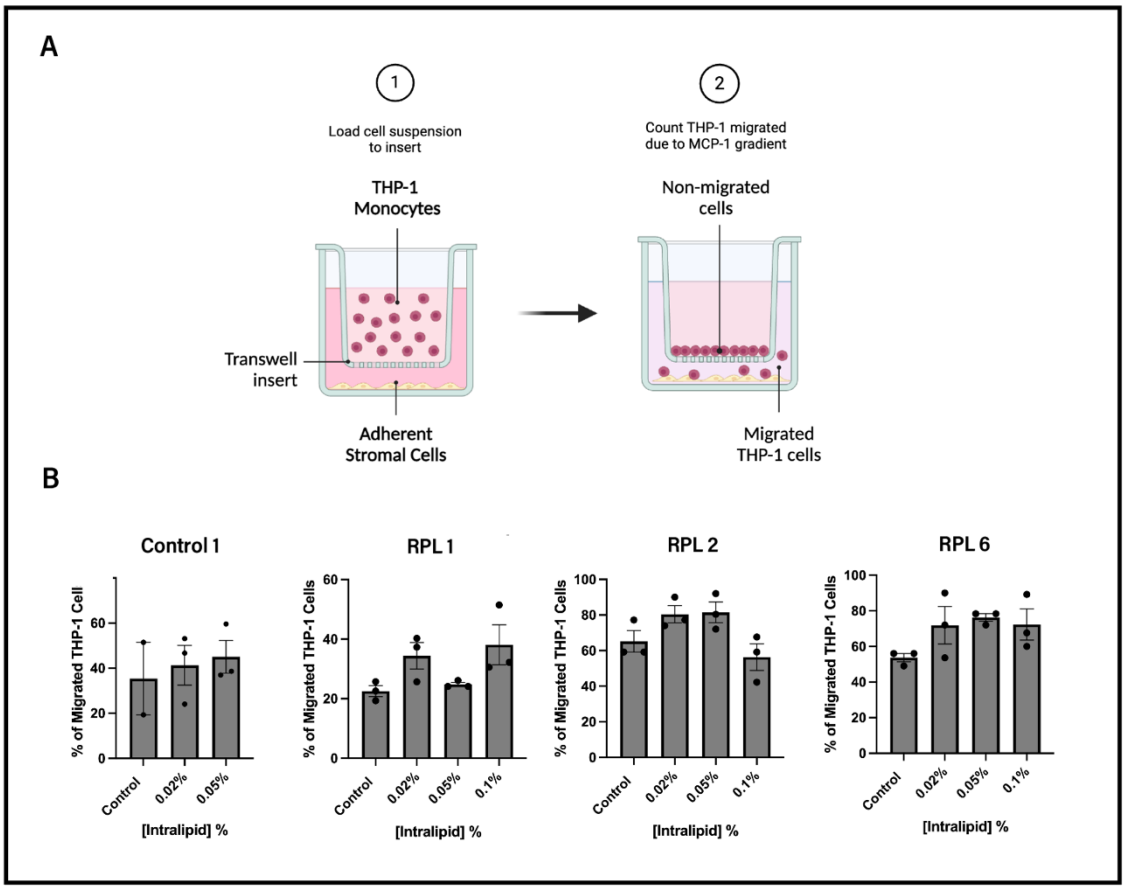


Figure 3.8: Percentage of Migrated THP-1 Monocytes in Transwell co-culture with intralipid-treated stromal cells. A. Transwell Setup of Upper and Lower Chambers. This schematic figure depicts the two chamber Transwell migration assay that was used to approximate how intralipid-treated stromal cells affect rates of THP-1 monocyte migration across the Transwell insert with 0.8 µm pores. The rendering includes the two types of cells before and after the 24-hour incubation

period. This figure was made using BioRender. **B. Migrated THP-1 Monocytes.** The figure shows the percentage of migrated THP-1 in a Transwell migration assay of each cell line in separate bar graph plots. Each stromal cell lines (n = 4) (C1, RPL1, RPL2, RPL6) was seeded and treated with no intralipid (control), 0.02%, 0.05%, and 0.1% intralipid in decidualisation growth medium (1 μM progesterone, 0.5 mM dbcAMP) in 24-well plates. On Day 4, 1 x 10^5 THP-1 monocytes were added to the upper chamber of the insert of each well. After 24 hours, the supernatant containing the migrated THP-1 cells was removed, spun down and counted using a haemocytometer. The bar graphs represent the average percentage of migrated cells across three repeats, normalized to 100% as the initial number of THP-1 cells added to the upper chamber of the Transwell insert. Mean is plotted with error bars of the SEM.

Across the four stromal cell lines seed in the migration assays, the monocytes co-cultured with the RPL1 and C1 cell lines have the lowest percentage of migration in the control and intralipid treatments, <50%. The highest levels of migration were of the monocytes cultured with RPL2 and RPL6 stromal cells, all greater than 50%. A two-way ANOVA analysis of the migration rates across each intralipid concentration shows significant differences between the untreated wells and the treated wells ($p=0.0443$). The analysis also shows significant differences in migration rates between the different cell lines ($p < 0.01$).

Accounting for the standard error using the error bars, there is a subtle difference in migration in intralipid-treat wells compared to control in RPL1, RPL2, and RPL6. As shown in Figure 3.9, this difference is not greater than 5 percentage points when including the range of potential error set by the error bars in RPL2 and RPL6. The most significant difference was in RPL1 between the Control and 0.02%, 0.1% intralipid groups.

In the C1 migration assays, the three repeats were not entirely consistent with the seemingly greatest variance. The results of these assays did not reflect the slight increase in MCP-1 shown in Figure 3.7 in the C1 cell line with significance. The average percent in the 0.02% and 0.05% treated cells are slightly higher than the control. However, in the 0.1% intralipid treated group, the number of migrated cells was unable to be determined by the haemocytometer, due to concentration being too low even after an attempt to concentrate the cells suspension. Opposite to Figure 3.7 showing the greatest level of MCP-1 in RPL2 under the 0.1% treatment, this group had a lower percentage of

migrated cells compared to the control in RPL2. Overall, this experiment was able to show slight differences in THP-1 migration after treating stromal cells with intralipid in 3 of the 4 cell lines.

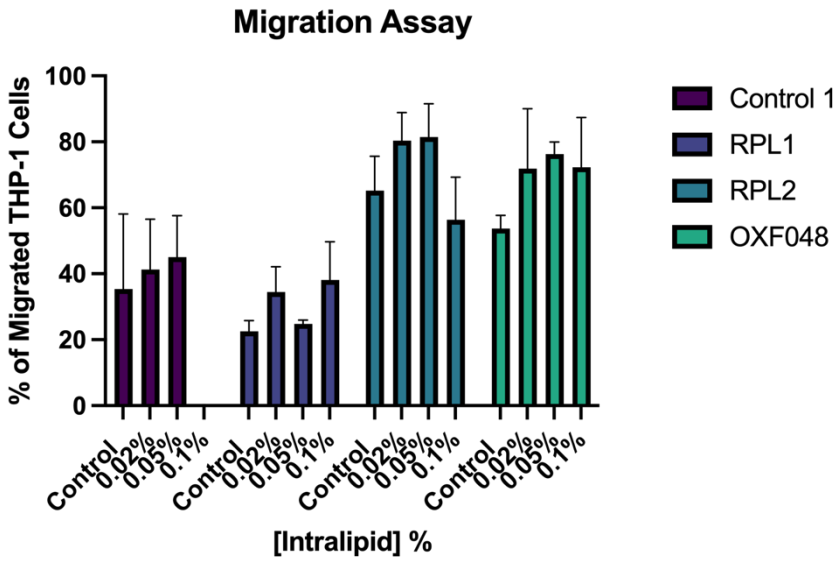


Figure 3.9: Percentage of Migrated THP-1 Monocytes co-cultured with control and intralipid-treated stromal cells. The figure shows the percentage of migrated THP-1 in a Transwell migration assay. Bar graphs represent the average percentage of migrated cells across three repeats, normalized to 100% (1 x 10^5) as the number of THP-1 cells added to the upper chamber of the Transwell insert. Mean is plotted with error bars of the SEM. The graph compares migrations levels between four cell lines (N = 4) and across three intralipid drugs concentrations in comparison to the control (untreated) cells.

3.5. Discussion

3.5.1. Summary

This chapter summarizes the laboratory work component of my thesis, aimed at contributing to the limited literature on the effect of immunomodulators in RPL patients and in an *in vitro* model. I established my model using human endometrial stromal cells derived from endometrial biopsies, confirming each cell line's ability to decidaulise with a standardized protocol. In an initial exploratory experiment with the immunoassay, I identified MCP-1 as a key inflammatory cytokine influenced by the Intralipid treatment. To validate this finding and test my MCP-1 hypothesis, I repeated the intralipid decidualisation experiment on three control and six RPL cell lines, demonstrating a significant increase in MCP-1 in the 0.2% Intralipid group during decidualisation compared to the control and other Intralipid groups. In this discussion, I will apply my findings to relevant clinical applications and further interpret my results. Regarding the objectives outlined in Chapter 3, I have successfully (1) established model cell lines, (2) explored inflammatory cytokine expression in drug-treated cells, and (3) conducted co-cultures of stromal cells and THP-1 cells to investigate the effects of intralipid.

3.5.2. Overview of Decidualisation in hESCs

As outlined in the introduction, decidualisation is essential for preparing the endometrium for implantation and maintaining early pregnancy. I characterized the decidual process through the expression of IGFBP-1 and prolactin, as well as morphological changes. The transformation of the cells from spindle-shaped to cobblestone-like decidual cells across all nine cell lines aligns with existing literature citing this defining feature of decidualisation (Pan-Castillo et al., 2018). However, the degree of rounding and complete morphological transformation varied by cell line and day of observation. For example, Control1 displayed the most pronounced decidual morphology on Day 8, while Control2, RPL2, RPL5, and RPL6 showed significant rounding between Days 6 and 8. In contrast,

cell lines Control3, RPL3, and RPL4 remained less rounded even on Day 8. Notably, RPL1 achieved the cobblestone shape earliest, between Days 4 and 6. These observations, based on the microscopy images (Appendix), indicate differences in the timing of decidualisation across cell lines, yet no clear correlations emerged between response times and whether the cell lines were from control or recurrent pregnancy loss groups.

Variations in decidualisation efficiency are also reflected in the differing levels of secreted prolactin (Figure 3.3). While each cell line exhibited distinct prolactin concentrations, the highest generally occurred on Day 8. The exception was RPL5, which peaked on Day 6, exhibiting levels 2 to 4 times higher than prolactin. This discrepancy between how prolactin and IGFBP-1 (Appendix) trends may be due to IGFBP-1 levels rising earlier during decidualisation (Bell et al., 1991), and the supporting literature that indicates that prolactin tends to increase slightly later in the process (Ben-Jonathan et al., 1996). In terms of timing, the relationship between prolactin and IGFBP-1 aligns with known patterns among decidualisation markers. In the regulatory pathway, IGFBP-1 is highly responsive to cAMP and is activated early (Schweizer-Groyer et al., 2006), whereas prolactin expression is more dependent on progesterone signalling (Brosens et al., 1999). By modeling decidualisation *in vitro,* we can closely examine potential impairments and consider supplementary treatments with progesterone—a well-studied approach to treating RPL.

Although prolactin expression in the control versus RM cell lines did not differ significantly in range and baseline secretion, the pattern in which the prolactin levels changed falls into two trends. In the control group, particularly Control2 and Control3, there was no significant increase in prolactin across the 8 days. In contrast, the RPL group showed minimal secretion of prolactin until Days 6 or 8, with RPL6 being an exception, reaching a concentration of over 100 pg/mL on Day 2 and peaking on Day 6. On average, RPL cell lines appeared to take 2-4 days longer to release significant prolactin levels compared to the control group. These differences in delayed morphological changes and

secreted markers suggest that decidualisation may be impaired in some cell lines and corresponding patient samples. However, a clear correlation between the morphological changes and marker secretion was not established, leaving no definitive criteria for decidualisation efficiency in response to dbcAMP and progesterone. For instance, RPL1 achieved the cobblestone shape earliest in the 8-day protocol but secreted the lowest amounts of prolactin until Day 8. Overall, the delay in decidualisation in some of the RPL aligns with previous studies showing incomplete morphological changes in stromal cells from RPL patients (ie. RPL3 and RPL4). This impaired decidualisation is also signified by less pronounced transformations and production of markers (Salker et al., 2010, Lucas et al., 2020). The extension of the decidualisation protocol from 6 to 8 days was implemented to optimize the *in vitro* model and adapt to the delayed decidual process. These results support this protocol modification and could inform future studies at decidualisation.

3.5.3. Inflammatory Cytokine Expression

Using the pre-designed BioLegend immunoassay, I observed how different inflammatory cytokines are expressed in intralipid- and HCQ-treated stromal cells. This study demonstrates that intralipid treatment modulates cytokine expression in human endometrial stromal cells, particularly MCP-1. Monocyte chemoattractant protein-1, also known as CCL2, is a chemokine involved in the recruitment of monocytes and macrophages, along with other immune cells, to sites of inflammation (ScienceDirect, MCP1). It is known that MCP-1 expression increases during decidualisation, an inflammatory-driven process, due to tumour necrosis factor-alpha (TNF-alpha) and interleukin-1β (IL-1β) (Lockwood et al., 2006). However, in this cell line model, stromal cells and decidualised stromal cells are cultured in isolation, without the broader endometrial immune environment, including epithelial, endothelial cells, and various immune cells that typically contribute to cytokine signalling. The lack of significant TNF-alpha and IL-1β detection in the immunoassay is likely due to this lack of cellular crosstalk and interaction. These cytokines are secreted by immune cells such as

macrophages and epithelial cells *in vivo,* and their low or undetectable levels here may reflect the limitations of a stromal-based culture system. Further experimentation using co-culture could provide further investigation and explanation to this limitation (Wang et al., 1999).

Furthermore, by decidualising the stromal cells in my MCP-1 experiments, the signal should also be stronger than when observing non-decidualised cells. In the context of RPL and the endometrium, elevated MCP-1 levels have been associated with increased inflammation and altered immune responses. MCP-1 may play a critical role in the pathophysiology of RPL due to disruptions in MCP-1 signalling contributing to impaired decidualisation and less than favourable environment for implantation (Caballero-Campo et al., 2002). Although there are not previous studies linking Intralipid to increased MCP-1 in stromal cells derived from RPL patients, the significant increase in MCP-1 expression compared to the non-treated cells supports the finding in adenomyosis patients that following Intralipid infusion, there is a significant increase in plasma MCP-1/CCL2 (Foyle et al., 2021). This relationship between Intralipid and MCP-1 suggests that this treatment further heightens the inflammatory response in RPL patients.

Clinical Applications

There is notable variability in how MCP-1 levels respond to Intralipid across the cell lines, which reflect the phenotypic heterogeneity of RPL patients and their underlying causes. Since all six RPL patient samples represent the 50% of RPL cases that are unexplained, immune dysregulation in the endometrium is a potential factor of recurring miscarriages. However, the inconsistent MCP-1 response suggests that not all RPL patients will react uniformly to immunomodulatory treatments. This variability in MCP-1 trends supports the idea that personalized approaches to immunomodulation might be necessary for effective RPL treatment. This is seen in some approaches to RPL treatment where dosage or the combination of drugs is modulated to fit the patient's needs. In

IVIG trials, several studies have adjusted dosage based on characteristics like elevated NK cell levels higher than 12% (Kim et al., 2022). Studies have also looked at combining IVIG with prednisone (corticosteroid), or the latter with progesterone (US Clinical Trials, Kim et al., 2022). Specific subsets of RPL patients such as antiphospholipid syndrome or acquired/inherited thrombophilia seem to benefit from various combinations of anticoagulants (ie. heparin), further showing the importance of knowing the individual cause of RPL and the mechanism of immune therapy (Abu-Heija, 2014).

These findings could inform immunomodulatory strategies for treating recurrent pregnancy loss, particularly by targeting cytokine-mediated pathways. For example, it has been shown that the upregulation of MCP-1 may play a role in implantation and the rapid recruitment of NK cells in the luteal phase (Ulukus et al., 2005). In some RPL patients, where NK cell levels are elevated, the treatment of Intralipid as an immunomodulatory treatment could result in a more aberrant endometrium. Therefore, in some RPL patients the treatment of Intralipid could hinder embryo implantation and increase the risk of miscarriage, rather than facilitate decidualisation and suppress the maternal immune system. This study could inform clinical designation of Intralipid as effective or ineffective in some cases--measuring NK cell or monocyte activation as a diagnostic marker in RPL before Intralipid treatment.

In these experiments, the stromal cells were treated with different dosages of Intralipid, however the levels of MCP-1 did not increase as Intralipid concentration increased. It seems that the optimal Intralipid concentration to increase MCP-1 is 0.02%, with an exception in RPL2, where the highest MCP-1 was measured in 0.1% Intralipid-treated stromal cells. This is likely due to different samples having varying sensitivities to Intralipid, reflecting the difference amongst RPL patients. A modification I would make is to start the range of Intralipid concentrations at a lower percentage to see if MCP-1 changes across a titration of Intralipid. These results could indicate that lower dosages

of Intralipid should be administered to increase MCP-1 levels, however a higher amount might have the opposite effect and result in suppression of MCP-1 secretion.

Other Inflammatory Cytokines

Another cytokine of interest, IL-8, showed significant expression in all but was not further explored due to unclear immunoassay results and time constraints. Optimization issues during the immunoassay caused the IL-8 fluorescent signal to exceed the kit's detectable range, with readings of over >12000 pg/mL despite a top standard of 12 ng/mL. Dilution was not feasible, as it would reduce sensitivity and concentration for the twelve other analytes. To further study IL-8, ELISAs could be used with 2:1, 5:1, or 10:1 diluted supernatant to clarify how HCQ and Intralipid affect IL-8 secretion in stromal cells. One of the most prominent roles of IL-8 is the attraction of neutrophils to sites of inflammation, as well as promoting monocyte-macrophage growth and differentiation (Corre et al., 1999). Based on the increase of MCP-1 and the role of MCP-1 in attracting monocytes, it is possible that the mechanism by which Intralipid increases MCP-1 could also have downstream effects on IL-8 expression. There is currently no information linking Intralipid and IL-8, however it has been shown that this pro-inflammatory cytokine is increased in decidual tissue of patients with RPL compared to control pregnancies (Krieg et al., 2012). If Intralipid or HCQ does influence IL-8 expression, these treatments could play a role in modulating this cytokine-mediated pathway in the endometrial immune system.

Although this thesis focused on Intralipid, the identification of IL-8 as a possible key cytokine in the mechanism of HCQ is supported by data showing that serum levels of IL-8 increase after oral HCQ was ingested. In the same study by Wakiya et al., no significant changes were observed in the serum MCP-1 levels due to HCQ administration. The results of these experiments are limited to patients with systemic lupus erythematosus (SLE), rather than conditions related to fertility. However,

SLE is a chronic autoimmune disease that is associated with a higher risk of pregnancy loss (Wakiya et al., 2022, Valeff et al., 2023). This provides relevant and valuable context for understanding the potential role of IL-8 in the immune pathways modulated by HCQ, particularly in RPL patients who may have overlap in immunological dysregulations as patients with SLE. IL-8 as a biomarker for treatment response in RPL could be further investigated to address more inflammatory pathways involved in RPL.

3.5.4. Monocyte Migration in Stromal Cell Co-Culture

Based on our knowledge of MCP-1 (CCL2) in modulating monocyte and macrophage activity during pregnancy, I conducted a Transwell migration assay by co-culturing the stromal cells with an immortalized monocyte cell line, THP-1. THP-1 is a valuable tool and model for studying monocyte structure and function and can be differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA) (Starr et al., 2018). I hypothesized that the cells treated with 0.02% Intralipid will have a greater percentage of migrated THP-1 monocytes, compared to the control no treatment and other Intralipid concentrations. Although MCP-1 is not the only cytokine involved in monocyte and macrophage recruitment, there are seemingly significant differences in MCP-1 expression in some cell lines when treated with 0.2% Intralipid. This experiment is designed to reflect this difference in MCP-1 through varying percentages of migrated THP-1 monocytes. RPL1 showed more significant effects than the other cell lines at 37% migrated cells in 0.02% Intralipid treated stromal cells compared to 21% in the untreated group ($p < 0.05$). For Control1, RPL2, and RPL6, the change in percentage was within the standard error bars and therefore insignificant. According to the MCP-1 heatmap, MCP-1 levels increased the most compared to its control, and this response aligns with the results of the migration assay. This shows that even if MCP-1 level increases, it is not always sufficient in modulating the monocyte or even macrophage population. This assay suggests that the role of MCP-1 in immune cell recruitment works in tandem or is attenuated by cytokines and gene pathways.

These findings further prove that personalizing treatments to the specific RPL patient is vital to achieving effectiveness and avoiding unnecessary interventions. This approach not only enhances treatment outcomes but also minimizes potential risks and puts RPL patients through further hardship and negative psychological effects.

Although limited, there are studies showing increased or altered monocyte subsets in RPL patients when compared to control samples (Comins-Boo et al., 2022). For patients exhibiting this profile of immune cells, intralipid as an immune therapy may be beneficial or further inflame the endometrium immune balance. Overall, given that MCP-1 is involved in recruitment of monocytes/macrophages, and this study shown intralipid increases MCP-1 levels, this suggests that intralipid might influence monocyte populations in the endometrium.

3.5.5. Limitations

There are currently no published studies investigating how intralipid modulates the inflammatory cytokines expressed by endometrial stromal cells from RPL patients. The work in this thesis represents my efforts to build our understanding of immunomodulators in the context of Recurrent Pregnancy Loss outside of the limited RCTs and retrospective studies and using relevant *in vitro* models. However, with this being a yearlong master's thesis, there were limitations concerning patient sample size, depth of experimentation, and the scope of the results.

Firstly, MCP-1 was the only cytokine I was able to identify as being differentially expression in response to either HCQ or Intralipid. The range of inflammatory cytokines that I tested my samples for was limited to a 13-analyte panel. There are a host of inflammatory cytokines that are involved in the immune regulation of the endometrium. Within the LEGENDplex panels, there is even a second inflammation analysing thirteen different cytokines, showcasing a need for more extensive cytokine

profiling of the Intralipid mechanism and of other immune-modulatory treatments. Since these results are limited to MCP-1 expression, my conclusions are contradictory to other studies on intralipid. Intralipids have been shown to decrease pro-inflammatory cytokines, such as TNF-α and IL-1β (Canelle et al., 2021). In this study, the increase in MCP-1 in intralipid-treated stromal cells might provide evidence that this immune therapy shifts or worsens the anti-inflammatory endometrial environment in RPL patients, rather than balance against a pro-inflammatory state. The study examined TNF-α and IL-1β secretion in NK cells and the other literature in support of the hypothesis that intralipid dampens inflammation is specifically observed in peripheral blood mononuclear cells (PBMCs) and T cells (Foyle et al., 2021). These contradictory findings point to a need for there to be a consensus on a pathologically and physiologically relevant model for testing immunomodulators. Given the wide range of immunomodulatory therapies and the different immune mechanisms involved in RPL, refining the models will enable more targeted treatment, improve reproducibility, and ultimately enhance patient outcomes.

Furthermore, my study is limited by the relatively small number of patient samples derived into cell lines. Due to this total of N=9, the data does not fully capture the heterogeneity of the endometrial environment in vivo. This has implications for my conclusions since I am only able to draw conclusions about relative trends, rather show definitive differences in expression between the control and RPL samples. Within the raw MCP-1 concentration data measured by the immunoassay and ELISAs, it seems that control cell lines have a higher base level of MCP-1 without any treatment, when compared to the recurrent pregnancy loss cell lines. However, with a respective sample size of n = 3 and n = 6 for the control and RM samples, no significant conclusions about the innate ability of stromal cells to secrete MCP-1, prolactin, and IGFBP-1 can be made. According to past studies, it is well established that RPL stromal cells often have impaired decidualisation leading to lower production of the decidual markers and other inflammatory cytokines (IL-6, IL-8) (Yang et al., 2022). It

would be consistent for the results in this thesis to also display this decrease in secretory function compared to the control stromal cells.

To further examine the relationship between MCP-1 and intralipid, I conducted migration assays to see if the differences in MCP-1 can affect a physiologically relevant process of monocyte migration in the endometrium. This experiment further supports the likelihood that this increase in MCP-1 is accurate and reproducible since MCP-1 is directly involved in monocyte recruitment. This is a strength of this thesis as there is literature, although limited, elucidating the profile of monocytes in the pathology of RPL. As immune cells, monocytes and macrophages play critical roles in a successful pregnancy and live birth result. There are studies reporting a higher presence of intermediate monocytes populations in RPL than in fertile patients (Comins-Boo et al., 2022). With the intended purpose of Intralipid to dampen the pro-inflammatory state in the endometrium, specifically to decrease NK cell activity, this immune therapy may also have a contradictory effect by attracting and activating more monocytes—a different type of immune cell. By establishing an increase in MCP-1 and potentially increased monocyte migration, a strength of this thesis is that these finding reveal greater complexities must be considered to treat RPL patients with intralipid and its downstream effects to multiple immune pathways. These migration assays show that more experiments to examine the crosstalk between immune cells and stromal cells should be conducted to model how these cells interact *in vivo*.

3.5.6. Future Directions

This study highlights the need for more comprehensive understanding of how intralipid and HCQ influence the endometrium in RPL patients. One of the primary challenges I encountered was the lack of an established method or *in vitro* model to determine how effective an immunomodulator is in RPL patients. For every immune-related disease or fertility condition, there are multiple causes and aberrations that lead to the prognosis. Given this, future research should focus on developing

more sophisticated models that can replicate complex interactions in the reproductive immune environment.

To address this gap, a priority for future studies could be the development of an organoid model and eventually advanced co-culture systems with the plethora of immune cells. This would allow researchers to better observe the response to immunomodulatory treatments, such as intralipid and HCQ, on a molecular and cellular level. Similar to the use of derived cell lines in this study, further exploration of patient-derived cell lines can better inform personalized treatment approaches based on molecular and genetic profiling. RPL subgroups such as those with elevated NK levels, senescent decidual cells, or specific upregulation of inflammatory cytokines may respond differently based on their immune profile and tolerance to immune drugs.

Other growing areas of research, specifically transcriptomics and proteomics, should be integrated into future research to deepen our understanding of how an immune therapy can alter gene expression and protein activity in the endometrium. This study is limited to observing the downstream effects of any changes in gene expression; however, transcriptomics and proteomics can allow for the identification of biomarkers that can predict treatment response. With overviews of global gene expressions, complimented with protein profiles, we can analyse how the profile of a RPL patient is different from those of fertile patients (Li et al., 2022).

Our general lack of understanding of how immunomodulators work is due to the many different types and how they specifically affect different conditions. For example, HCQ is commonly used as an anti-malarian drug for its immunomodulator effects, but those effects may not be translated to RPL. As randomised clinical trials remain the gold standard for generating high-quality clinical evidence (Hariton & Locascio, 2018), all immunomodulators must go through this rigorous process to be established as an effective mode of treatment. However, RCTs can be time-consuming

and require a lot of resources to complete. Future research should aim to bridge the gap between *in vitro* experiments and clinical application. Therefore, combining insights from bioinformatics and -omics studies with insights from *in vitro* experiment models could enhance our understanding of the stratification of RPL patients (ie. subgroups, age groups, unexplained) while advocating for further RCTs. As shown in Chapter 2 in the systematic review, the current evidence for intralipids in RPL is limited to very few RCTs. These are potential future directions that could expand on my findings and strength the limitations of this study.

3.6. Conclusions

This chapter aimed to investigate the effects of intralipid and HCQ on endometrial stromal cells during decidualisation, specifically cytokine expression in control and RPL patients. The primary finding is that intralipid treatment, at an optimal concentration of 0.02%, significantly increases MCP-1 expression, offering potential therapeutic insights for RPL. A secondary result is that both intralipid and HCQ influence the secretion of two decidualisation biomarkers, prolactin and IGFBP-1, indicating a potential role in modulating the immune environment through this pathway and possibly by altering monocyte populations. The findings suggest that immunomodulatory treatments may be beneficial for RPL patients with underlying immune system dysfunctions. These conclusions are a result of a monolayer cell culture and co-culture assays. With these two being simple *in vitro* models, there are limitations in how this effect might be seen in the *in vivo* environment. Considering these limitations, along with sample size and variance in results, these results must be further stratified with other investigations of intralipid. This chapter provides a foundation for future work emphasized by the results of the previous chapter.

Final Conclusions

This thesis aimed to explore immunomodulators in the treatment of Recurrent Pregnancy Loss by summarising the available evidence in a systematic review and examine their potential immune effects during human endometrial stromal cell decidualisation. By integrating this review with laboratory investigations into cytokine expression and monocyte activity, this work provides understanding of both theoretical and clinical facets of RPL research and management.

In Chapter 1, I introduced the significance of decidualisation in successful pregnancy outcomes and the evidence on its dysregulation in RPL patients. This introduction outlined the physiological importance of a balanced immune system as the maternal-fetal interface, specifically through hormonal signalling, cytokine regulation, and immune cell interactions. Chapter 1 provides the background and reasoning for an in-depth investigation into administering therapeutic interventions to improve clinical outcomes (ie. Live birth rate, miscarriage rate) in RPL patients.

Chapter 2 presented a systematic review with meta-analyses of the existing evidence on “pure” immunomodulators, such as intralipid, hydroxychloroquine, IVIG, G-CSF, and steroids for RPL. The findings varied across the different interventions, with steroids being the most promising and only significant result. The other findings were insignificant and reflects the variability in study designs, treatment protocols, and patient populations. These findings reaffirm a need for more robust, large-scale, quality RCTs to validate the efficacy of immunomodulators in RPL. More specifically, the one included study for intralipid showed promising results, however there was insufficient evidence to run an analysis. This prompted further laboratory investigations into intralipid’s potential effect in modulating inflammatory cytokines and the maternal immune tolerance.

Chapter 3 built upon these findings by conducting laboratory experiment primarily on intralipid in hESC decidualisation to elucidate its effects on immune signalling. The results demonstrated that

intralipid treatment modulates MCP-1 expression, with implications for monocyte recruitment and activation during decidualisation. Additionally, prolactin secretion pattern reaffirmed the importance of decidualisation markers in assessing therapeutic outcomes. These findings can help contribute to insights into the molecular pathways influenced by intralipid, bridging gaps in our current understanding of how immunomodulators work mechanistically.

Overall, this thesis underscores the multifaceted nature of RPL and highlights the potential of targeted or combinatory immunomodulatory treatments to address its underlying pathophysiology. While intralipid shows promise, further clinical trials and translational studies are needed to optimise its application, both in term of drug regimen and administration to specific RPL subgroups. Future research should work towards refining patient stratification, standardising treatment protocols, and integrating novel biomarkers to personalise therapy for all RPL patients.

References

Abdolmohammadi Vahid, S., Ghaebi, M., Ahmadi, M., Nouri, M., Danaei, S., Aghebati-Maleki, L., Mousavi Ardehaie, R., Yousefi, B., Hakimi, P., Hojjat-Farsangi, M., Rikhtegar, R., & Yousefi, M. (2019). Altered T-cell subpopulations in recurrent pregnancy loss patients with cellular immune abnormalities. *Journal of Cellular Physiology*, *234*(4), 4924–4933. https://doi.org/10.1002/jcp.27290

Abu-Heija, A. (2014). Thrombophilia and Recurrent Pregnancy Loss. *Sultan Qaboos University Medical Journal*, *14*(1), e26–e36.

Acacio, B., Coulam, C., Rinehart, J., Rinehart, L., Ng, S. C., Roussev, R. G., & Parrett, S. (2008). Pregnancy Outcome After Intralipid Infusion Among Women Experiencing Recurrent Pregnancy Loss. *Fertility and Sterility*, *89*(4), S11. https://doi.org/10.1016/j.fertnstert.2008.02.034

Agostinis, C., Mangogna, A., Bossi, F., Ricci, G., Kishore, U., & Bulla, R. (2019). Uterine Immunity and Microbiota: A Shifting Paradigm. *Frontiers in Immunology*, *10*, 2387. https://doi.org/10.3389/fimmu.2019.02387

Ahmadi, M., Abdolmohammadi-Vahid, S., Ghaebi, M., Aghebati-Maleki, L., Afkham, A., Danaii, S., Abdollahi-Fard, S., Heidari, L., Jadidi-Niaragh, F., Younesi, V., Nouri, M., & Yousefi, M. (2017). Effect of Intravenous immunoglobulin on Th1 and Th2 lymphocytes and improvement of pregnancy outcome in recurrent pregnancy loss (RPL). *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie*, *92*, 1095–1102. https://doi.org/10.1016/j.biopha.2017.06.001

Ahmadi, M., aghdam, S. A., Nouri, M., Babaloo, Z., Farzadi, L., Ghasemzadeh, A., Hamdi, K., Movassaghpour, A. A., Jadidi-Niaragh, F., Afkham, A., Motallebnezhad, M., Eghbal-Fard, S., Dolati, S., Younesi, V., & Yousefi, M. (2017). Intravenous immunoglobulin (IVIG) treatment modulates peripheral blood Th17 and regulatory T cells in recurrent miscarriage patients: Non randomized, open-label clinical trial. *Immunology Letters*, *192*, 12–19. https://doi.org/10.1016/j.imlet.2017.10.003

Ahmadi, M., Ghaebi, M., Abdolmohammadi-Vahid, S., Abbaspour-Aghdam, S., Hamdi, K., Abdollahi-Fard, S., Danaii, S., Mosapour, P., Koushaeian, L., Dolati, S., Rikhtegar, R., Oskouei, F. D., Aghebati-Maleki, L., Nouri, M., & Yousefi, M. (2019). NK cell frequency and cytotoxicity in correlation to pregnancy outcome and response to IVIG therapy among women with recurrent pregnancy loss. *Journal of Cellular Physiology*, *234*(6), 9428–9437. https://doi.org/10.1002/jcp.27627

Ali, S., Majid, S., Niamat Ali, Md., & Taing, S. (2020). Evaluation of T cell cytokines and their role in recurrent miscarriage. *International Immunopharmacology*, *82*, 106347. https://doi.org/10.1016/j.intimp.2020.106347

Ashkar AA, Black GP, Wei Q, et al. Assessment of requirements for IL-15 and IFN regulatory factors in uterine NK cell differentiation and function during pregnancy. J Immunol. 2003;171:2937–2944.

B, T., S, G., & E, G. (1993). Mechanisms involved in the decidualization of human endometrial stromal cells. *Acta Europaea Fertilitatis*, *24*(5). https://pubmed.ncbi.nlm.nih.gov/7985467/

Balasundaram, P., & Farhana, A. (2024). Immunology at the Maternal-Fetal Interface. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK574542/

Bansal, P., Goyal, A., Cusick, A., Lahan, S., Dhaliwal, H. S., Bhyan, P., Bhattad, P. B., Aslam, F., Ranka, S., Dalia, T., Chhabra, L., Sanghavi, D., Sonani, B., & Davis, J. M. (n.d.). Hydroxychloroquine: A comprehensive review and its controversial role in coronavirus disease 2019. *Annals of Medicine*, *53*(1), 117–134. https://doi.org/10.1080/07853890.2020.1839959

Bátorfi, J., Kotlán, B., Padányi, A., Réti, M., Gyódi, E., Rajczy, K., Miklós, K., Németh, J., Melicher, F., Vályi-Nagy, I., Petrányi, G., & Fülöp, V. (2005). [Intravenous immunoglobulin treatment of recurrent spontaneous abortion with immunopathological background]. *Orvosi Hetilap*, *146*(45), 2297–2302.

Ben-Jonathan N, Mershon JL, Allen DL, et al. Extrapituitary prolactin: distribution, regulation, functions, and clinical aspects. Endocr Rev. 1996;17(6):639–669Bell, S. C., Jackson, J. A., Ashmore, J., Zhu, H. H., & Tseng, L. (1991). Regulation of insulin-like growth factor-binding protein-1 synthesis and secretion by progestin and relaxin in long term cultures of human endometrial stromal cells. *The Journal of Clinical Endocrinology and Metabolism*, *72*(5), 1014–1024. https://doi.org/10.1210/jcem-72-5-1014

Bender Atik, R., Christiansen, O. B., Elson, J., Kolte, A. M., Lewis, S., Middeldorp, S., Nelen, W., Peramo, B., Quenby, S., Vermeulen, N., & Goddijn, M. (2018a). ESHRE guideline: Recurrent pregnancy loss. *Human Reproduction Open*, *2018*(2), hoy004. https://doi.org/10.1093/hropen/hoy004

Bender Atik, R., Christiansen, O. B., Elson, J., Kolte, A. M., Lewis, S., Middeldorp, S., Nelen, W., Peramo, B., Quenby, S., Vermeulen, N., & Goddijn, M. (2018b). ESHRE guideline: Recurrent pregnancy loss. *Human Reproduction Open*, *2018*(2), hoy004. https://doi.org/10.1093/hropen/hoy004

Brar AK, Frank GR, Kessler CA, Cedars MI, Handwerger S. Progesterone-dependent decidualization of the human endometrium is mediated by cAMP. Endocrine. 1997 Jun;6(3):301-7. doi: 10.1007/BF02820507. PMID: 9368687.

Brinsden PR, Alam V, de Moustier B, Engrand P. Recombinant human leukemia inhibitory factor does not improve implantation and pregnancy outcomes after assisted reproductive techniques in women with recurrent unexplained implantation failure. Fertil Steril. 2009;91:1445– 1447.

Brosens, J. J., Bennett, P. R., Abrahams, V. M., Ramhorst, R., Coomarasamy, A., Quenby, S., Lucas, E. S., & McCoy, R. C. (2022). Maternal selection of human embryos in early gestation: Insights from recurrent miscarriage. *Seminars in Cell & Developmental Biology*, *131*, 14–24. https://doi.org/10.1016/j.semcdb.2022.01.007

Brosens, J. J., Hayashi, N., & White, J. O. (1999). Progesterone Receptor Regulates Decidual Prolactin Expression in Differentiating Human Endometrial Stromal Cells1. *Endocrinology*, *140*(10), 4809–4820. https://doi.org/10.1210/endo.140.10.7070

Brosens, J. J., Salker, M. S., Teklenburg, G., Nautiyal, J., Salter, S., Lucas, E. S., Steel, J. H., Christian, M., Chan, Y.-W., Boomsma, C. M., Moore, J. D., Hartshorne, G. M., Sućurović, S., Mulac-Jericevic, B., Heijnen, C. J., Quenby, S., Koerkamp, M. J. G., Holstege, F. C. P., Shmygol, A., & Macklon, N. S. (2014). Uterine selection of human embryos at implantation. *Scientific Reports*, *4*, 3894. https://doi.org/10.1038/srep03894

Burton, G. J., Woods, A. W., Jauniaux, E., & Kingdom, J. C. P. (2009). Rheological and physiological consequences of conversion of the maternal spiral arteries for uteroplacental blood flow during human pregnancy. *Placenta*, *30*(6), 473–482. https://doi.org/10.1016/j.placenta.2009.02.009

Caballero-Campo P, Dominguez F, Coloma J, Meseguer M, Remohi J, Pellicer A, Simon C. Hormonal and embryonic regulation of chemokines IL-8, MCP-1 and RANTES in the human endometrium during the window of implantation . Mol Hum Reprod 2002;8:375–384.

Canadian Hydroxychloroquine Study Group. (1991). A randomized study of the effect of withdrawing hydroxychloroquine sulfate in systemic lupus erythematosus. *The New England Journal of Medicine*, *324*(3), 150–154. https://doi.org/10.1056/NEJM199101173240303

Canella, P. R. B. C., Barini, R., Carvalho, P. de O., & Razolli, D. S. (2021a). Lipid emulsion therapy in women with recurrent pregnancy loss and repeated implantation failure: The role of abnormal natural killer cell activity. *Journal of Cellular and Molecular Medicine*, *25*(5), 2290–2296. https://doi.org/10.1111/jcmm.16257

Canella, P. R. B. C., Barini, R., Carvalho, P. de O., & Razolli, D. S. (2021b). Lipid emulsion therapy in women with recurrent pregnancy loss and repeated implantation failure: The role of abnormal natural killer cell activity. *Journal of Cellular and Molecular Medicine*, *25*(5), 2290–2296. https://doi.org/10.1111/jcmm.16257

Canella, P. R. B. C., Barini, R., Carvalho, P. de O., & Razolli, D. S. (2021c). Lipid emulsion therapy in women with recurrent pregnancy loss and repeated implantation failure: The role of abnormal natural killer cell activity. *Journal of Cellular and Molecular Medicine*, *25*(5), 2290–2296. https://doi.org/10.1111/jcmm.16257

Cao, C., Bai, S., Zhang, J., Sun, X., Meng, A., & Chen, H. (n.d.). Understanding recurrent pregnancy loss: Recent advances on its etiology, clinical diagnosis, and management. *Medical Review*, *2*(6), 570–589. https://doi.org/10.1515/mr-2022-0030

Carp, H. j. a., Ahiron, R., Mashiach, S., Carp, H. j. a., Toder, V., Torchinski, A., Shoenfeld, Y., & Gazit, E. (2001). Further Experience with Intravenous Immunoglobulin in Women with Recurrent Miscarriage and a Poor Prognosis. *American Journal of Reproductive Immunology*, *46*(4), 268–273. https://doi.org/10.1034/j.1600-0897.2001.d01-12.x

Carp, H. J., Ahiron, R., Mashiach, S., Schonfeld, Y., Gazit, E., & Toder, V. (1996). Intravenous immunoglobulin in women with five or more abortions. *American Journal of Reproductive Immunology (New York, N.Y.: 1989)*, *35*(4), 360–362. https://doi.org/10.1111/j.1600-0897.1996.tb00494.x

Cavalcante, M. B., Costa, F. D. S., Barini, R., & Araujo Júnior, E. (2015a). Granulocyte colony-stimulating factor and reproductive medicine: A review. *Iranian Journal of Reproductive Medicine*, *13*(4), 195–202.

Cavalcante, M. B., Costa, F. D. S., Barini, R., & Araujo Júnior, E. (2015b). Granulocyte colony-stimulating factor and reproductive medicine: A review. *Iranian Journal of Reproductive Medicine*, *13*(4), 195–202.

Cavalcante, M. B., Sarno, M., & Barini, R. (2021a). Lymphocyte immunotherapy in recurrent miscarriage and recurrent implantation failure. *American Journal of Reproductive Immunology (New York, N.Y.: 1989)*, *85*(4), e13408. https://doi.org/10.1111/aji.13408

Cavalcante, M. B., Sarno, M., & Barini, R. (2021b). Lymphocyte immunotherapy in recurrent miscarriage and recurrent implantation failure. *American Journal of Reproductive Immunology (New York, N.Y.: 1989)*, *85*(4), e13408. https://doi.org/10.1111/aji.13408

Cha, J., Sun, X., & Dey, S. K. (2012). Mechanisms of implantation: Strategies for successful pregnancy. *Nature Medicine*, *18*(12), 1754–1767. https://doi.org/10.1038/nm.3012

Champion, H., Innes, B. A., Robson, S. C., Lash, G. E., & Bulmer, J. N. (2012). Effects of interleukin-6 on extravillous trophoblast invasion in early human pregnancy. *Molecular Human Reproduction*, *18*(8), 391–400. https://doi.org/10.1093/molehr/gas010

Chan, R. W. S., Schwab, K. E., & Gargett, C. E. (2004). Clonogenicity of Human Endometrial Epithelial and Stromal Cells1. *Biology of Reproduction*, *70*(6), 1738–1750. https://doi.org/10.1095/biolreprod.103.024109

Chen, J., Liu, B., Zhang, Y., Ao, L., Li, Z., Qu, B., Li, X., & Yang, J. (2020). Effect of immunotherapy on patients with unexplained recurrent spontaneous abortion. *Annals of Palliative Medicine*, *9*(5), Article 5. https://doi.org/10.21037/apm-19-440b

Chen, K., Yu, Q., Sha, Q., Wang, J., Fang, J., Li, X., Shen, X., Fu, B., & Guo, C. (2024). Single-cell transcriptomic analysis of immune cell dynamics in the healthy human endometrium. *Biochemistry and Biophysics Reports*, *39*, 101802. https://doi.org/10.1016/j.bbrep.2024.101802

Chen, S., Yang, G., Wu, P., Sun, Y., Dai, F., He, Y., Qian, H., Liu, Y., & Shi, G. (2020). Antinuclear antibodies positivity is a risk factor of recurrent pregnancy loss: A meta-analysis. *Seminars in Arthritis and Rheumatism*, *50*(4), 534–543. https://doi.org/10.1016/j.semarthrit.2020.03.016

Christiansen, O. B. (1998). Intravenous immunoglobulin in the prevention of recurrent spontaneous abortion: The European experience. *American Journal of Reproductive Immunology (New York, N.Y.: 1989)*, *39*(2), 77–81. https://doi.org/10.1111/j.1600-0897.1998.tb00338.x

Christiansen, O. B., Larsen, E. C., Egerup, P., Lunoee, L., Egestad, L., & Nielsen, H. S. (2015). Intravenous immunoglobulin treatment for secondary recurrent miscarriage: A randomised, double-blind, placebo-controlled trial. *BJOG: An International Journal of Obstetrics and Gynaecology*, *122*(4), 500–508. https://doi.org/10.1111/1471-0528.13192

Christiansen, O. B., Mathiesen, O., Husth, M., Lauritsen, J. G., & Grunnet, N. (1994). Placebo-controlled trial of active immunization with third party leukocytes in recurrent miscarriage. *Acta Obstetricia Et Gynecologica Scandinavica*, *73*(3), 261–268. https://doi.org/10.3109/00016349409023451

Christiansen, O. B., Mathiesen, O., Husth, M., Rasmussen, K. L., Ingerslev, H. J., Lauritsen, J. G., & Grunnet, N. (1995). Placebo-controlled trial of treatment of unexplained secondary recurrent spontaneous abortions and recurrent late spontaneous abortions with i.v. Immunoglobulin. *Human Reproduction (Oxford, England)*, *10*(10), 2690–2695. https://doi.org/10.1093/oxfordjournals.humrep.a135769

Clark, D. A. (2011). Intravenous immunoglobulin and idiopathic secondary recurrent miscarriage: Methodological problems. *Human Reproduction (Oxford, England)*, *26*(9), 2586–2587; author reply 2587-2589. https://doi.org/10.1093/humrep/der185

Clifford K, Flanagan AM, Regan L. Endometrial CD56 natural killer cells in women with recurrent miscarriage: a histomorphometric study. Hum Reprod. 1999;14:2727– 2730.

Comins-Boo, A., Valdeolivas, L., Pérez-Pla, F., Cristóbal, I., Subhi-Issa, N., Domínguez-Soto, Á., Pilar-Suárez, L., Gasca-Escorial, P., Calvo-Urrutia, M., Fernández-Arquero, M., Herráiz, M. Á., Corbí, Á., & Sánchez-Ramón, S. (2022a). Immunophenotyping of peripheral blood monocytes could help identify a baseline pro-inflammatory profile in women with recurrent reproductive failure. *Journal of Reproductive Immunology*, *154*, 103735. https://doi.org/10.1016/j.jri.2022.103735

Comins-Boo, A., Valdeolivas, L., Pérez-Pla, F., Cristóbal, I., Subhi-Issa, N., Domínguez-Soto, Á., Pilar-Suárez, L., Gasca-Escorial, P., Calvo-Urrutia, M., Fernández-Arquero, M., Herráiz, M. Á., Corbí, Á., & Sánchez-Ramón, S. (2022b). Immunophenotyping of peripheral blood monocytes could help identify a baseline pro-inflammatory profile in women with recurrent reproductive failure. *Journal of Reproductive Immunology*, *154*, 103735. https://doi.org/10.1016/j.jri.2022.103735

Comins-Boo, A., Valdeolivas, L., Pérez-Pla, F., Cristóbal, I., Subhi-Issa, N., Domínguez-Soto, Á., Pilar-Suárez, L., Gasca-Escorial, P., Calvo-Urrutia, M., Fernández-Arquero, M., Herráiz, M. Á., Corbí, Á., & Sánchez-Ramón, S. (2022c). Immunophenotyping of peripheral blood monocytes could help identify a baseline pro-inflammatory profile in women with recurrent reproductive failure. *Journal of Reproductive Immunology*, *154*, 103735. https://doi.org/10.1016/j.jri.2022.103735

Coomarasamy, A., Williams, H., Truchanowicz, E., Seed, P. T., Small, R., Quenby, S., Gupta, P., Dawood, F., Koot, Y. E., Atik, R. B., Bloemenkamp, K. W., Brady, R., Briley, A., Cavallaro, R., Cheong, Y. C., Chu, J., Eapen, A., Essex, H., Ewies, A., … Rai, R. (2016a). PROMISE: First-trimester progesterone therapy in women with a history of unexplained recurrent miscarriages - a randomised, double-blind, placebo-controlled, international multicentre trial and economic evaluation. *Health Technology Assessment (Winchester, England)*, *20*(41), 1–92. https://doi.org/10.3310/hta20410

Coomarasamy, A., Williams, H., Truchanowicz, E., Seed, P. T., Small, R., Quenby, S., Gupta, P., Dawood, F., Koot, Y. E., Atik, R. B., Bloemenkamp, K. W., Brady, R., Briley, A., Cavallaro, R., Cheong, Y. C., Chu, J., Eapen, A., Essex, H., Ewies, A., … Rai, R. (2016b). PROMISE: First-trimester progesterone therapy in women with a history of unexplained recurrent miscarriages - a randomised, double-blind, placebo-controlled, international multicentre trial and economic evaluation. *Health Technology Assessment (Winchester, England)*, *20*(41), 1–92. https://doi.org/10.3310/hta20410

Coombs, P. E. (n.d.). *Research Subject Guides: Systematic Reviews and Evidence Syntheses: Databases*. Retrieved 5 January 2025, from https://subjectguides.lib.neu.edu/systematicreview/databases

Corre I., Pineau D., Hermouet S. Interleukin-8: An Autocrine/Paracrine Growth Factor for Human Hematopoietic Progenitors Acting in Synergy with Colony Stimulating Factor-1 to Promote Monocyte-Macrophage Growth and Differentiation. Exp. Hematol. 1999;27:28–36. doi: 10.1016/S0301-472X(98)00032-0.

Coulam, C. B., Krysa, L., Stern, J. J., & Bustillo, M. (1995). Intravenous immunoglobulin for treatment of recurrent pregnancy loss. *American Journal of Reproductive Immunology (New York, N.Y.: 1989)*, *34*(6), 333–337. https://doi.org/10.1111/j.1600-0897.1995.tb00960.x

Coulam, C. B., Stern, J. J., & Bustillo, M. (1994). Ultrasonographic findings of pregnancy losses after treatment for recurrent pregnancy loss: Intravenous immunoglobulin versus placebo. *Fertility and Sterility*, *61*(2), 248–251. https://doi.org/10.1016/s0015-0282(16)56511-x

Critchley, H. O. D., Maybin, J. A., Armstrong, G. M., & Williams, A. R. W. (2020). Physiology of the Endometrium and Regulation of Menstruation. *Physiological Reviews*, *100*(3), 1149–1179. https://doi.org/10.1152/physrev.00031.2019

de Ziegler D, Fanchin R, de Moustier B, Bulletti C. The hormonal control of endometrial receptivity: estrogen (E2) and progesterone, J Reprod Immunol, 1998, vol. 39 (pg. 149-166)

Daher, S., Denardi, K. de A. G., Blotta, M. H. S. L., Mamoni, R. L., Reck, A. P. M., Camano, L., & Mattar, R. (2004). Cytokines in recurrent pregnancy loss. *Journal of Reproductive Immunology*, *62*(1), 151–157. https://doi.org/10.1016/j.jri.2003.10.004

Dai, S., Jia, R., Zhang, X., Fang, Q., & Huang, L. (2014). The PD-1/PD-Ls pathway and autoimmune diseases. *Cellular Immunology*, *290*(1), 72–79. https://doi.org/10.1016/j.cellimm.2014.05.006

Dakhly, D. M. R., Bayoumi, Y. A., Sharkawy, M., Gad Allah, S. H., Hassan, M. A., Gouda, H. M., Hashem, A. T., Hatem, D. L., Ahmed, M. F., & El-Khayat, W. (2016). Intralipid supplementation in women with recurrent spontaneous abortion and elevated levels of natural killer cells. *International Journal of Gynaecology and Obstetrics: The Official Organ of the International Federation of Gynaecology and Obstetrics*, *135*(3), 324–327. https://doi.org/10.1016/j.ijgo.2016.06.026

Dan, S., Wei, W., Yichao, S., Hongbo, C., Shenmin, Y., Jiaxiong, W., & Hong, L. (2015a). Effect of Prednisolone Administration on Patients with Unexplained Recurrent Miscarriage and in Routine Intracytoplasmic Sperm Injection: A Meta-Analysis. *American Journal of Reproductive Immunology (New York, N.Y.: 1989)*, *74*(1), 89–97. https://doi.org/10.1111/aji.12373

Dan, S., Wei, W., Yichao, S., Hongbo, C., Shenmin, Y., Jiaxiong, W., & Hong, L. (2015b). Effect of Prednisolone Administration on Patients with Unexplained Recurrent Miscarriage and in Routine Intracytoplasmic Sperm

Injection: A Meta-Analysis. *American Journal of Reproductive Immunology (New York, N.Y.: 1989)*, *74*(1), 89–97. https://doi.org/10.1111/aji.12373

Dakhly DMR, Bayoumi YA, Sharkawy M, et al. Intralipid supplementation in women with recurrent spontaneous abortion and elevated levels of natural killer cells. *Int J Gynaecol Obstet*. 2016;135(3):324-327. 10.1016/j.ijgo.2016.06.026

de Moreuil, C., Alavi, Z., & Pasquier, E. (2020). Hydroxychloroquine may be beneficial in preeclampsia and recurrent miscarriage. *British Journal of Clinical Pharmacology*, *86*(1), 39–49. https://doi.org/10.1111/bcp.14131

Dekel, N., Gnainsky, Y., Granot, I., & Mor, G. (2010). Inflammation and implantation. *American Journal of Reproductive Immunology (New York, N.Y.: 1989)*, *63*(1), 17–21. https://doi.org/10.1111/j.1600-0897.2009.00792.x

Dendrinos, S., Sakkas, E., & Makrakis, E. (2009). Low-molecular-weight heparin versus intravenous immunoglobulin for recurrent abortion associated with antiphospholipid antibody syndrome. *International Journal of Gynaecology and Obstetrics: The Official Organ of the International Federation of Gynaecology and Obstetrics*, *104*(3), 223–225. https://doi.org/10.1016/j.ijgo.2008.11.010

Dernoncourt, A., Hedhli, K., Abisror, N., Cheloufi, M., Cohen, J., Kolanska, K., McAvoy, C., Selleret, L., Ballot, E., Mathieu d'Argent, E., Chabbert Buffet, N., Fain, O., Kayem, G., & Mekinian, A. (2024). Hydroxychloroquine in recurrent pregnancy loss: Data from a French prospective multicenter registry. *Human Reproduction (Oxford, England)*, *39*(9), 1934–1941. https://doi.org/10.1093/humrep/deae146

Devall, A. J., Papadopoulou, A., Podesek, M., Haas, D. M., Price, M. J., Coomarasamy, A., & Gallos, I. D. (2021). Progestogens for preventing miscarriage: A network meta-analysis. *The Cochrane Database of Systematic Reviews*, *4*(4), CD013792. https://doi.org/10.1002/14651858.CD013792.pub2

Diejomaoh, M. F., Al-Azemi, M. M., Bandar, A., Egbase, P. E., Jirous, J., Al-Othman, S., Bukhadour, N., & Al-Sweih, N. (2002). A favorable outcome of pregnancies in women with primary and secondary recurrent pregnancy loss associated with antiphospholipid syndrome. *Archives of Gynecology and Obstetrics*, *266*(2), 61–66. https://doi.org/10.1007/s004040100179

Ding, J., Wang, J., Cai, X., Yin, T., Zhang, Y., Yang, C., & Yang, J. (2022). Granulocyte colony-stimulating factor in reproductive-related disease: Function, regulation and therapeutic effect. *Biomedicine & Pharmacotherapy*, *150*, 112903. https://doi.org/10.1016/j.biopha.2022.112903

Efficacy and safety of recombinant human granulocyte colony-stimulating factor in patients with unexplained recurrent spontaneous abortion: A systematic review and meta-analysis—ScienceDirect. (n.d.). Retrieved 6 January 2025, from https://www.sciencedirect.com/science/article/pii/S0165037823000360

Elenkov, I. J. (2004). Glucocorticoids and the Th1/Th2 balance. *Annals of the New York Academy of Sciences, 1024*(1), 138–146. https://doi.org/10.1196/annals.1321.010

Encyclopedia of Reproduction. (n.d.). ScienceDirect. Retrieved 1 January 2025, from http://www.sciencedirect.com:5070/referencework/9780128151457/encyclopedia-of-reproduction

Epland, K., Suez, D., & Paris, K. (2022). A clinician's guide for administration of high-concentration and facilitated subcutaneous immunoglobulin replacement therapy in patients with primary immunodeficiency diseases. *Allergy, Asthma & Clinical Immunology*, *18*(1), 87. https://doi.org/10.1186/s13223-022-00726-7

ESHRE Guideline Group on RPL, Bender Atik, R., Christiansen, O. B., Elson, J., Kolte, A. M., Lewis, S., Middeldorp, S., Mcheik, S., Peramo, B., Quenby, S., Nielsen, H. S., van der Hoorn, M.-L., Vermeulen, N., & Goddijn, M. (2023). ESHRE guideline: Recurrent pregnancy loss: an update in 2022. *Human Reproduction Open*, *2023*(1), hoad002. https://doi.org/10.1093/hropen/hoad002

European Society of Human Reproduction and Embryology. Recurrent Pregnancy Loss – Guideline of the European Society of Human Reproduction and Embryology. Belgium: ESHRE, 2017.

Evain-Brion, D., & Malassine, A. (2003). Human placenta as an endocrine organ. *Growth Hormone & IGF Research: Official Journal of the Growth Hormone Research Society and the International IGF Research Society*, *13 Suppl A*, S34-37. https://doi.org/10.1016/s1096-6374(03)00053-4

Expensive immunotherapy shows no advantage over placebo for recurrent miscarriage patients—UChicago Medicine. (n.d.). Retrieved 25 December 2024, from https://www.uchicagomedicine.org/forefront/news/2010/august/expensive-immunotherapy-shows-no-advantage-over-placebo-for-recurrent-miscarriage-patients

Fernainy, P., Cohen, A. A., Murray, E., Losina, E., Lamontagne, F., & Sourial, N. (2024). Rethinking the pros and cons of randomized controlled trials and observational studies in the era of big data and advanced methods: A panel discussion. *BMC Proceedings*, *18*(2), 1. https://doi.org/10.1186/s12919-023-00285-8

Ford, H. B., & Schust, D. J. (2009). Recurrent Pregnancy Loss: Etiology, Diagnosis, and Therapy. *Reviews in Obstetrics and Gynecology*, *2*(2), 76–83.

Foyle, K. L., Sharkey, D. J., Moldenhauer, L. M., Green, E. S., Wilson, J. J., Roccisano, C. J., Hull, M. L., Tremellen, K. P., & Robertson, S. A. (2021). Effect of Intralipid infusion on peripheral blood T cells and plasma cytokines in women undergoing assisted reproduction treatment. *Clinical & Translational Immunology*, *10*(8), e1328. https://doi.org/10.1002/cti2.1328

Friedman, T., & Gath, D. (1989). The psychiatric consequences of spontaneous abortion. *The British Journal of Psychiatry: The Journal of Mental Science*, *155*, 810–813. https://doi.org/10.1192/bjp.155.6.810

G-CSF (Granulocyte-Colony Stimulating Factor) Treatment. (n.d.). Cleveland Clinic. Retrieved 3 January 2025, from https://my.clevelandclinic.org/health/treatments/24126-g-csf-treatment

Gellersen, B., Brosens, I. A., & Brosens, J. J. (2007). Decidualization of the human endometrium: Mechanisms, functions, and clinical perspectives. *Seminars in Reproductive Medicine*, *25*(6), 445–453. https://doi.org/10.1055/s-2007-991042

Gellersen, B., & Brosens, J. J. (2014b). Cyclic Decidualization of the Human Endometrium in Reproductive Health and Failure. *Endocrine Reviews*, *35*(6), 851–905. https://doi.org/10.1210/er.2014-1045

Gellersen B, Brosens J 2003 Cyclic AMP and progesterone receptor crosstalk in human endometrium: a decidualizing affair. J Endocrinol 178:357–372

Genest, G., Almasri, W., Banjar, S., Beauchamp, C., Buckett, W., Dzineku, F., Demirtas, E., Gold, P., Dahan, M. H., Jamal, W., Kadoch, I. J., Lapensée, L., Mahutte, N., Miron, P., Sylvestre, C., Tulandi, T., Piccirillo, C. A., & Laskin, C. A. (2022b). Immunotherapy for recurrent pregnancy loss: A reappraisal. *F&S Reviews*, *3*(1), 24–41. https://doi.org/10.1016/j.xfnr.2021.11.002

Glucocorticoids Improve the Pregnancy Rate and Outcome in Women With Unexplained Positive Autoantibodies: A Systematic Review and Meta-Analysis—PMC. (n.d.). Retrieved 6 January 2025, from https://pmc.ncbi.nlm.nih.gov/articles/PMC9131042/

Gołąbek-Grenda, A., & Olejnik, A. (2022). *In vitro* modeling of endometriosis and endometriotic microenvironment – Challenges and recent advances. *Cellular Signalling*, *97*, 110375. https://doi.org/10.1016/j.cellsig.2022.110375

Gomaa, M. F., Elkholy, A. G., El-Said, M. M., & Abdel-Salam, N. E. (2014). Combined oral prednisolone and heparin versus heparin: The effect on peripheral NK cells and clinical outcome in patients with unexplained recurrent miscarriage. A double-blind placebo randomized controlled trial. *Archives of Gynecology and Obstetrics*, *290*(4), 757–762. https://doi.org/10.1007/s00404-014-3262-0

GRADE approach. (n.d.). Retrieved 1 January 2025, from https://training.cochrane.org/grade-approach

Grandi, S. M., Hinkle, S. N., Mumford, S. L., Sjaarda, L. A., Grantz, K. L., Mendola, P., Mills, J. L., Pollack, A. Z., Yeung, E., Zhang, C., & Schisterman, E. F. (2022). Long-Term Mortality in Women With Pregnancy Loss and Modification by Race/Ethnicity. *American Journal of Epidemiology*, *191*(5), 787–799. https://doi.org/10.1093/aje/kwac023

Granulocyte colony stimulating factor (G-CSF). (n.d.). Retrieved 3 January 2025, from https://www.cancerresearchuk.org/about-cancer/treatment/drugs/g-csf

Graphou, O., Chioti, A., Pantazi, A., Tsukoura, C., Kontopoulou, V., Guorgiadou, E., Balafoutas, C., Koussoulakos, S., Margaritis, L. H., & Varla-Leftherioti, M. (2003). Effect of intravenous immunoglobulin treatment on the Th1/Th2 balance in women with recurrent spontaneous abortions. *American Journal of Reproductive Immunology (New York, N.Y.: 1989)*, *49*(1), 21–29. https://doi.org/10.1034/j.1600-0897.2003.01169.x

Greenbaum, S., Averbukh, I., Soon, E., Rizzuto, G., Baranski, A., Greenwald, N. F., Kagel, A., Bosse, M., Jaswa, E. G., Khair, Z., Kwok, S., Warshawsky, S., Piyadasa, H., Goldston, M., Spence, A., Miller, G., Schwartz, M., Graf, W., Van Valen, D., … Angelo, M. (2023). A spatially resolved timeline of the human maternal–fetal interface. *Nature*, *619*(7970), 595–605. https://doi.org/10.1038/s41586-023-06298-9

Group, T. G. R. (1994). Intravenous immunoglobulin in the prevention of recurrent miscarriage. *BJOG: An International Journal of Obstetrics & Gynaecology*, *101*(12), 1072–1077. https://doi.org/10.1111/j.1471-0528.1994.tb13584.x

Guo, C., Cai, P., Jin, L., Sha, Q., Yu, Q., Zhang, W., Jiang, C., Liu, Q., Zong, D., Li, K., Fang, J., Lu, F., Wang, Y., Li, D., Lin, J., Li, L., Zeng, Z., Tong, X., Wei, H., & Qu, K. (2021). Single-cell profiling of the human decidual immune

microenvironment in patients with recurrent pregnancy loss. *Cell Discovery*, *7*(1), 1–15. https://doi.org/10.1038/s41421-020-00236-z

Guo W., Li P, Zhao G, Fan H, Hu Y, Hou Y.Glucocorticoid receptor mediates the effect of progesterone on uterine natural killer cells.Am J Reprod Immunol. 2012;67:463– 473.

Hanna J, Goldman-Wohl D, Hamani Y, et al.Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. Nat Med. 2006;12:1065–1074.

Hariton, E., & Locascio, J. J. (2018). Randomised controlled trials—The gold standard for effectiveness research. *BJOG : An International Journal of Obstetrics and Gynaecology*, *125*(13), 1716. https://doi.org/10.1111/1471-0528.15199

He, Y., Su, X., Li, H., Tang, R., Ju, Y., Chen, S., & Wang, X. (2024). Subcutaneous injection granulocyte colony-stimulating factor (G-CSF) is superior to intrauterine infusion on patients with recurrent implantation failure: A systematic review and network meta-analysis. *Journal of Reproductive Immunology*, *163*, 104250. https://doi.org/10.1016/j.jri.2024.104250

Hedegaard, S., Landersoe, S. K., Olsen, L. R., Krog, M. C., Kolte, A. M., & Nielsen, H. S. (2021). Stress and depression among women and men who have experienced recurrent pregnancy loss: Focusing on both sexes. *Reproductive BioMedicine Online*, *42*(6), 1172–1180. https://doi.org/10.1016/j.rbmo.2021.03.012

Heilmann, L., Kriechbaum, A., Hojnacki, B., Bode, V., & Wolf, H. (1993). [Clinical experiences with passive immunotherapy in habitual abortion]. *Zeitschrift Fur Geburtshilfe Und Perinatologie*, *197*(2), 55–58.

Hirahara F., Andoh N., Sawai K., Hirabuki T., Uemura T., Minaguchi H. Hyperprolactinemic recurrent miscarriage and results of randomized bromocriptine treatment trials. Fertil. Steril. 1998;70:246–252. doi: 10.1016/S0015-0282(98)00164-2.

Hong Li, Y., & Marren, A. (2018). Recurrent pregnancy loss: A summary of international evidence-based guidelines and practice. *Australian Journal of General Practice*, *47*(7), 432–436. https://doi.org/10.31128/AJGP-01-18-4459

Hsieh, L.-E., Song, J., Tremoulet, A. H., Burns, J. C., & Franco, A. (2022). Intravenous immunoglobulin induces IgG internalization by tolerogenic myeloid dendritic cells that secrete IL-10 and expand Fc-specific regulatory T cells. *Clinical and Experimental Immunology*, *208*(3), 361–371. https://doi.org/10.1093/cei/uxac046

Hutton, B., Sharma, R., Fergusson, D., Tinmouth, A., Hebert, P., Jamieson, J., & Walker, M. (2007). Use of intravenous immunoglobulin for treatment of recurrent miscarriage: A systematic review. *BJOG: An International Journal of Obstetrics & Gynaecology*, *114*(2), 134–142. https://doi.org/10.1111/j.1471-0528.2006.01201.x

Hyde KJ, Schust DJ. Genetic considerations in recurrent pregnancy loss. Cold Spring Harb Perspect Med. 2015 Feb 6;5(3):a023119. doi: 10.1101/cshperspect.a023119. PMID: 25659378; PMCID: PMC4355257.

Immunoglobulin replacement therapy | Immune Deficiency Foundation. (n.d.). Retrieved 25 December 2024, from https://primaryimmune.org/understanding-primary-immunodeficiency/treatment/immunoglobulin-replacement-therapy

In vitro decidualisation of canine uterine stromal cells | Reproductive Biology and Endocrinology | Full Text. (n.d.). Retrieved 6 January 2025, from https://rbej.biomedcentral.com/articles/10.1186/s12958-015-0066-4

Intralipid therapy and adverse reproductive outcome: Is there any evidence? - PubMed. (n.d.). Retrieved 6 January 2025, from https://pubmed.ncbi.nlm.nih.gov/35118388/

Intravenous immunoglobulin in the prevention of recurrent miscarriage. The German RSA/IVIG Group. (1994). *British Journal of Obstetrics and Gynaecology*, *101*(12), 1072–1077. https://doi.org/10.1111/j.1471-0528.1994.tb13584.x

Intravenous Immunoglobulin (IVIG). (n.d.). Retrieved 25 December 2024, from https://rheumatology.org/patients/intravenous-immunoglobulin-ivig

Jablonowska, B., Palfi, M., Matthiesen, L., Selbing, A., Kjellberg, S., & Ernerudh, J. (2002). T and B lymphocyte subsets in patients with unexplained recurrent spontaneous abortion: IVIG versus placebo treatment. *American Journal of Reproductive Immunology (New York, N.Y.: 1989)*, *48*(5), 312–318. https://doi.org/10.1034/j.1600-0897.2002.01010.x

Jafarzadeh, S., Ahmadi, M., Dolati, S., Aghebati-Maleki, L., Eghbal-Fard, S., Kamrani, A., Behrad, B., Roshangar, L., Jadidi-Niaragh, F., Yousefi, B., Mehdipour, M., Farzadi, L., & Yousefi, M. (2019). Intravenous immunoglobulin G treatment increases live birth rate in women with recurrent miscarriage and modulates regulatory and exhausted regulatory T cells frequency and function. *Journal of Cellular Biochemistry*, *120*(4), 5424–5434. https://doi.org/10.1002/jcb.27821

Jeremić, K., Pervulov, M., Gojnić, M., Dukanac, J., Ljubić, A., & Stojnić, J. (2005). Comparison of two therapeutic protocols in patients with antiphospholipid antibodies and recurrent miscarriages. *Vojnosanitetski Pregled*, *62*(6), 435–439. https://doi.org/10.2298/vsp0506435j

Jin, L.-P., Fan, D.-X., Zhang, T., Guo, P.-F., & Li, D.-J. (2011). The costimulatory signal upregulation is associated with Th1 bias at the maternal-fetal interface in human miscarriage. *American Journal of Reproductive Immunology (New York, N.Y.: 1989)*, *66*(4), 270–278. https://doi.org/10.1111/j.1600-0897.2011.00997.x

Jones, R. L., Stoikos, C., Findlay, J. K., & Salamonsen, L. A. (2006). *TGF-β superfamily expression and actions in the endometrium and placenta*. https://doi.org/10.1530/rep.1.01076

Justus, C. R., Marie, M. A., Sanderlin, E. J., & Yang, L. V. (2023). Transwell In Vitro Cell Migration and Invasion Assays. In O. Friedrich & D. F. Gilbert (Eds.), *Cell Viability Assays: Methods and Protocols* (pp. 349–359). Springer US. https://doi.org/10.1007/978-1-0716-3052-5_22

Kaandorp, S. P., Goddijn, M., Post, J. A. M. van der, Hutten, B. A., Verhoeve, H. R., Hamulyák, K., Mol, B. W., Folkeringa, N., Nahuis, M., Papatsonis, D. N. M., Büller, H. R., Veen, F. van der, & Middeldorp, S. (2010). Aspirin plus Heparin or Aspirin Alone in Women with Recurrent Miscarriage. *New England Journal of Medicine*, *362*(17), 1586–1596. https://doi.org/10.1056/NEJMoa1000641

Kessel, A., Ammuri, H., Peri, R., Pavlotzky, E. R., Blank, M., Shoenfeld, Y., & Toubi, E. (2007). Intravenous immunoglobulin therapy affects T regulatory cells by increasing their suppressive function. *Journal of Immunology (Baltimore, Md.: 1950)*, *179*(8), 5571–5575. https://doi.org/10.4049/jimmunol.179.8.5571

Kim, J. H., Kim, S. H., Yang, N., Ko, Y., Lee, S. R., & Chae, H. D. (2022a). Outcomes of Empirical Treatment With Intravenous Immunoglobulin G Combined With Low-Dose Aspirin in Women With Unexplained Recurrent Pregnancy Loss. *Journal of Korean Medical Science*, *37*(25), e200. https://doi.org/10.3346/jkms.2022.37.e200

Kim, J. H., Kim, S. H., Yang, N., Ko, Y., Lee, S. R., & Chae, H. D. (2022b). Outcomes of Empirical Treatment With Intravenous Immunoglobulin G Combined With Low-Dose Aspirin in Women With Unexplained Recurrent Pregnancy Loss. *Journal of Korean Medical Science*, *37*(25), e200. https://doi.org/10.3346/jkms.2022.37.e200

Kiprov, D. D., Nachtigall, R. D., Weaver, R. C., Jacobson, A., Main, E. K., & Garovoy, M. R. (1996). The use of intravenous immunoglobulin in recurrent pregnancy loss associated with combined alloimmune and autoimmune abnormalities. *American Journal of Reproductive Immunology (New York, N.Y.: 1989)*, *36*(4), 228–234. https://doi.org/10.1111/j.1600-0897.1996.tb00168.x

Kitaya K, Yasuda J, Yagi I, Tada Y, Fushiki S, Honjo H. IL-15 expression at human endometrium and decidua.Biol Reprod. 2000;63:683– 687.

Kniotek, M., Zych, M., Roszczyk, A., Szafarowska, M., & Jerzak, M. M. (2021). Decreased Production of TNF-α and IL-6 Inflammatory Cytokines in Non-Pregnant Idiopathic RPL Women Immunomodulatory Effect of Sildenafil Citrate on the Cellular Response of Idiopathic RPL Women. *Journal of Clinical Medicine*, *10*(14), 3115. https://doi.org/10.3390/jcm10143115

Krieg S.A., Fan X., Hong Y., Sang Q.-X., Giaccia A., Westphal L.M., Lathi R.B., Krieg A.J., Nayak N.R. Global Alteration in Gene Expression Profiles of Deciduas from Women with Idiopathic Recurrent Pregnancy Loss. MHR Basic Sci. Reprod. Med. 2012;18:442–450. doi: 10.1093/molehr/gas017.

Kobayashi, H. (2023). Endometrial Inflammation and Impaired Spontaneous Decidualization: Insights into the Pathogenesis of Adenomyosis. *International Journal of Environmental Research and Public Health*, *20*(4), 3762. https://doi.org/10.3390/ijerph20043762

Koert, E., Malling, G. M. H., Sylvest, R., Krog, M. C., Kolte, A. M., Schmidt, L., & Nielsen, H. S. (2019). Recurrent pregnancy loss: Couples' perspectives on their need for treatment, support and follow up. *Human Reproduction*, *34*(2), 291–296. https://doi.org/10.1093/humrep/dey362

Kumar, A. (2014). Immunomodulation in Recurrent Miscarriage. *Journal of Obstetrics and Gynaecology of India*, *64*(3), 165–168. https://doi.org/10.1007/s13224-014-0541-4

Kuon, R., Müller, F., Vomstein, K., Weber, M., Markert, U., Strowitzki, T., Daniel, V., & Toth, B. (2016). First steps towards a targeted immunomodulatory therapy in patients with recurrent miscarriage and elevated natural killer cells. *Journal of Reproductive Immunology*, *115*, 61. https://doi.org/10.1016/j.jri.2016.04.190

Kuroda, K., Nakagawa, K., Horikawa, T., Moriyama, A., Ojiro, Y., Takamizawa, S., Ochiai, A., Matsumura, Y., Ikemoto, Y., Yamaguchi, K., & Sugiyama, R. (2021). Increasing number of implantation failures and pregnancy losses

associated with elevated Th1/Th2 cell ratio. *American Journal of Reproductive Immunology*, *86*(3), e13429. https://doi.org/10.1111/aji.13429

Kutteh, W. H. (1996). Antiphospholipid antibody-associated recurrent pregnancy loss: Treatment with heparin and low-dose aspirin is superior to low-dose aspirin alone. *American Journal of Obstetrics and Gynecology*, *174*(5), 1584–1589. https://doi.org/10.1016/s0002-9378(96)70610-5

Kwak, J. Y., Gilman-Sachs, A., Beaman, K. D., & Beer, A. E. (1992). Reproductive outcome in women with recurrent spontaneous abortions of alloimmune and autoimmune causes: Preconception versus postconception treatment. *American Journal of Obstetrics and Gynecology*, *166*(6 Pt 1), 1787–1795; discussion 1795-1798. https://doi.org/10.1016/0002-9378(92)91570-z

Kwak-Kim, J. Y. H., Wang, K., Ahn, L., Toth, B., Gilman-Sachs, A., & Lee, T. (2022). Natural killer cell-mediated immunopathology in recurrent pregnancy losses. *Exploration of Immunology*, *2*, 693–722. https://doi.org/10.37349/ei.2022.00077

Lai, T.-J., Teng, S.-W., Chang, C.-K., & Huang, C.-Y. (2024). Progesterone in Pregnancy: Evidence-Based Strategies to Reduce Miscarriage and Enhance Assisted Reproductive Technology. *Medical Science Monitor : International Medical Journal of Experimental and Clinical Research*, *30*, e943400-1-e943400-9. https://doi.org/10.12659/MSM.943400

Laijawala, R. A. (2024). Recurrent Pregnancy Loss: Immunological aetiologies and associations with mental health. *Brain, Behavior, & Immunity - Health*, *41*, 100868. https://doi.org/10.1016/j.bbih.2024.100868

Lash GE, Schiessl B, Kirkley M, et al. Expression of angiogenic growth factors by uterine natural killer cells during early pregnancy. J Leukoc Biol. 2006;80:572–580.

Lee, S. K., Kim, J. Y., Han, A. R., Hur, S. E., Kim, C. J., Kim, T. H., Cho, B. R., Han, J. W., Han, S. G., Na, B. J., & Kwak-Kim, J. (2016). Intravenous Immunoglobulin G Improves Pregnancy Outcome in Women with Recurrent Pregnancy Losses with Cellular Immune Abnormalities. *American Journal of Reproductive Immunology (New York, N.Y.: 1989)*, *75*(1), 59–68. https://doi.org/10.1111/aji.12442

Li, J., Wang, L., Ding, J., Cheng, Y., Diao, L., Li, L., Zhang, Y., & Yin, T. (2022). Multiomics Studies Investigating Recurrent Pregnancy Loss: An Effective Tool for Mechanism Exploration. *Frontiers in Immunology*, *13*, 826198. https://doi.org/10.3389/fimmu.2022.826198

Li, T., Yuan, Y., Liu, H., Lu, Q., & Mu, R. (2022b). Glucocorticoids Improve the Pregnancy Rate and Outcome in Women With Unexplained Positive Autoantibodies: A Systematic Review and Meta-Analysis. *Frontiers in Medicine*, *9*, 819406. https://doi.org/10.3389/fmed.2022.819406

Ling, Q., Xu, J., Tian, Y., Chen, D., Mo, C., & Peng, B. (2024). Effect of IVIG therapy on pregnant women with unexplained recurrent spontaneous abortion: A systematic review and meta-analysis. *Frontiers in Endocrinology*, *15*. https://doi.org/10.3389/fendo.2024.1381461

Lockwood, C. J., Matta, P., Krikun, G., Koopman, L. A., Masch, R., Toti, P., Arcuri, F., Huang, S.-T. J., Funai, E. F., & Schatz, F. (2006). Regulation of Monocyte Chemoattractant Protein-1 Expression by Tumor Necrosis Factor-α and Interleukin-1β in First Trimester Human Decidual Cells. *The American Journal of Pathology*, *168*(2), 445–452. https://doi.org/10.2353/ajpath.2006.050082

Lok, I. H., & Neugebauer, R. (2007). Psychological morbidity following miscarriage. *Best Practice & Research. Clinical Obstetrics & Gynaecology*, *21*(2), 229–247. https://doi.org/10.1016/j.bpobgyn.2006.11.007

Lucas, E. S., Dyer, N. P., Fishwick, K., Ott, S., & Brosens, J. J. (2016). Success after failure: The role of endometrial stem cells in recurrent miscarriage. *Reproduction (Cambridge, England)*, *152*(5), R159-166. https://doi.org/10.1530/REP-16-0306

Lucas, E. S., Dyer, N. P., Murakami, K., Lee, Y. H., Chan, Y.-W., Grimaldi, G., Muter, J., Brighton, P. J., Moore, J. D., Patel, G., Chan, J. K. Y., Takeda, S., Lam, E. W.-F., Quenby, S., Ott, S., & Brosens, J. J. (2016). Loss of Endometrial Plasticity in Recurrent Pregnancy Loss. *Stem Cells (Dayton, Ohio)*, *34*(2), 346–356. https://doi.org/10.1002/stem.2222

Lucas, E. S., Vrljicak, P., Muter, J., Diniz-da-Costa, M. M., Brighton, P. J., Kong, C.-S., Lipecki, J., Fishwick, K. J., Odendaal, J., Ewington, L. J., Quenby, S., Ott, S., & Brosens, J. J. (2020). Recurrent pregnancy loss is associated with a pro-senescent decidual response during the peri-implantation window. *Communications Biology*, *3*(1), 1–14. https://doi.org/10.1038/s42003-020-0763-1

Ma, J., Gao, W., & Li, D. (2023). Recurrent implantation failure: A comprehensive summary from etiology to treatment. *Frontiers in Endocrinology*, *13*, 1061766. https://doi.org/10.3389/fendo.2022.1061766

Ma, N., Qin, R., Qin, W., Liao, M., Zhao, Y., Hang, F., & Qin, A. (2022). Oral immunosuppressants improve pregnancy outcomes in women with idiopathic recurrent miscarriage: A meta-analysis. *Journal of Clinical Pharmacy and Therapeutics*, *47*(7), 870–878. https://doi.org/10.1111/jcpt.13629

Mahmoud, F., Diejomaoh, M., Omu, A., Abul, H., & Haines, D. (2004). Effect of IgG therapy on lymphocyte subpopulations in the peripheral blood of Kuwaiti women experiencing recurrent pregnancy loss. *Gynecologic and Obstetric Investigation*, *58*(2), 77–83. https://doi.org/10.1159/000078154

Mamoun, F. (n.d.). *Placenta holds answers to many unexplained pregnancy losses, study finds*. Retrieved 30 December 2024, from https://medicine.yale.edu/news-article/dr-harvey-kliman-study-finds-the-placenta-holds-answers-to-many-unexplained-pregnancy-losses/

Manfredi, G., Dell'Aera, L., & Liguori, R. (2015). Overcoming recurrent spontaneous abortions in women suffering from IgG subclass deficiency: High efficiency of low dose intravenous immunoglobulins treatment. *European Annals of Allergy and Clinical Immunology*, *47*(3), 91–94.

Marečková, M., Garcia-Alonso, L., Moullet, M., Lorenzi, V., Petryszak, R., Sancho-Serra, C., Oszlanczi, A., Icoresi Mazzeo, C., Wong, F. C. K., Kelava, I., Hoffman, S., Krassowski, M., Garbutt, K., Gaitskell, K., Yancheva, S., Woon, E. V., Male, V., Granne, I., Hellner, K., … Vento-Tormo, R. (2024). An integrated single-cell reference atlas of the human endometrium. *Nature Genetics*, *56*(9), 1925–1937. https://doi.org/10.1038/s41588-024-01873-w

Marozio, L., Nuzzo, A. M., Gullo, E., Moretti, L., Canuto, E. M., Tancredi, A., Goia, M., Cosma, S., Revelli, A., Rolfo, A., & Benedetto, C. (2023). Immune Checkpoints in Recurrent Pregnancy Loss: New Insights into a Detrimental and Elusive Disorder. *International Journal of Molecular Sciences*, *24*(17), Article 17. https://doi.org/10.3390/ijms241713071

Martini, A. E., Jasulaitis, S., Fogg, L. F., Uhler, M. L., & Hirshfeld-Cytron, J. E. (2018). Evaluating the Utility of Intralipid Infusion to Improve Live Birth Rates in Patients with Recurrent Pregnancy Loss or Recurrent Implantation Failure. *Journal of Human Reproductive Sciences*, *11*(3), 261–268. https://doi.org/10.4103/jhrs.JHRS_28_18

Maruyama, T., Makino, T., Iwasaki, K., Sugi, T., Saito, S., Umeuchi, M., Ozawa, N., Matsubayashi, H., & Nozawa, S. (1994). The influence of intravenous immunoglobulin treatment on maternal immunity in women with unexplained recurrent miscarriage. *American Journal of Reproductive Immunology (New York, N.Y.: 1989)*, *31*(1), 7–18. https://doi.org/10.1111/j.1600-0897.1994.tb00841.x

Marzusch, K., Dietl, J., Klein, R., Hornung, D., Neuer, A., & Berg, P. A. (1996). Recurrent first trimester spontaneous abortion associated with antiphospholipid antibodies: A pilot study of treatment with intravenous immunoglobulin. *Acta Obstetricia Et Gynecologica Scandinavica*, *75*(10), 922–926. https://doi.org/10.3109/00016349609055029

McGowan, J., & Sampson, M. (2005). Systematic reviews need systematic searchers. *Journal of the Medical Library Association*, *93*(1), 74–80.

McIntire, R. H., Ganacias, K. G., & Hunt, J. S. (2008a). Programming of human monocytes by the uteroplacental environment. *Reproductive Sciences (Thousand Oaks, Calif.)*, *15*(5), 437–447. https://doi.org/10.1177/1933719107314065

McIntire, R. H., Ganacias, K. G., & Hunt, J. S. (2008b). Programming of Human Monocytes by the Uteroplacental Environment. *Reproductive Sciences (Thousand Oaks, Calif.)*, *15*(5), 437. https://doi.org/10.1177/1933719107314065

Mekinian, A., Lazzaroni, M. G., Kuzenko, A., Alijotas-Reig, J., Ruffatti, A., Levy, P., Canti, V., Bremme, K., Bezanahary, H., Bertero, T., Dhote, R., Maurier, F., Andreoli, L., Benbara, A., Tigazin, A., Carbillon, L., Nicaise-Roland, P., Tincani, A., Fain, O., & SNFMI and the European Forum on Antiphospholipid Antibodies. (2015). The efficacy of hydroxychloroquine for obstetrical outcome in anti-phospholipid syndrome: Data from a European multicenter retrospective study. *Autoimmunity Reviews*, *14*(6), 498–502. https://doi.org/10.1016/j.autrev.2015.01.012

Melo P, Thornton T, Coomarasamy A, Granne I. Evidence for the effectiveness of immunologic therapies in women with subfertility and/or undergoing assisted reproduction. Fertil Steril. 2022 Jun;117(6):1144-1159. doi: 10.1016/j.fertnstert.2022.04.015. PMID: 35618357.

Melo, P., Dhillon-Smith, R., Islam, M. A., Devall, A., & Coomarasamy, A. (2023b). Genetic causes of sporadic and recurrent miscarriage. *Fertility and Sterility*, *120*(5), 940–944. https://doi.org/10.1016/j.fertnstert.2023.08.952

Meng, L., Lin, J., Chen, L., Wang, Z., Liu, M., Liu, Y., Chen, X., Zhu, L., Chen, H., & Zhang, J. (2016a). Effectiveness and potential mechanisms of intralipid in treating unexplained recurrent spontaneous abortion. *Archives of Gynecology and Obstetrics*, *294*(1), 29–39. https://doi.org/10.1007/s00404-015-3922-8

Meng, L., Lin, J., Chen, L., Wang, Z., Liu, M., Liu, Y., Chen, X., Zhu, L., Chen, H., & Zhang, J. (2016b). Effectiveness and potential mechanisms of intralipid in treating unexplained recurrent spontaneous abortion. *Archives of Gynecology and Obstetrics*, *294*(1), 29–39. https://doi.org/10.1007/s00404-015-3922-8

Meng, L., Lin, J., Chen, L., Wang, Z., Liu, M., Liu, Y., Chen, X., Zhu, L., Chen, H., & Zhang, J. (2016c). Effectiveness and potential mechanisms of intralipid in treating unexplained recurrent spontaneous abortion. *Archives of Gynecology and Obstetrics*, *294*(1), 29–39. https://doi.org/10.1007/s00404-015-3922-8

Miko, E., Moocyteyes, M., Doba, K., Barakonyi, A., & Szereday, L. (2019). Immune Checkpoint Molecules in Reproductive Immunology. *Frontiers in Immunology*, *10*, 846. https://doi.org/10.3389/fimmu.2019.00846

Mol, B. W., Lai, S., Rahim, A., Bordewijk, E. M., Wang, R., van Eekelen, R., Gurrin, L. C., Thornton, J. G., van Wely, M., & Li, W. (2023). Checklist to assess Trustworthiness in RAndomised Controlled Trials (TRACT checklist): Concept proposal and pilot. *Research Integrity and Peer Review*, *8*(1), 6. https://doi.org/10.1186/s41073-023-00130-8

Monocyte Chemotactic Protein 1—An overview | ScienceDirect Topics. (n.d.). Retrieved 5 January 2025, from https://www.sciencedirect.com/topics/neuroscience/monocyte-chemotactic-protein-1

Mor, G., & Cardenas, I. (2010). The Immune System in Pregnancy: A Unique Complexity. *American Journal of Reproductive Immunology (New York, N.Y. : 1989)*, *63*(6), 425–433. https://doi.org/10.1111/j.1600-0897.2010.00836.x

Mor, G., Romero, R., Aldo, P. B., & Abrahams, V. M. (2005). Is the trophoblast an immune regulator? The role of Toll-like receptors during pregnancy. *Critical Reviews in Immunology*, *25*(5), 375–388. https://doi.org/10.1615/critrevimmunol.v25.i5.30

Mori, M., Bogdan, A., Balassa, T., Csabai, T., & Szekeres-Bartho, J. (2016). The decidua—The maternal bed embracing the embryo—Maintains the pregnancy. *Seminars in Immunopathology*, *38*(6), 635–649. https://doi.org/10.1007/s00281-016-0574-0

Morikawa, M., Yamada, H., Kato, E. H., Shimada, S., Kishida, T., Yamada, T., Fujimoto, S., & Kobashi, G. (2001). Massive Intravenous Immunoglobulin Treatment in Women with Four or More Recurrent Spontaneous Abortions of Unexplained Etiology: Down-Regulation of NK Cell Activity and Subsets. *American Journal of Reproductive Immunology*, *46*(6), 399–404. https://doi.org/10.1034/j.1600-0897.2001.d01-31.x

Mowbray, J. F., Gibbings, C., Liddell, H., Reginald, P. W., Underwood, J. L., & Beard, R. W. (1985). Controlled trial of treatment of recurrent spontaneous abortion by immunisation with paternal cells. *Lancet (London, England)*, *1*(8435), 941–943. https://doi.org/10.1016/s0140-6736(85)91723-4

Mu, F., Huang, J., Zeng, X., Liu, L., & Wang, F. (2023). Efficacy and safety of recombinant human granulocyte colony-stimulating factor in patients with unexplained recurrent spontaneous abortion: A systematic review and meta-analysis. *Journal of Reproductive Immunology*, *156*, 103830. https://doi.org/10.1016/j.jri.2023.103830

Mueller-Eckhardt, G., Heine, O., Neppert, J., Künzel, W., & Mueller-Eckhardt, C. (1989a). Prevention of recurrent spontaneous abortion by intravenous immunoglobulin. *Vox Sanguinis*, *56*(3), 151–154. https://doi.org/10.1111/j.1423-0410.1989.tb02018.x

Mueller-Eckhardt, G., Heine, O., Neppert, J., Künzel, W., & Mueller-Eckhardt, C. (1989b). Prevention of Recurrent Spontaneous Abortion by Intravenous Immunoglobulin. *Vox Sanguinis*, *56*(3), 151–154. https://doi.org/10.1111/j.1423-0410.1989.tb02018.x

Natural Killer Cells in Pregnancy and Recurrent Pregnancy Loss: Endocrine and Immunologic Perspectives | Endocrine Reviews | Oxford Academic. (n.d.). Retrieved 30 December 2024, from https://academic.oup.com/edrv/article-abstract/26/1/44/2355218?redirectedFrom=fulltext&login=false

Nazari, Z., Ghaffari, J., & Ebadi, A. (2015). Comparison of the effect of aspirin and heparin with or without intravenous immunoglobulin in treatment of recurrent abortion with unknown etiology: A clinical study. *Journal of Natural Science, Biology, and Medicine*, *6*(Suppl 1), S17–S21. https://doi.org/10.4103/0976-9668.166054

Ng, S.-W., Norwitz, G. A., Pavlicev, M., Tilburgs, T., Simón, C., & Norwitz, E. R. (2020). Endometrial Decidualization: The Primary Driver of Pregnancy Health. *International Journal of Molecular Sciences*, *21*(11), 4092. https://doi.org/10.3390/ijms21114092

Noyes RW, Hertig AT, Rock J. Dating the endometrial biopsy. Fertil Steril 1: 3–25, 1950. doi:10.1016/S0015-0282(16)30062-0.

Okada, H., Tsuzuki, T., & Murata, H. (2018a). Decidualization of the human endometrium. *Reproductive Medicine and Biology*, *17*(3), 220–227. https://doi.org/10.1002/rmb2.12088

Okada, H., Tsuzuki, T., & Murata, H. (2018b). Decidualization of the human endometrium. *Reproductive Medicine and Biology*, *17*(3), 220–227. https://doi.org/10.1002/rmb2.12088

Pan-Castillo, B., Gazze, S. A., Thomas, S., Lucas, C., Margarit, L., Gonzalez, D., Francis, L. W., & Conlan, R. S. (2018). Morphophysical dynamics of human endometrial cells during decidualization. *Nanomedicine: Nanotechnology, Biology and Medicine*, *14*(7), 2235–2245. https://doi.org/10.1016/j.nano.2018.07.004

Parhizkar, F., Parhizkar, Z., Mojahedi, M., Chakari-Khiavi, A., Salehnia, F., Chakari-Khiavi, F., Danaii, S., & Yousefi, M. (2022). The impact of IVIG therapy on live birth rates in women with RPL: A systematic review and meta-analysis. *Gene Reports*, *26*, 101490. https://doi.org/10.1016/j.genrep.2021.101490

Patriarca, A., Piccioni, V., Gigante, V., & Benedetto, C. (2000). The use of intravenous immunoglobulin in sine causa or alloimmune recurrent spontaneous abortion (RSA). *Panminerva Medica*, *42*(3), 193–195.

(PDF) An integrated single-cell reference atlas of the human endometrium. (n.d.). ResearchGate. Retrieved 30 December 2024, from https://www.researchgate.net/publication/375404303_An_integrated_single-cell_reference_atlas_of_the_human_endometrium

Perricone, R., De Carolis, C., Kröegler, B., Greco, E., Giacomelli, R., Cipriani, P., Fontana, L., & Perricone, C. (2008). Intravenous immunoglobulin therapy in pregnant patients affected with systemic lupus erythematosus and recurrent spontaneous abortion. *Rheumatology (Oxford, England)*, *47*(5), 646–651. https://doi.org/10.1093/rheumatology/ken046

Plaçais, L., Kolanska, K., Kraiem, Y. B., Cohen, J., Suner, L., Bornes, M., Sedille, L., Rosefort, A., D'Argent, E. M., Selleret, L., Abisror, N., Johanet, C., Buffet, N. C., Darai, E., Antoine, J. M., Fain, O., Kayem, G., & Mekinian, A. (2020). Intralipid therapy for unexplained recurrent miscarriage and implantation failure: Case-series and literature review. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, *252*, 100–104. https://doi.org/10.1016/j.ejogrb.2020.06.017

Practice Committee of the American Society for Reproductive Medicine. Evaluation and treatment of recurrent pregnancy loss: A committee opinion. Fertil Steril 2012;98:1103–11. doi: 10.1016/j.fertnstert.2012.06.048.

Progesterone and Prednisone and Folic Acid in Recurrent Miscarriage and Recurrent Pregnancy Loss(RPL)—Clinical Trials Registry—ICH GCP. (n.d.). Retrieved 5 January 2025, from https://ichgcp.net/clinical-trials-registry/NCT04558268

Psychoyos A. Uterine receptivity for nidation, Ann N Y Acad Sci, 1986, vol. 476 (pg. 36-42)

PubChem. (n.d.). *Hydroxychloroquine*. Retrieved 3 January 2025, from https://pubchem.ncbi.nlm.nih.gov/compound/3652

Quenby, S., Bates, M., Doig, T., Brewster, J., Lewis-Jones, D. I., Johnson, P. M., & Vince, G. (1999). Pre-implantation endometrial leukocytes in women with recurrent miscarriage. *Human Reproduction (Oxford, England)*, *14*(9), 2386–2391. https://doi.org/10.1093/humrep/14.9.2386

Quenby, S., Gallos, I. D., Dhillon-Smith, R. K., Podesek, M., Stephenson, M. D., Fisher, J., Brosens, J. J., Brewin, J., Ramhorst, R., Lucas, E. S., McCoy, R. C., Anderson, R., Daher, S., Regan, L., Al-Memar, M., Bourne, T., MacIntyre, D. A., Rai, R., Christiansen, O. B., … Coomarasamy, A. (2021a). Miscarriage matters: The epidemiological, physical, psychological, and economic costs of early pregnancy loss. *Lancet (London, England)*, *397*(10285), 1658–1667. https://doi.org/10.1016/S0140-6736(21)00682-6

Quenby S, Kalumbi C, Bates M, Farquharson R, Vince G. Prednisolone reduces preconceptual endometrial natural killer cells in women with recurrent miscarriage. Fertil Steril. 2005;84:980 –984

Quenby, S., Gallos, I. D., Dhillon-Smith, R. K., Podesek, M., Stephenson, M. D., Fisher, J., Brosens, J. J., Brewin, J., Ramhorst, R., Lucas, E. S., McCoy, R. C., Anderson, R., Daher, S., Regan, L., Al-Memar, M., Bourne, T., MacIntyre, D. A., Rai, R., Christiansen, O. B., … Coomarasamy, A. (2021b). Miscarriage matters: The epidemiological, physical, psychological, and economic costs of early pregnancy loss. *Lancet (London, England)*, *397*(10285), 1658–1667. https://doi.org/10.1016/S0140-6736(21)00682-6

Recombinant human granulocyte– colony stimulating factor in women with unexplained recurrent pregnancy losses: A randomized clinical trial | Human Reproduction | Oxford Academic. (n.d.). Retrieved 3 October 2024, from https://academic.oup.com/humrep/article/34/3/424/5334616

Ramathal CY, Bagchi IC, Taylor RN, Bagchi MK. Endometrial decidualization: Of mice and men. Semin Reprod Med. 2010;28:17–26. doi: 10.1055/s-0029-1242989.

Randall G. Richards, Anoop K. Brar, Graeme R. Frank, Scott M. Hartman, Hiroaki Jikihara, Fibroblast Cells from Term Human Decidua Closely Resemble Endometrial Stromal Cells: Induction of Prolactin and Insulin-Like Growth Factor Binding Protein-1 Expression, Biology of Reproduction, Volume 52, Issue 3, 1 March 1995, Pages 609–615, https://doi.org/10.1095/biolreprod52.3.609

Reed, B. G., & Carr, B. R. (2000). The Normal Menstrual Cycle and the Control of Ovulation. In K. R. Feingold, B. Anawalt, M. R. Blackman, A. Boyce, G. Chrousos, E. Corpas, W. W. de Herder, K. Dhatariya, K. Dungan, J. Hofland, S. Kalra, G. Kaltsas, N. Kapoor, C. Koch, P. Kopp, M. Korbonits, C. S. Kovacs, W. Kuohung, B. Laferrère, … D. P. Wilson (Eds.), *Endotext*. MDText.com, Inc. http://www.ncbi.nlm.nih.gov/books/NBK279054/

Retis-Resendiz, A. M., Cid-Cruz, Y., Velázquez-Hernández, D. M., Romero-Reyes, J., León-Juárez, M., García-Gómez, E., Camacho-Arroyo, I., & Vázquez-Martínez, E. R. (2024). cAMP regulates the progesterone receptor gene expression through the protein kinase A pathway during decidualization in human immortalized endometrial stromal cells. *Steroids*, *203*, 109363. https://doi.org/10.1016/j.steroids.2024.109363

Risk of Bias 2 (RoB 2) tool | Cochrane Methods. (n.d.). Retrieved 1 January 2025, from https://methods.cochrane.org/risk-bias-2

Romero, R., Espinoza, J., Gonçalves, L. F., Kusanovic, J. P., Friel, L. A., & Nien, J. K. (2006). Inflammation in preterm and term labour and delivery. *Seminars in Fetal & Neonatal Medicine*, *11*(5), 317–326. https://doi.org/10.1016/j.siny.2006.05.001

Rosner, J., Samardzic, T., & Sarao, M. S. (2023). *Physiology, female reproduction*. Academic Press.

RPh, R. B. (2022, October 27). *SCIG vs. IVIG: Which Treatment Is Best for You?* AmeriPharma® Specialty Care. https://ameripharmaspecialty.com/ivig/scig-vs-ivig/

S.A. Robertson, M.G. Petroff, J.S. Hunt, Immunology of pregnancy, T.M. Plant, A.J. Zeleznik (Eds.), Knobil and Neill's physiology of reproduction, Elsevier, Amsterdam (2015), pp. 1835-1874

Saab, W., Seshadri, S., Huang, C., Alsubki, L., Sung, N., & Kwak-Kim, J. (2021). A systemic review of intravenous immunoglobulin G treatment in women with recurrent implantation failures and recurrent pregnancy losses. *American Journal of Reproductive Immunology (New York, N.Y.: 1989)*, *85*(4), e13395. https://doi.org/10.1111/aji.13395

Sadasivam, S. R., Arumugam, I., & Sadasivam, K. (2023). P-505 Role of intramuscular immunoglobulin in improving the live birth rate in ANA positive women with recurrent pregnancy loss. *Human Reproduction*, *38*(Supplement_1), dead093.848. https://doi.org/10.1093/humrep/dead093.848

Sadu Singh, B. K., Narayanan, S. S., Khor, B. H., Sahathevan, S., Abdul Gafor, A. H., Fiaccadori, E., Sundram, K., & Karupaiah, T. (2020). Composition and Functionality of Lipid Emulsions in Parenteral Nutrition: Examining Evidence in Clinical Applications. *Frontiers in Pharmacology*, *11*, 506. https://doi.org/10.3389/fphar.2020.00506

Saito, S. (2024). Role of immune cells in the establishment of implantation and maintenance of pregnancy and immunomodulatory therapies for patients with repeated implantation failure and recurrent pregnancy loss. *Reproductive Medicine and Biology*, *23*(1), e12600. https://doi.org/10.1002/rmb2.12600

Salazar, M. D., Wang, W. J., Skariah, A., He, Q., Field, K., Nixon, M., Reed, R., Dambaeva, S., Beaman, K., Gilman-Sachs, A., & Kwak-Kim, J. (2022). Post-hoc evaluation of peripheral blood natural killer cell cytotoxicity in predicting the risk of recurrent pregnancy losses and repeated implantation failures. *Journal of Reproductive Immunology*, *150*, 103487. https://doi.org/10.1016/j.jri.2022.103487

Salker, M. S., Nautiyal, J., Steel, J. H., Webster, Z., Sućurović, S., Nicou, M., Singh, Y., Lucas, E. S., Murakami, K., Chan, Y.-W., James, S., Abdallah, Y., Christian, M., Croy, B. A., Mulac-Jericevic, B., Quenby, S., & Brosens, J. J. (2012). Disordered IL-33/ST2 activation in decidualizing stromal cells prolongs uterine receptivity in women with recurrent pregnancy loss. *PloS One*, *7*(12), e52252. https://doi.org/10.1371/journal.pone.0052252

Salker, M., Teklenburg, G., Molokhia, M., Lavery, S., Trew, G., Aojanepong, T., Mardon, H. J., Lokugamage, A. U., Rai, R., Landles, C., Roelen, B. A. J., Quenby, S., Kuijk, E. W., Kavelaars, A., Heijnen, C. J., Regan, L., Macklon, N. S., & Brosens, J. J. (2010b). Natural Selection of Human Embryos: Impaired Decidualization of Endometrium

Disables Embryo-Maternal Interactions and Causes Recurrent Pregnancy Loss. *PLoS ONE*, *5*(4), e10287. https://doi.org/10.1371/journal.pone.0010287

Salker MS, Nautiyal J, Steel JH, et al. Disordered IL-33/ ST2 activation in decidualizing stromal cells prolongs uterine receptivity in women with recurrent pregnancy loss. PLoS One. 2012;7:e52252.

Santjohanser, C., Knieper, C., Franz, C., Hirv, K., Meri, O., Schleyer, M., Würfel, W., & Toth, B. (2013). Granulocyte-colony stimulating factor as treatment option in patients with recurrent miscarriage. *Archivum Immunologiae Et Therapiae Experimentalis*, *61*(2), 159–164. https://doi.org/10.1007/s00005-012-0212-z

Scarpellini, F., & Sbracia, M. (2009). Use of granulocyte colony-stimulating factor for the treatment of unexplained recurrent miscarriage: A randomised controlled trial. *Human Reproduction (Oxford, England)*, *24*(11), 2703–2708. https://doi.org/10.1093/humrep/dep240

Scarrone, M., Salmeri, N., Buzzaccarini, G., Canti, V., Pasi, F., Papaleo, E., Rovere-Querini, P., Candiani, M., Alteri, A., Busnelli, A., & Vanni, V. S. (2024). Low-molecular-weight heparin in the prevention of unexplained recurrent miscarriage: A systematic review and meta-analysis. *Scientific Reports*, *14*(1), 14168. https://doi.org/10.1038/s41598-024-62949-5

Schreiber, K., Breen, K., Cohen, H., Jacobsen, S., Middeldorp, S., Pavord, S., Regan, L., Roccatello, D., Robinson, S. E., Sciascia, S., Seed, P. T., Watkins, L., & Hunt, B. J. (2017a). HYdroxychloroquine to Improve Pregnancy Outcome in Women with AnTIphospholipid Antibodies (HYPATIA) Protocol: A Multinational Randomized Controlled Trial of Hydroxychloroquine versus Placebo in Addition to Standard Treatment in Pregnant Women with Antiphospholipid Syndrome or Antibodies. *Seminars in Thrombosis and Hemostasis*, *43*(6), 562–571. https://doi.org/10.1055/s-0037-1603359

Schreiber, K., Breen, K., Cohen, H., Jacobsen, S., Middeldorp, S., Pavord, S., Regan, L., Roccatello, D., Robinson, S. E., Sciascia, S., Seed, P. T., Watkins, L., & Hunt, B. J. (2017b). HYdroxychloroquine to Improve Pregnancy Outcome in Women with AnTIphospholipid Antibodies (HYPATIA) Protocol: A Multinational Randomized Controlled Trial of Hydroxychloroquine versus Placebo in Addition to Standard Treatment in Pregnant Women with Antiphospholipid Syndrome or Antibodies. *Seminars in Thrombosis and Hemostasis*, *43*(6), 562–571. https://doi.org/10.1055/s-0037-1603359

Schreiber, K., & Hunt, B. J. (2016). Pregnancy and Antiphospholipid Syndrome. *Seminars in Thrombosis and Hemostasis*, *42*(7), 780–788. https://doi.org/10.1055/s-0036-1592336

Schweizer-Groyer, G., Fallot, G., Cadepond, F., Girard, C., & Groyer, A. (2006). The cAMP-responsive unit of the human insulin-like growth factor-binding protein-1 coinstitutes a functional insulin-response element. *Annals of the New York Academy of Sciences*, *1091*, 296–309. https://doi.org/10.1196/annals.1378.075

Sciascia, S., Hunt, B. J., Talavera-Garcia, E., Lliso, G., Khamashta, M. A., & Cuadrado, M. J. (2016). The impact of hydroxychloroquine treatment on pregnancy outcome in women with antiphospholipid antibodies. *American Journal of Obstetrics and Gynecology*, *214*(2), 273.e1-273.e8. https://doi.org/10.1016/j.ajog.2015.09.078

Scopus preview—Scopus—Document details—Intravenous immunoglobulin immunotherapy in immune mediated habitual abortions. (n.d.). Retrieved 9 December 2024, from https://www.scopus.com/record/display.uri?eid=2-s2.0-85010952295&origin=inward&txGid=a8d45b48df9b5395317d6f9ed7505996

Scopus preview—Scopus—Document details—Placebo-controlled trial of treatment of unexplained secondary recurrent spontaneous abortions and recurrent late spontaneous abortions with i.v.immunoglobulin. (n.d.). https://doi.org/10.1093/oxfordjournals.humrep.a135769

SELECTED ORAL COMMUNICATION SESSION, SESSION 34: EARLY PREGNANCY LOSS PROGNOSIS AND TREATMENT, Tuesday 5 July 2011 10:00 – 11:30. (2011). *Human Reproduction*, *26*(suppl_1), i48–i50. https://doi.org/10.1093/humrep/26.s1.34

Shi, Y., Tan, D., Hao, B., Zhang, X., Geng, W., Wang, Y., Sun, J., & Zhao, Y. (2022). Efficacy of intravenous immunoglobulin in the treatment of recurrent spontaneous abortion: A systematic review and meta-analysis. *American Journal of Reproductive Immunology*, *88*(5), e13615. https://doi.org/10.1111/aji.13615

Single-cell profiling of the human decidual immune microenvironment in patients with recurrent pregnancy loss | Cell Discovery. (n.d.). Retrieved 31 December 2024, from https://www.nature.com/articles/s41421-020-00236-z

Single-cell reconstruction of the early maternal–fetal interface in humans | Nature. (n.d.). Retrieved 31 December 2024, from https://www.nature.com/articles/s41586-018-0698-6

Starkey, P. M., Clover, L. M., & Rees, M. C. P. (1991). Variation during the menstrual cycle of immune cell populations in human endometrium. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, *39*(3), 203–207. https://doi.org/10.1016/0028-2243(91)90058-S

Starr T, Bauler TJ, Malik-Kale P, Steele-Mortimer O. The phorbol 12-myristate-13-acetate differentiation protocol is critical to the interaction of THP-1 macrophages with Salmonella Typhimurium. PLoS One. 2018 Mar 14;13(3):e0193601. doi: 10.1371/journal.pone.0193601. PMID: 29538403; PMCID: PMC5851575.

Stephenson, M. D., Awartani, K. A., & Robinson, W. P. (2002). Cytogenetic analysis of miscarriages from couples with recurrent miscarriage: A case-control study. *Human Reproduction (Oxford, England)*, *17*(2), 446–451. https://doi.org/10.1093/humrep/17.2.446

Stricker, R. B., Steinleitner, A., Bookoff, C. N., Weckstein, L. N., & Winger, E. E. (2000). Successful treatment of immunologic abortion with low-dose intravenous immunoglobulin. *Fertility and Sterility*, *73*(3), 536–540. https://doi.org/10.1016/s0015-0282(99)00572-5

Stricker, R. B., & Winger, E. E. (2005). Update on treatment of immunologic abortion with low-dose intravenous immunoglobulin. *American Journal of Reproductive Immunology (New York, N.Y.: 1989)*, *54*(6), 390–396. https://doi.org/10.1111/j.1600-0897.2005.00335.x

Sun, X., Liu, X., Zhu, R., Fan, G., Zhang, Y., & Chen, F. (2010). [Effectiveness of intravenous immunoglobulin therapy in treating unexplained recurrent spontaneous abortion and its effect on the level of serum soluble human leucocyte antigen G]. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao. Acta Academiae Medicinae Sinicae*, *32*(5), 483–487. https://doi.org/10.3881/j.issn.1000-503X.2010.05.002

Sung, N., Khan, S. A., Yiu, M. E., Jubiz, G., Salazar, M. D., Skariah, A., Dambaeva, S., & Kwak-Kim, J. (2021). Reproductive outcomes of women with recurrent pregnancy losses and repeated implantation failures are significantly improved with immunomodulatory treatment. *Journal of Reproductive Immunology*, *148*, 103369. https://doi.org/10.1016/j.jri.2021.103369

Svensson J, Jenmalm MC, Matussek A, Geffers R, Berg G, Ernerudh J. Macrophages at the fetal-maternal interface express markers of alternative activation and are induced by M-CSF and IL-10. J Immunol. 2011;187:3671–3682.

Taglauer ES, Trikhacheva AS, Slusser JG, Petroff MG. Expression and function of PDCD1 at the human maternal-fetal interface. Biol Reprod. (2008) 79:562–9. 10.1095/biolreprod.107.066324

Tamura, I., Asada, H., Maekawa, R., Tanabe, M., Lee, L., Taketani, T., Yamagata, Y., Tamura, H., & Sugino, N. (2012). Induction of IGFBP-1 expression by cAMP is associated with histone acetylation status of the promoter region in human endometrial stromal cells. *Endocrinology*, *153*(11), 5612–5621. https://doi.org/10.1210/en.2012-1420

Teklenburg, G., Salker, M., Molokhia, M., Lavery, S., Trew, G., Aojanepong, T., Mardon, H. J., Lokugamage, A. U., Rai, R., Landles, C., Roelen, B. A. J., Quenby, S., Kuijk, E. W., Kavelaars, A., Heijnen, C. J., Regan, L., Brosens, J. J., & Macklon, N. S. (2010). Natural selection of human embryos: Decidualizing endometrial stromal cells serve as sensors of embryo quality upon implantation. *PloS One*, *5*(4), e10258. https://doi.org/10.1371/journal.pone.0010258

The ESHRE Guideline Group on RPL, Bender Atik, R., Christiansen, O. B., Elson, J., Kolte, A. M., Lewis, S., Middeldorp, S., Nelen, W., Peramo, B., Quenby, S., Vermeulen, N., & Goddijn, M. (2018). ESHRE guideline: Recurrent pregnancy loss. *Human Reproduction Open*, *2018*(2), hoy004. https://doi.org/10.1093/hropen/hoy004

Thompson, B. B., Holzer, P. H., & Kliman, H. J. (2024). Placental Pathology Findings in Unexplained Pregnancy Losses. *Reproductive Sciences (Thousand Oaks, Calif.)*, *31*(2), 488–504. https://doi.org/10.1007/s43032-023-01344-3

Tomkiewicz, J., & Darmochwał-Kolarz, D. (2023). The Diagnostics and Treatment of Recurrent Pregnancy Loss. *Journal of Clinical Medicine*, *12*(14), 4768. https://doi.org/10.3390/jcm12144768

Tong, J., Lv, S., Yang, J., Li, H., Li, W., & Zhang, C. (2022). Decidualization and Related Pregnancy Complications. *Maternal-Fetal Medicine*, *4*(1), 24. https://doi.org/10.1097/FM9.0000000000000135

Triolo, G., Ferrante, A., Ciccia, F., Accardo-Palumbo, A., Perino, A., Castelli, A., Giarratano, A., & Licata, G. (2003a). Randomized study of subcutaneous low molecular weight heparin plus aspirin versus intravenous immunoglobulin in the treatment of recurrent fetal loss associated with antiphospholipid antibodies. *Arthritis and Rheumatism*, *48*(3), 728–731. https://doi.org/10.1002/art.10957

Triolo, G., Ferrante, A., Ciccia, F., Accardo-Palumbo, A., Perino, A., Castelli, A., Giarratano, A., & Licata, G. (2003b). Randomized study of subcutaneous low molecular weight heparin plus aspirin versus intravenous immunoglobulin

in the treatment of recurrent fetal loss associated with antiphospholipid antibodies. *Arthritis and Rheumatism*, *48*(3), 728–731. https://doi.org/10.1002/art.10957

Ugwumadu A (2014). Basic Sciences for Obstetrics and Gynaecology: Core Material for MRCOG. Oxford, England: Oxford University Press. ISBN 978-0-19-953508-8. OCLC 889303297

Ulukus EC, Ulukus M, Seval Y, et al. Expression of interleukin-8 and monocyte chemotactic protein-1 in adenomyosis. Hum Reprod 2005; 20: 2958–2963.

Vacca P, Cantoni C, Vitale M, et al. Crosstalk between decidual NK and CD14 myelomonocytic cells results in induction of Tregs and immunosuppression. Proc Natl Acad Sci U S A. 2010;107:11918 –11923.

Valeff, N. J., Ventimiglia, M. S., Diao, L., & Jensen, F. (2023). Lupus and recurrent pregnancy loss: The role of female sex hormones and B cells. *Frontiers in Endocrinology*, *14*, 1233883. https://doi.org/10.3389/fendo.2023.1233883

Van Noorden, R. (2023). Medicine is plagued by untrustworthy clinical trials. How many studies are faked or flawed? *Nature*, *619*(7970), 454–458. https://doi.org/10.1038/d41586-023-02299-w

Vaquero, E., Lazzarin, N., Valensise, H., Menghini, S., Di Pierro, G., Cesa, F., & Romanini, C. (2001). Pregnancy outcome in recurrent spontaneous abortion associated with antiphospholipid antibodies: A comparative study of intravenous immunoglobulin versus prednisone plus low-dose aspirin. *American Journal of Reproductive Immunology (New York, N.Y.: 1989)*, *45*(3), 174–179. https://doi.org/10.1111/j.8755-8920.2001.450309.x

Valeff NJ, Ventimiglia MS, Diao L, Jensen F. Lupus and recurrent pregnancy loss: the role of female sex hormones and B cells. Front Endocrinol (Lausanne). 2023 Oct 3;14:1233883. doi: 10.3389/fendo.2023.1233883. PMID: 37859991; PMCID: PMC10584304.

Waitzberg DL, Torrinhas RS, Jacintho TM. New parenteral lipid emulsions for clinical use. JPEN J Parenter Enteral Nutr. 2006;30(4):351-367. 10.1177/0148607106030004351

Wakiya R, Ueeda K, Nakashima S, Shimada H, Kameda T, Mansour MMF, Kato M, Miyagi T, Sugihara K, Mizusaki M, Mino R, Kadowaki N, Dobashi H. Effect of add-on hydroxychloroquine therapy on serum proinflammatory cytokine levels in patients with systemic lupus erythematosus. Sci Rep. 2022 Jun 17;12(1):10175. doi: 10.1038/s41598-022-14571-6. PMID: 35715525; PMCID: PMC9205904.

Wang, H., Bloom, O., Zhang, M., Vishnubhakat, J. M., Ombrellino, M., Che, J., Frazier, A., Yang, H., Ivanova, S., Borovikova, L., Manogue, K. R., Faist, E., Abraham, E., Andersson, J., Andersson, U., Molina, P. E., Abumrad, N. N., Sama, A., & Tracey, K. J. (1999). Proinflammatory cytokines (tumor necrosis factor and interleukin 1) stimulate release of high mobility group protein-1 by pituicytes. *Surgery, 126*(2), 389–392. https://doi.org/10.1016/S0039-6060(99)70182-0

Wang, L., Wang, H., Luo, J., Xie, T., Mor, G., & Liao, A. (2022). Decorin promotes decidual M1-like macrophage polarization via mitochondrial dysfunction resulting in recurrent pregnancy loss. *Theranostics*, *12*(17), 7216–7236. https://doi.org/10.7150/thno.78467

Wang, Q., Li, J., Wang, S., Deng, Q., An, Y., Xing, Y., Dai, X., Li, Z., Ma, Q., Wang, K., Liu, C., Yuan, Y., Dong, G., Zhang, T., Yang, H., Du, Y., Hou, Y., Ke, W., & Shang, Z. (2022). Single-cell transcriptional profiling reveals cellular and molecular divergence in human maternal–fetal interface. *Scientific Reports*, *12*(1), 10892. https://doi.org/10.1038/s41598-022-14516-z

Wang, S.-W., Zhong, S.-Y., Lou, L.-J., Hu, Z.-F., Sun, H.-Y., & Zhu, H.-Y. (2016). The effect of intravenous immunoglobulin passive immunotherapy on unexplained recurrent spontaneous abortion: A meta-analysis. *Reproductive Biomedicine Online*, *33*(6), 720–736. https://doi.org/10.1016/j.rbmo.2016.08.025

Wang, W., Vilella, F., Alama, P., Moreno, I., Mignardi, M., Isakova, A., Pan, W., Simon, C., & Quake, S. R. (2020a). Single-cell transcriptomic atlas of the human endometrium during the menstrual cycle. *Nature Medicine*, *26*(10), 1644–1653. https://doi.org/10.1038/s41591-020-1040-z

Wang, W.-J., Hao, C.-F., Yi-Lin, null, Yin, G.-J., Bao, S.-H., Qiu, L.-H., & Lin, Q.-D. (2010). Increased prevalence of T helper 17 (Th17) cells in peripheral blood and decidua in unexplained recurrent spontaneous abortion patients. *Journal of Reproductive Immunology*, *84*(2), 164–170. https://doi.org/10.1016/j.jri.2009.12.003

Wang X , Chen C , Wang L , Chen D , Guang W , French J . Conception, early pregnancy loss, and time to clinical pregnancy: a population-based prospective study . Fertil Steril 2003 ; 79 : 577 – 84 .

Wegmann, T. G. (1984). Fetal protection against abortion: Is it immunosuppression or immunostimulation? *Annales D'immunologie*, *135D*(3), 309–312. https://doi.org/10.1016/s0769-2625(84)81196-4

Wetendorf M, DeMayo FJ. The progesterone receptor regulates implantation, decidualization, and glandular development via a complex paracrine signalling network. Mol Cell Endocrinol. 2012;357:108-118.

Wilcox AJ , Weinberg CR , O'Connor JF , Baird DD , Schlatterer JP , Canfield RE , et al . Incidence of early loss of pregnancy . N Engl J Med 1988 ; 319 : 189 – 94

Wong, L. F., Porter, T. F., & Scott, J. R. (2014a). Immunotherapy for recurrent miscarriage. *The Cochrane Database of Systematic Reviews*, *2014*(10), CD000112. https://doi.org/10.1002/14651858.CD000112.pub3

Wong, L. F., Porter, T. F., & Scott, J. R. (2014b). Immunotherapy for recurrent miscarriage. *The Cochrane Database of Systematic Reviews*, *2014*(10), CD000112. https://doi.org/10.1002/14651858.CD000112.pub3

Wu, H.-M., Chen, L.-H., Hsu, L.-T., & Lai, C.-H. (2022). Immune Tolerance of Embryo Implantation and Pregnancy: The Role of Human Decidual Stromal Cell- and Embryonic-Derived Extracellular Vesicles. *International Journal of Molecular Sciences*, *23*(21), 13382. https://doi.org/10.3390/ijms232113382

Würfel, W. (2015). Treatment with granulocyte colony-stimulating factor in patients with repetitive implantation failures and/or recurrent spontaneous abortions. *Journal of Reproductive Immunology*, *108*, 123–135. https://doi.org/10.1016/j.jri.2015.01.010

Yamada, H., Deguchi, M., Maesawa, Y., Nakajima, Y., Nishino, Y., Tanimura, K., & Ebina, Y. (2015). Medium-dose intravenous immunoglobulin therapy for women with six or more recurrent miscarriages. *Journal of Reproductive Immunology*, *109*, 48–51. https://doi.org/10.1016/j.jri.2015.01.008

Yamada, H., Deguchi, M., Saito, S., & Takeshita, T. (2022). Author's reply – Intravenous immunoglobulin treatment in women with four or more recurrent pregnancy losses: A double-blind, randomised, placebo-controlled trial. *eClinicalMedicine*, *52*. https://doi.org/10.1016/j.eclinm.2022.101617

Yamada, H., Deguchi, M., Saito, S., Takeshita, T., Mitsui, M., Saito, T., Nagamatsu, T., Takakuwa, K., Nakatsuka, M., Yoneda, S., Egashira, K., Tachibana, M., Matsubara, K., Honda, R., Fukui, A., Tanaka, K., Sengoku, K., Endo, T., & Yata, H. (2022). Intravenous immunoglobulin treatment in women with four or more recurrent pregnancy losses: A double-blind, randomised, placebo-controlled trial. *EClinicalMedicine*, *50*, 101527. https://doi.org/10.1016/j.eclinm.2022.101527

Yang, K. M., Park, C. W., Kim, H. S., Cha, S. H., Kim, H. O., & Jun, J. Y. (2004). Effectiveness of intravenous immunoglobulin therapy in women with recurrent spontaneous abortions with elevated pre-conceptional peripheral blood CD56+Natural Killer cell percentage. *Fertility and Sterility*, *82*, S321. https://doi.org/10.1016/j.fertnstert.2004.07.867

Yang, K., Park, C., Yoo, K., Song, I., Kim, J., & Park, A. (2003). The level of preconceptional peripheral blood natural killer cells which expected a successful treatment outcome using low-dose intravenous gamma immunoglobulin (IVIg) infusion in patients with recurrent spontaneous abortion. *Fertility and Sterility*, *80*, 288–289. https://doi.org/10.1016/S0015-0282(03)01738-2

Yang, X., Tian, Y., Zheng, L., Luu, T., & Kwak-Kim, J. (2022). The Update Immune-Regulatory Role of Pro- and Anti-Inflammatory Cytokines in Recurrent Pregnancy Losses. *International Journal of Molecular Sciences*, *24*(1), 132. https://doi.org/10.3390/ijms24010132

Zafardoust, S., Akhondi, M. M., Sadeghi, M. R., Mohammadzadeh, A., Karimi, A., Jouhari, S., & Ansaripour, S. (2017). Efficacy of Intrauterine Injection of Granulocyte Colony Stimulating Factor (G-CSF) on Treatment of Unexplained Recurrent Miscarriage: A Pilot RCT Study. *Journal of Reproduction & Infertility*, *18*(4), 379–385.

Zegers-Hochschild, F., Adamson, G. D., de Mouzon, J., Ishihara, O., Mansour, R., Nygren, K., Sullivan, E., Vanderpoel, S., International Committee for Monitoring Assisted Reproductive Technology, & World Health Organization. (2009). International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) revised glossary of ART terminology, 2009. *Fertility and Sterility*, *92*(5), 1520–1524. https://doi.org/10.1016/j.fertnstert.2009.09.009

Zeng, S., Liang, Y., Lai, S., Bi, S., Huang, L., Li, Y., Deng, W., Xu, P., Liu, M., Xiong, Z., Chen, J., Tu, Z., Chen, D., & Du, L. (2023). TNFα/TNFR1 signal induces excessive senescence of decidua stromal cells in recurrent pregnancy loss. *Journal of Reproductive Immunology*, *155*, 103776. https://doi.org/10.1016/j.jri.2022.103776

Appendix

Search Design for All Databases

	Search Terms for EMBASE	No. Of Searches
1	exp immunomodulating agent/ or exp immunoglobulin/ or exp intralipid/ or exp acetylsalicylic acid/ or exp granulocyte/ or exp steroid/ or exp immunotherapy/ or exp heparin derivative/ or exp lymphocyte/	4958864
2	(immunomod* or immunoglob* or Immunologi* or aspirin* or heparin* or glucocorti* or steroid* or prednisolone or prednisone or intralipid or granulocyte or lymphocyte).ti,ab,kf.	1618854
3	1 or 2	5396625
4	recurrent abortion/	8076
5	((recur* or habitual or refractory) adj3 (miscarr* or pregn* or abort* or gestat* or obstet*)).ti,ab,kf.	17648
6	4 or 5	19230
7	3 and 6	7824
8	limit 7 to "remove medline records"	3410

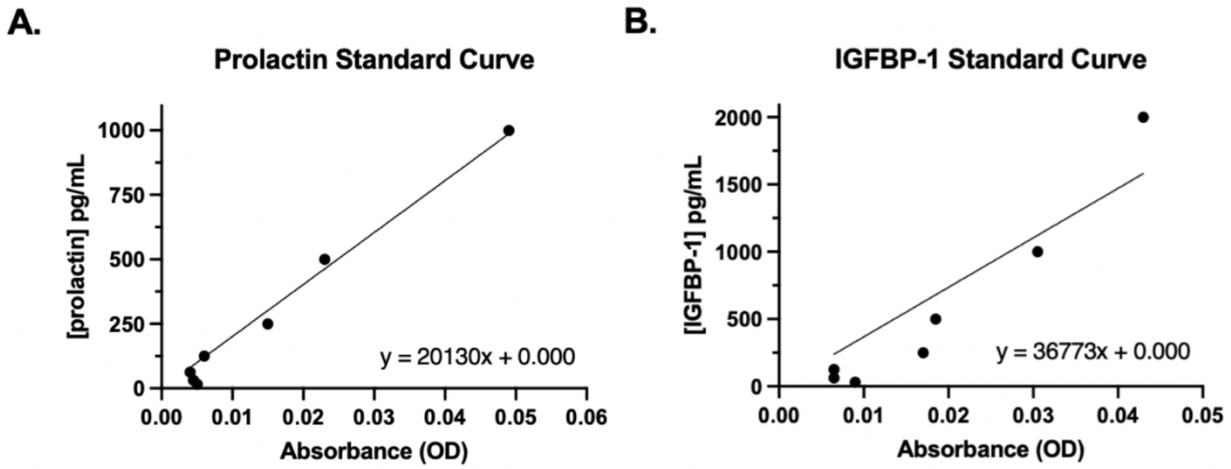
	Search Terms for Medline	No. Of Searches
1	exp Immunologic Factors/ or exp Immunoglobulins/ or exp Aspirin/ or exp Granulocytes/ or exp Immunomodulation/ or exp Heparin/ or exp Lymphocytes/ or exp Steroids/	3199013
2	(immunomod* or immunoglob* or Immunologi* or aspirin* or heparin* or glucocorti* or steroid* or prednisolone or prednisone or intralipid or granulocyte or lymphocyte).ti,ab,kf.	1184192
3	1 or 2	3708557
4	((recur* or habitual or refractory) adj3 (miscarr* or pregn* or abort* or gestat* or obstet*)).ti,ab,kf.	11886
5	exp Abortion, Habitual/	9441
6	4 or 5	15433
7	3 and 6	5108

	Search Terms for SCOPUS	No. Of Searches
1	((((recur* OR habitual OR refractory) W/3 (miscarr* OR pregn* OR abort* OR gestat* OR obstet*))) AND ((immunomod* OR immunoglob* OR	3710

	immunologi* OR aspirin* OR heparin* OR glucocorti* OR steroid* OR prednisolone OR prednisone OR intralipid OR granulocyte OR lymphocyte)))	

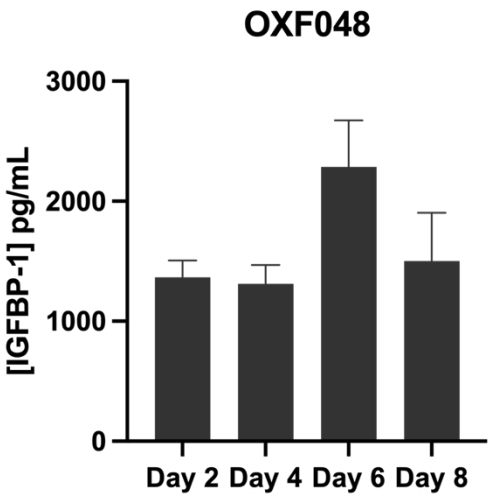
	Search Terms for Web of Science	No. Of Searches
1	TS=(((recur* OR habitual OR refractory) NEAR/3 (miscarr* OR pregn* OR abort* OR gestat* OR obstet*)) AND (immunomod* OR immunoglob* OR immunologi* OR aspirin* OR heparin* OR glucocorti* OR steroid* OR prednisolone OR prednisone OR intralipid OR granulocyte OR lymphocyte))	3755

ELISA Standard Curves



A. Prolactin. $R^2 = 0.988$, p-value < 0.0001. The seven-point standard curve includes known concentrations from 15.6 to 1000 pg/mL. The equation of the line-of-best-fit is displayed above. **B. IGFBP-1.** $R^2 = 0.978$, p-value < 0.0001. The line plots display a seven-point standard curve ranging from 31.25 to 2000 pg/mL. he equation of the line-of-best-fit is displayed above. The transmitted wavelengths were recorded at 540 nm and the MARS data analysis software calculated the raw data, given in optical density (OD), from mOD. Each standard was run in duplicate and averaged. The standard curves all used x=0, y=0 since all y-intercepts were less than one standard deviation from zero.

IGFBP-1 levels in cell line RPL5 show successful decidualisation



Methods for processing endometrium tissue biopsies

1. After washing, break up tissue with scalpel to ~1 mm3 pieces
2. Digest tissues with collagenase (1mg/mL) supplemented with DNase I (40µg/mL) in PBS at 37°C for 30 min-1 hour in shaking thermostat to obtain single stromal cells.
3. Centrifuge at 120g for 10 min at 4°C, wash with medium.
4. Filter cell suspensions through a 70µM cell strainer and then a 40µM cell strainer. (filtering out glands)
5. Plate or freeze.
6. Cell viability was monitored using Trypan blue staining.