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Intravenous immunoglobulin and prednisolone to women with unexplained recurrent pregnancy loss after assisted reproductive technology treatment: a randomised, double-blind, placebo-controlled trial

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Key words: recurrent pregnancy loss, habitual abortion, reproductive immunology, immunotherapy, artificial reproductive technology

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Abstract:

Introduction: Recurrent pregnancy loss (RPL), defined as two or more consecutive pregnancy losses in the first trimester, affects around 5 % of fertile women. The underlying causes remain unknown in up to 60% of patients; however, most studies point at an immunological pathology in uRPL, and therefore, an effective treatment may be immunomodulatory. This study aims to evaluate the effect of intravenous immunoglobulin (IVIg) and prednisolone on reproductive outcome and the immune system in women with uRPL undergoing assisted reproductive technology (ART) treatment.

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Methods and analysis: This randomized, placebo-controlled trial with double-blinded randomization to two parallel arms evaluate if immunomodulatory (active) treatment is superior to placebo in increasing the chance of ongoing pregnancy assessed at nuchal translucency scan in gestational week (GW) 11-13 after ART treatment in 74 uRPL patients with ≥2 pregnancy losses as its primary objective. The active treatment consists of IVIg (one infusion 1-5 days before embryo transfer [ET] and in gestational week 5, 6, and 7) and prednisolone (5 mg/day from first day of menstrual bleeding until ET and 10 mg/day from ET to GW 8+0) while the comparator consists of intravenous human albumin (5%) and placebo tablets. Allocation is concealed for participants, caregivers, and investigators until trial termination and is performed in a 1:1 ratio. The secondary objective is to evaluate treatment safety, and the tertiary objective is exploration of the association between treatment, reproductive outcome after ET, and the lymphocyte subset distribution in peripheral blood collected before and after intravenous infusion(s). Excess biological material is stored in a biobank for future research.

Ethics and dissemination: The North Denmark Region Committee on Health Research Ethics (N-20200066) approved this RCT. The results will be published in peer-reviewed scientific journals and presented to relevant patient associations, at relevant academic conferences and to key stakeholders.

Trial registration: Clinicaltrials.gov ID: NCT04701034. WHO unique trial Number: U1111-1273-8585.

Protocol version: this article was written per the study protocol version 7, dated 01.12.2021.

Strengths and limitations of this study

- The pragmatic, randomized, double-blinded study design reflecting the contemporary practice strengthens the study results' external validity.
- This is the first RCT to evaluate immunomodulatory treatment with PRS&IVIg in uRPL patients who have all underwent ART, and possessing a combined clinical and immunochemical perspective, this trial offers a unique opportunity to identify uRPL patients responsive to immunomodulatory treatment in whom an increased LBR after ART is expected, as no prior RCT has evaluated such a wide range of leukocyte subsets simultaneously in uRPL patients twice during the implantation window.

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- The combination of two active drugs in the active treatment group does not allow differentiation of whether one of the active drugs or only the combination possesses the effect on reproductive outcome observed in the study.
- Participants are not selected based on a biomarker associated with an aberrant immune function, which was done in some of the previous studies reporting a significant effect of IVIg on uRPL. However, since the evidence of such an association is still sparse, further elaboration on this topic is needed and therefore, immunological analyses on peripheral blood from participants in this trial will search for immune biomarkers characterizing uRPL patients who have a beneficial response to the treatment.

INTRODUCTION

Background

Recurrent pregnancy loss (RPL) defined as ≥2 consecutive pregnancy losses (PLs)[1] affects around 5 % of women in reproductive age.[2] In Denmark, >25 % of all RPL cases happens after assisted reproductive technologies (ART) including in-vitro fertilization (IVF), intracytoplasmatic sperm injection (ICSI), and frozen embryo/blastocyst transfer (FET), and >10 % children born after 2019 in Denmark are conceived using ART. In up to 60 % of RPL patients, no risk factor is found, and for unexplained RPL (uRPL) no treatment with proven benefit has been found yet.[1] Among women with uRPL, immunological aberrations are thought to be at least partly responsible. For that reason, many treatment regimens used in autoimmune diseases have been tested on uRPL patients. A meta-analysis of RCTs evaluating the efficacy of IVIg treatment for women with RPL found no overall significant effect on live birth rate; however, the acquired sample size was not obtained. Since subgroup analyses did show a significant effect in secondary RPL, the authors suggested that further RCTs were required to obtain sufficient evidence.[3] A pilot study suggested that a combination of prednisone (PRS) and IVIg starting before pregnancy improves the chance of live birth in women with RPL after ART.[4,5] Additionally, previous studies of PRS supplement to ART treatment have shown improved pregnancy rate, especially for women with immune aberrations.[6–9] The underlying mechanism for an improved ART outcome after immunomodulatory treatment may be the regulation of immunological processes occurring particularly in early pregnancy. Some prior studies have suggested different biomarkers for an aberrant immune system in the uterus[10–14] and peripheral blood [15–19] in uRPL patients, which

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have been associated with unsuccessful pregnancy outcome in some small studies[11,15,20] but not in others.[12]

Previous studies have observed a counterbalanced lymphocyte distribution in uRPL patients treated with PRS; thus, the treatment re-established the immune system presumed to be beneficial for embryo implantation.[21–24] The same effects have been observed in uRPL patients treated with IVIg.[22,25–27] For that reason, treatment before pregnancy with PRS and IVIg (PRS&IVIg) is expected to contribute to a favourable immunological environment in peripheral blood and the uterus which is expected to increase the chance of an uncomplicated pregnancy.

STUDY OBJECTIVES

Primary objective

- To investigate whether treatment with PRS&IVIg before and in early pregnancy improves the reproductive outcome in RPL patients undergoing ART treatment.

Secondary objective

- To evaluate if PRS&IVIg treatment is associated with decreased incidence of pregnancy complications, negative pregnancy outcome, and negative perinatal outcomes.

Tertiary explorative objective

- To investigate how PRS&IVIg affect the leukocyte subset distribution (LSD) in peripheral blood and whether a specific LSD can predict which uRPL patients benefit from PRS&IVIg

METHODS

Study design

In a randomized 1:1 double-blinded, placebo-controlled trial of uRPL patients undergoing ART, the effect of PRS&IVIg treatment is compared to treatment with placebo (placebo tablets and human albumin). The study also possesses an explorative capacity by examining the association between treatment, reproductive outcome, and LSD.

Population

The population consist of RPL patients with ≥2 consecutive PLs after ART referred to The Center for Recurrent Pregnancy Loss of Western Denmark (RPL Center) at Aalborg University Hospital (AaUH). In Danish public clinics, the costs for a couple's first child are covered by tax-financed

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public health insurance. Also, all Danish citizens with RPL can be referred to The RPL Center without costs.

The public and private fertility clinics in Denmark have received a detailed description of the RCT to inform their personnel and ask for referral of potentially eligible patients with ≥2 PLs to the RPL Center. All referred patients undergoing ART are screened for eligibility.

Inclusion criteria:

1. Women with ≥2 consecutive PLs (biochemical pregnancies or clinical miscarriages) before completed GW 10 after ART with the same partner or with an egg/semen donor*

Exclusion criteria:

1. BMI ≥35
2. Age ≥41 years
3. Significant uterine malformation(s)
4. Known parental balanced chromosomal translocations
5. ≥2 previous pregnancies with fetuses with known abnormal karyotype
6. Patients with IgA deficiency, IgA-autoantibodies or hyperprolinaemia
7. Anti-Müllerian hormone (AMH) <4 pmol/l if the planned IVF/ICSI cycle does not imply the use of donor egg. If IVF/ICSI with egg donation is planned, a low AMH value is not an exclusion criterion.
8. Treatment with medication interacting with PRS:
    - CYP3A4-inhibitors (e.g., erythromycin, itraconazole, ritonavir, and lopinavir), CYP3A4-inductors (e.g., phenobarbital, phenytoin, and rifampicin), loop diuretics, thiazides, amphotericin B, β2-agonists, antidiabetics (metformin is acceptable), interleukin-2, somatropins, anticholinergics, and regular treatment with NSAIDs.
9. Patients with moderate/severe hypertension, diabetes mellitus, heart insufficiency, severe mental disorders, Cushing syndrome, myasthenia gravis, ocular herpes simplex, pheochromocytoma, systemic sclerosis, and moderate/severe renal dysfunction.
10. Patients with a clinical or biochemical profile indicating need for heparin or levothyroxine treatment during pregnancy
11. Previous treatment with IVIg
12. Allergy to PRS and/or IVIg

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* If the participant plans to use egg donation in the study cycle, the previous two PLs must also have happened with the use of egg donation; however, it is not required to use the same egg donor in all three embryo transfers (ET).

Allocation, randomization, and blinding

Investigators at the RPL Center screen for inclusion and exclusion criteria, deliver the written and oral study information, and obtain a signed written consent form. The recruited participant is assigned an ID-number, which is given in continuous order, and she receives a tablet container with treatment corresponding to the randomisation list and labelled with the matching ID-number. Also, a written information folder is given after randomisation with a summary of known adverse reactions (AR) for study drugs, boxes in which the participant can describe her ARs and adverse events (AE), a description on how to respond to such ARs/AEs, and a telephone number available 24/7. It also contains a check-list with ticking boxes to tick off each day the participant takes her tablet(s). It describes when to change the dose and serves as a reminder improving treatment compliance. The folder is examined at every appointment to aid high compliance and safety.

To ensure blinding of participants, investigators, outcome assessors, and care providers, a computer-generated simple randomisation list is created by the Hospital Pharmacy North Denmark Region (HPNDR) and kept confidential. The list contains 74 ID-numbers with an allocation ratio 1:1. It is made before the first patient is included and located at the HPNDR. Only the personnel at the Department of Clinical Immunology (DCI) preparing intravenous (IV) infusion medicine have a copy. No personnel with access to the randomisation list will be in contact with participants nor be involved in data collection. The allocation sequence is concealed as ID-numbers are assigned to participants in consecutive order by the blinded chief investigator.
Randomisation is in blocks of different sizes arranged to ensure a 1:1 ratio when conducting the interim analysis. For every participant who does not meet the criteria for per-protocol (PP) analysis, an extra patient will be included. The additional ID-numbers on the randomisation list will be generated by the HPNDR in correspondence with the number of participants missing in each treatment group.
The investigator will authorize the pharmacist to break the code in order to reveal the participant's allocation group in case of serious illness and the code will only be broken if it is of substantial

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importance to a participant´s health or functional capacity and only the chief investigator and sponsor can make this decision.

The randomisation code will be disclosed when the primary outcome is collected on 74 participants with no protocol deviations and a pre-analysis blind review on all outcomes described in the SAP has been performed which is expected to happen in December 2022.

Study medicine preparation

PRS and the placebo comparator are in tablet form with identical appearance and prepacked for each participant by the HPNDR in identical containers labelled with ID-numbers corresponding to the randomisation list. The chief investigator informs the DCI personnel about when and to whom they should prepare IV infusion medicine for. Within 4hours before administration, the relevant volume and content is transferred into a transparent, yellow (UV-protected) ethylene vinyl acetate (EVA) bag labelled with volume and ID-number. Human albumin is chosen as IVIg comparator due to the similar physical form and appearance e.g., both drugs form foam. The yellow EVA bags and IV sets make visual distinction even more difficult.

Intervention

Within the first three days of the participant's menstrual cycle, she starts administration of one tablet daily (5 mg of prednisolone or placebo) until ET on which day the dose increases to two tablets daily. At the time of the ET (from five working days before to two working days after), the participant receives the first IV infusion of study medicine and have the first study-specific blood sample taken. Participants with a pre-pregnancy body weight (BW) $\leq$ 70 kg will receive 250 ml, participants with BW 70-85 kg will receive 300 ml, and participants with a BW $\geq$85 kg will receive 350 ml in each infusion. Around 14 days after ET, plasma-hCG is measured twice with 24-48hrs interval. If plasma-hCG increases sufficiently (here defined as $\geq$30 % per 24hrs), the treatment continues (see Figure 1).

IV infusions containing the same volume is repeated in gestational week (GW) 5, 6, and 7, respectively; however, no IV infusion is administered after a negative pregnancy test or a PL. The tablet treatment discontinues gradually over four days when the participant has a negative pregnancy test, a PL, or when she reaches GW 8+0, whichever comes first.

Before each infusion, a plasma-hCG measurement or ultrasound scan is performed to confirm that the pregnancy continues as expected. Initial infusion rate of 0.3 ml/kg BW/hr and if well-tolerated,

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the infusion rate may gradually increase. Blood pressure and pulse are monitored before, during, and after the treatment. In case of anaphylaxis, the treatment is discontinued, adrenaline (0.1 %) is administered, and the participant is excluded.

On the day of the first IV infusion and again about four weeks later (corresponding to GW 6, i.e., the day of her third IV infusion), a blood sample is taken.

After the last infusion, routine monitoring at the RPL Center or at her local hospital is offered. A NT scan is offered in GW 11-13. The participant receives an e-mail with a unique link to a questionnaire two weeks after the NT scan and two weeks after her due date. After GW 8, the participant is offered the same treatment and follow-up as any other Danish pregnant women except from two additional fetal growth scans in the third trimester due to her history of RPL.

Active treatment

Prednisolone: 5 mg of prednisolone per tablet. One tablets daily rising to two tablets daily after ET.

Intravenous immunoglobulin (IVIg): Privigen 100 mg/ml (10 %) (CSL Behring). The three optional volumes to be administered depend on BW as previously described; thus, the dose corresponds to approximately 0.4 g IVIg per kg BW.

Placebo

Prednisolone comparator: Placebo tablets contain 85 mg lactose monohydrate, 86 mg potato starch, 8.1 mg talc, 3 mg gelatine, and 0.9 mg magnesium stearate. One tablets daily rising to two tablets daily after ET.

IVIg comparator: Human albumin 50 mg/ml (5 %) (CSL Behring).

Other medication:

The study medicine does not affect the participant's ART treatment decided solely by her fertility clinic; however, no immunotherapy is accepted. Thus, hormone-replacement-therapy and natural cycles with fresh or frozen embryos and blastocyst transfers are accepted.

Data collection and management

Study-relevant data is recorded in an electronic case report form (eCRF) managed in REDCap; an electronic data capture tool hosted at AaUH. The information is obtained from electronic medical records, the information folder, and directly from the participant. Demographic and medical

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baseline data is collected before randomisation during the diagnostic workup. After randomisation, data related to the index ART cycle is collected. When the treatment is terminated, remaining tablets and the information folder are returned which enables the investigators to assess compliance and drug safety. The remaining tablets from each participant are counted by the unblinded DCI personnel.

The two questionnaires collect data after NT scan and birth, respectively, about pregnancy complications, AEs/ARs, the participant's and her fetus/baby's wellbeing, and perinatal outcome. The second questionnaire also requests a copy of the hospital´s delivery record.

The chief investigator is the primary data manager responsible for the validity of data in the eCRF. An audit trail ensures data traceability and validity to maintain transparency and accountability. During the study period, continuous review and evaluation of the audit trail and the data accuracy are performed by the chief investigator and the GCP board. The eCRF have range checks, description of codes, and valid options of choices where applicable. After one week, it automatically sends a reminder to participants who have not replied to the questionnaires and notifies the investigators. Personal identifier variables are marked which allows anonymous data export. The same data is collected in participants with and without major protocol deviations, and the reason for any protocol deviation is described. Data is protected according to the GDPR (the General Data Protection Regulation), the Danish Data Protection Act, and the Danish Health Act. Specific authorized persons from The North Denmark Region Committee on Health Research Ethics or the Danish Medicines Agency have unrestricted access throughout the trial to monitor, audit, and inspect the source data regarding the study participants.

Blood sample analysis

The study-specific blood sample analyses are performed by the DCI, and the results will be shared with the investigators after the randomisation code is disclosed. In each fresh EDTA blood sample, the total leucocyte cell count, and the fractions of T helper cells (Th) 1, Th2, Th17, cytotoxic T cells (Tc), regulatory T cells (Tregs), B cells, and natural killer (NK) cells ($CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16$) are measured with flow cytometry. A TruCulture analysis is performed on both blood samples from only 25 participants as this RCT serve as a pilot study exploring the relevance of measuring stimuli-specific cytokine production in RPL patients treated with immunomodulatory

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medicine. The appendices contain a detailed description of the methods of the flow cytometric and TruCulture analyses used (see Appendix A and B).

Remaining biological material is saved in a research biobank for later analysis of small extracellular vesicles (sEV) with different phenotypes and for other future studies on immunological risk factors to RPL. The biological material is stored at -80^{0}C and consists of three 6 ml collection tubes containing serum, EDTA plasma, and citrate plasma, respectively.

Endpoints

The primary endpoint is the percentage of participants with ≥1 normal, viable fetus at NT scan in GW 11-13 among all participants in each treatment group, and subsequently among participants pregnant after ET. Also, the percentage participants with ≥1 normal, viable fetus at NT scan among all participants except those pregnant with a fetus having a confirmed chromosomal abnormality will be reported. Furthermore, the relative risk, absolute risk reduction, and an adjusted risk ratio for this primary outcome will describe the primary outcome. The primary endpoint is also measured in subgroups based on diagnosis of primary and secondary RPL, respectively, i.e., no prior birth or a history of ≥1 previous birth after 22 GW. The secondary endpoints are the incidence of AE/ARs and pregnancy complications (miscarriage rate, negative pregnancy rate, preeclampsia, gestational hypertension, gestational diabetes mellitus, acute instrumental delivery), and the perinatal outcomes (congenital deformations, prematurity, small for gestational age, low birth weight, admission to neonatal care unit, sex, birth weight and gestational age), while the tertiary endpoints are the distribution of leukocyte subsets. A thoroughly description can be found in the statistical analysis plan (SAP) (see supplemental material).

Safety considerations, safety monitoring, and reporting

An AE is defined as any untoward medical occurrence (including any unfavourable or unintended sign, symptom, or disease) in a participant during the study (from the day of randomization until childbirth or 6 months after last IV infusion) and which does not necessarily have a causal relationship with the treatment in contrast to an AR. A serious AE (SAE) is defined as an AE that results in death, is life-threatening, requires hospitalization or prolongation of existing hospitalisation, results in persistent or significant disability or incapacity, or is a congenital anomaly or birth defect. Some medical events may jeopardize the subject or may require an intervention to prevent one of the above characteristics/consequences. Such events will also be considered as

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serious ARs (SARs). A suspected unexpected serious adverse reaction (SUSAR) is defined as an adverse reaction, the nature or severity of which is not consistent with the applicable product information (EUR-Lex, CT-3). For each case, it is considered whether it is serious, unexpected, and possibly, probably, or definitely related to an intervention based on the Summery of Product Characteristics (SmPC) and timing.

Participants is encouraged to describe all AEs in their information folder, and they are followed-up on each appointment at the RPL Center. The questionnaires follow-up on AEs in the participants and their foetuses/children after treatment cessation. If a new AE is described in the questionnaire, an investigator contacts the participant to assess the AE's character and whether follow-up consultations or therapy is required. Non-pregnant participants are informed to contact the investigators in case of AEs after treatment cessation. All AEs are reported from the investigator to the sponsor immediately (<24hours) and described in detail in a follow-up report to the sponsor. The sponsor reports SUSARs to The National Board of Health and The North Denmark Region Committee on Health Research Ethics during the entire study period within seven days. In addition, an annual report on SAE will be sent to these authorities. Thus, the study follows the EudraLex Clinical Trials Guidelines Chapter II - safety reporting. Some AEs linked to pregnancy, ART, or RPL are expected and includes amenorrhea, vaginal haemorrhage, weight gain, backpain, pelvic pain, drowsiness, nycturia, increased hunger, nausea, and vomiting. These AEs are not recorded unless they are serious; likewise, hospitalisation due to symptoms of a threatened miscarriage, induced abortion, or surgical abortion (evacuation), is not assessed as a SAR/SAE.

Sample size

Based on previous studies[4,5,28] and an expectation on similar treatment effects in women with 2 and ≥3 PLs, a minimum LBR of 40 % in the active treatment group and 12 % in placebo group is anticipated. Based on these expectations, a type I error of 0.05, and a type II error of 0.20, the study will need a sample of 74 participants. Based on experience from previous trials, <2 % dropouts, and <10 % protocol deviations (see Figure 2) are expected.

Statistical analysis plan

The statistical analyses will be carried out by the investigators in collaboration with professional statisticians. A $p \leq 0.05$ is considered statistically significant. All randomized participants will be

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included in the intention-to-treat (ITT) population, while the PP population will only include participants with no major protocol deviations listed on Figure 2.

When the primary outcome is collected in 38 participants, who fulfil criteria for the PP analysis, a interim analysis will be conducted by an independent group of statisticians who get access to data on the primary outcome; SARs and SUSARs; and a simple-coded (A/B) randomisation list. The statisticians will not get data on ARs/AEs, since it will increase the risk of breaking the code. The interim analysis will provide information of differences in efficacy and safety between groups. If a difference of ≥3 SUSARs or SARs between groups is observed, the study will discontinue. The study will not terminate in case of futility, and it will not implement any modifications to trial procedures. Therefore, no correction of the reported P value will be performed in the final analysis. The sponsor has the ultimate authority to stop the study.

After completion of the study inclusion and before breaking the blind, a pre-analysis blind review on data will be caried out by the statisticians to perform data validation, detect outliers, assess distribution of variables, and measure the outcomes. Afterwards, the unblinded data analysis will proceed.

The analyses on primary outcome will be performed as an ITT and a PP analysis, while the secondary outcomes are performed on the ITT population, and the tertiary outcomes are performed on the PP population. A $\chi 2$ test will compare the primary, secondary, and tertiary categorical outcome variables between treatment groups. A Fisher's Exact Test will be used when less than five participants are expected in one group. An adjusted relative risk for a normal viable pregnancy at NT scan between the two treatment groups is estimated using Poisson regression and is adjusted for relevant confounders including BMI, smoking, and age.

For the analysis of the tertiary outcomes, including the concentration and fraction (continuous) of leukocyte subsets, an unpaired two-sample Mann-Whitney U test or t-test will be used for between group comparisons depending on data distribution while paired Wilcoxon signed-rank test or paired t-test will be used for within group comparisons. When comparing more than two groups, i.e., subgroups according to reproductive outcome after ET or percentile subgroups, an ANOVA or Kruskal-Wallis test will be used.

The analyses will compare

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1. The outcomes from blood sample 1 with blood sample 2 within each two treatment groups, respectively.
2. The outcomes from each two blood samples, respectively, between treatment groups
3. The outcome changes from blood sample 1 to 2 between treatment groups and between subgroups based on pre-treatment cell fraction (3 percentile subgroups).

Also, receiver operation characteristic curve analyses will determine the optimal cut-off values for the Th1/Th2 ratio, Th17/Treg ratio, and the NK cell fraction in blood sample 1 to identify participants with a positive reproductive outcome in the active treatment group.
Further information on the statistical analysis is described in detail in the SAP in supplemental material.

Data quality and monitoring

The study protocol, trial master file (TMF), and eCRF have been reviewed by the regional GCP-board. The GCP-board comprises experts in clinical trials who are not involved in other aspects of the study and have no competing interests. They make systematic inspections including examinations of the TMF, study procedures, safety, and data collection, accuracy, completeness, and validity to ensure it is performed in accordance with the protocol and comply with the Danish legislation and GCP guidelines. The trial will also be open for inspection from the Danish Health Authority.

Patient and public involvement:

The active treatment was given to ten RPL patients according to a preliminary protocol proposal as well as presented to stakeholders in the field of ART. In a post-treatment semi-structured interview with the patients, proposals for optimizations according to treatment plan and compliance as well as motivational factors and barriers were performed. Following this, stakeholders submitted their proposals for optimisation of the feasibility of the treatment protocol, which is highly dependent on cooperation with fertility clinics and their treatments. These proposals were all used to revise the protocol.

DISCUSSION

Ethics and dissemination

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The study protocol, participant material, and informed consent are approved by The North Denmark Region Committee on Health Research Ethics(N-20200066), The National Board of Health (EudraCT number: 2020-000256-35), and the Data Protection Agency (2020-156). It was registered at ClinicalTrials.gov before the study was initiated (NCT04701034). In case of protocol modifications, a formal amendment will be approved by these authorities before implementation and when it is approved, it will be sent by e-mail to all collaborating parties in the study. The study adheres to the Declaration of Helsinki II, local regulations, and GCP guidelines.

Participation is voluntary, and the written informed consent can be withdrawn at any time without consequences for the subsequent treatment at the RPL Center. Future studies on the research biobank material not already described will require a new approval from ethical committee and if required, an additional consent provision from the participants. The study is covered by the national patient health insurance. Study drugs are given to participants without expenses. None of the study investigators will get any economic or professional advantages before, during, or after the study. The findings will be submitted for publication in internationally acknowledged peer-reviewed journals in obstetrics and gynaecology or general medicine and will be described in at least two scientific papers: one manuscript focusing on the reproductive outcome and minimum one manuscript focusing on changes of immune biomarkers according to treatment and the reproductive outcome. Data will also be presented at national and international congresses. Future research based on the biobank will subsequently be performed and shared in scientific papers. Authorship eligibility will be based on the International Committee of Medical Journal Editors (ICMJE) recommendations 2018. An anonymous dataset may be shared upon reasonable request after agreement and approval by the sponsor and chief investigator. Study results and the allocation group of each participant will be shared with the participants who requested this information after the complete data analysis. We will publish both positive, negative, and inconclusive results. No publication restrictions are imposed.

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Competing interest statement

USK received consulting fees from Merck for Update of patient information leaflet and from IBSA Nordic for Clinical instruction and support for attending meeting/congress from Merck.
All other authors have no conflicts of interest to declare.

Author contributorship statement

OBC is sponsor of the study. CNP and OBC made the study design, and planned, conceived, initiated, and conducted the study and the clinical implementation. They are both grant holders. CNP is the chief investigator and wrote the protocol, made the statistical analysis plan, and made the eCRF and arranged all data collection, data management, data reporting, data analysis. OBC and USK made critical protocol revisions. KRN, LE, MMJ, and RS provided detailed information on immunological analyses on blood samples and planned and performed all laboratory analyses on participants’ blood samples. USK provided information on ART and supported CNP and OBC in patient and stakeholder involvement. CNP decided to submit protocol for publication. CNP, USK, and OBC will interpret data after final data analysis. All authors contributed to refinement of the study protocol and approved the final manuscript.

Abbreviations:

AaUH: Aalborg University hospital
AE: adverse event
AMH: Anti-Müllerian hormone
AR: adverse reaction
ART: artificial reproductive technology
BMI: body mass index
BW: body weight
DCI: department of clinical immunology
eCRF: electronic case report form
ET: embryo transfer

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EVA: ethylene vinyl acetate

FET: frozen embryo/blastocyst transfer

GCP: good clinical practice

GDPR: General Data Protection Regulation

GW: gestational week

ICMJE: International Committee of Medical Journal Editors

ICSI: intracytoplasmatic sperm injection

ITT: intention-to-treat

PP: per-protocol

HPNDR: The Hospital Pharmacy North Denmark Region

IV: intravenous

IVF: in intro fertilisation

IVIg: intravenous Immunoglobulin

LBR: live birth rate

LSD: lymphocyte subset distribution

NK: natural killer

NT: nuchal translucency

PL: pregnancy loss

PRS&IVIg: prednisolone and intravenous Immunoglobulin

RCT: randomised controlled trial

RPL: recurrent pregnancy loss

SAR: serious adverse reaction

SmPC: Summery of Product Characteristics

SUSAR: suspected unexpected serious adverse reaction

Tc: T cytotoxic

Th: T helper

TMF. Trial master file

Treg: T regulatory

uRPL: unexplained recurrent pregnancy loss

Appendices and supplemental material:

- Appendix A: Blood sample analysis in flow cytometry

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- Appendix B: Blood sample analysis in TruCulture
- SAP
- Information folder
- Consent form

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Figures:

Figure 1: A flow-diagram on the treatment for each participant.

Figure 2: Diagram over the criteria for major protocol deviations.

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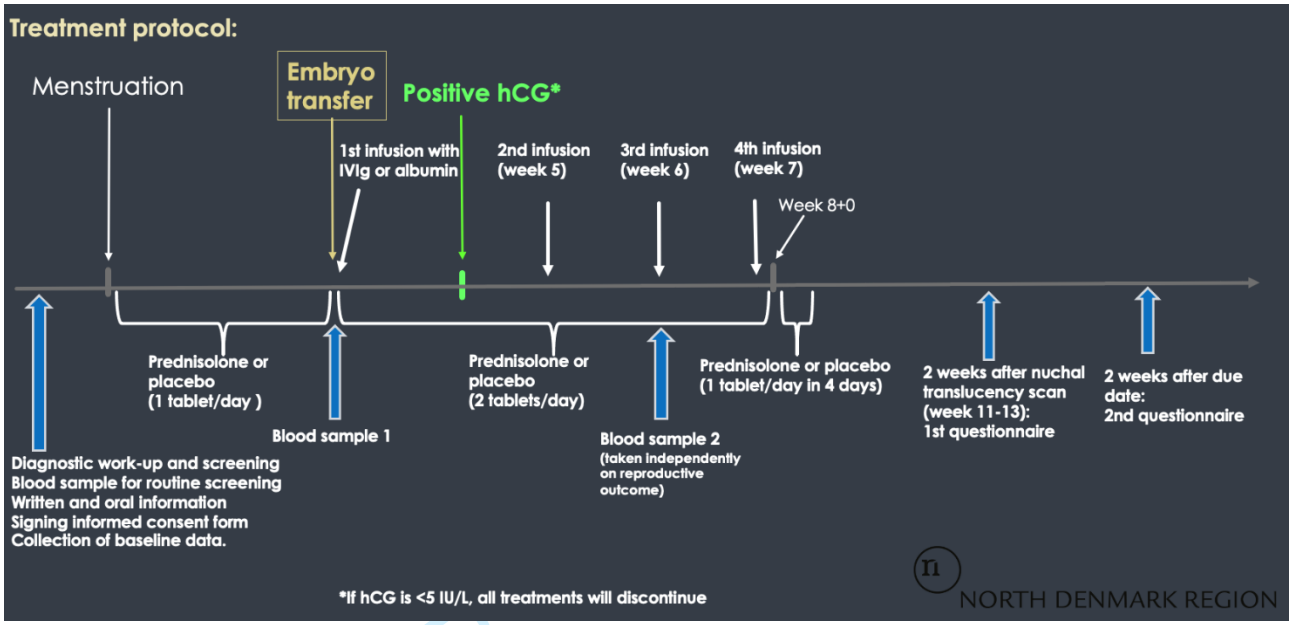

Figure 1: A flow-diagram on the treatment for each participant.

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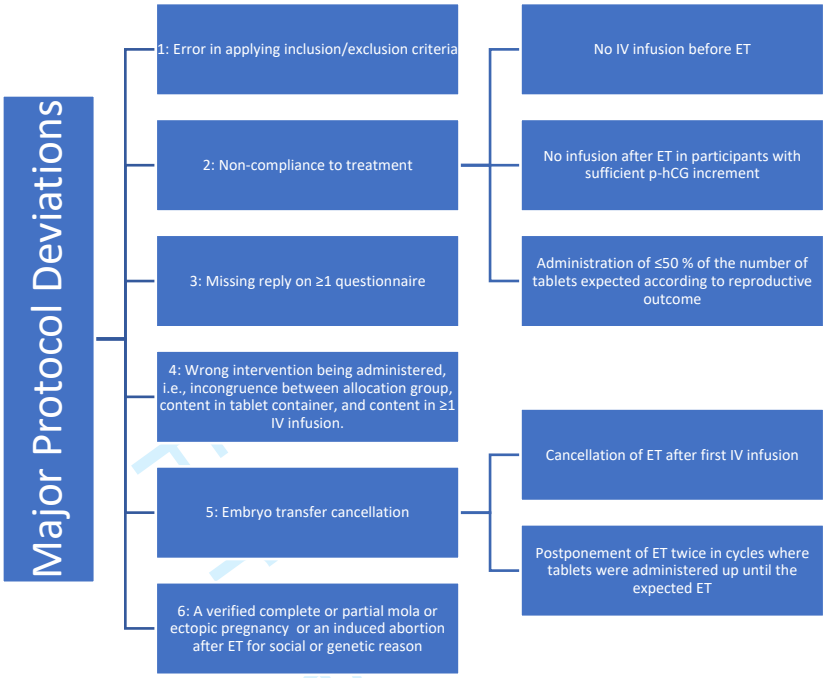

Figure 2: Diagram over the criteria for major protocol deviations.

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Analysis method for the analysis of the leukocyte subset distribution in peripheral blood

Appendix A

The participant's blood sample 1 and 2, which are collected before and after intravenous treatment with albumin or immunoglobulin, respectively, are analysed in the exact same manner.
First, a total leucocyte count (TLC) will be measured using the Sysmex XN-1000 blood analyser. The Sysmex provides absolute count for white blood cells (WBC), lymphocytes, monocytes, and neutrophil granulocytes.

The EDTA blood sample collected from the participant will be prepared for flow cytometric analysis using two different protocols. These flow cytometer analyses will be performed throughout the study and the analyses are initiated within 1 hour from blood samples were collected. The gating will be checked in the end of the study on all analyses.

The first flow cytometric protocol will provide the concentration of T helper cells (Th), cytotoxic T cells (Tc), B cells, and natural killer (NK) cells, and the fraction of each subgroup of the total lymphocyte population using two different antibody panels. These two panels contain 10 µL of fluorochrome-conjugated-antibodies against CD3, CD4, CD8, and CD45 (BD Bioscience) (tube 1) or CD3, CD16, CD56, and CD45 (BD Bioscience) and CD19 (BD Bioscience) (tube 2) which will be added to FACS tube 1 and 2, respectively, followed by 50 µL EDTA blood from the participant. Then the tubes will incubate in the dark in room temperature 15 minutes before a lysing solution (BD Biosciences) will be added to the tubes removing the red blood cells (RBC). The samples are run through the Novocyte 3000 flow cytometer (Acea Biosciences, Inc., San Diego, USA).
The collected data includes all WBC. Thus, to analyse only the lymphocytes, the $CD45^{+}SSC^{low}$ cells will be gated. In the tube 1, the $CD3^{+}$ T cells will further be divided into $CD3^{+}CD8^{+}$ Tc-cells and $CD3^{+}CD4^{+}$ Th-cells. In the tube 2, $CD3^{-}CD56^{+}CD16^{+}$ NK cells and $CD3^{-}CD19^{+}$ B cells are gated.

The second protocol will provide the percentage of Th1-, Th2-, Th17-cells, T regulatory (Treg) cells, and $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{-}$ NK cells of the total peripheral blood mononuclear cell (PBMC) population using two different antibody panels.
The first panel includes fluorochrome-conjugated-antibodies against CD3, CD4, CD16, CD25, CD56, and CD127 (all from BD Bioscience). These markers can differentiate $CD56^{dim}CD16^{+}$ NK cells from $CD56^{bright}CD16^{-}$ NK cells. This antibody panel have CD16 and CD56 conjugated to different fluorochromes to be able to distinguish the NK subgroups, while the panel used in tube 2 in the first flow cytometric protocol contained anti-CD16 and anti-CD56 antibodies conjugated to the same fluorochrome to provide the total NK cell count. Anti-CD25 and anti-CD127 antibodies will be used to gate Treg cells ($CD25^{+}CD127^{low}$).
The second panel will differentiate Th1, Th2 and Th17 cells. These groups will be distinguished using the CCR4 (Biolegend), CCR6(BD Bioscience), CCR10(BD Bioscience) and CXCR3(BD Bioscience) chemokine receptors in combination with the anti-CD3 and anti-CD4 antibodies. This analysis will provide the percentage of each Th-cell subgroup.
All antibody concentrations will be optimized for this second protocol before the antibodies will be included in this project. The optimization is lot number specific, which means different dilution factors are

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used throughout the project. Of the optimized antibody concentration, we will use 5µL of all antibodies except CD25, of which we will use 20 µL.

300 µL EDTA blood will be added to each FACS tube labelled full panel 1, full panel 2 or necessary controls for each panel. The blood will be lysed and washed to remove RBCs. Then each antibody panel will be added to different tubes and the samples will incubate in the dark in room temperature 15 minutes followed by two washes. The samples will be resuspended in 300µL wash buffer containing phosphate buffered saline (PBS) (VWR Chemicals) + 1% FCS (Gibco) before running the samples on the flow cytometer within 6 hours after the blood sample was taken.

The remaining of the blood sample will be frozen and saved for future analysis. This research biobank for future research will consist of three 6 ml collection tubes containing serum, EDTA plasma, and citrate plasma, respectively. After immediate centrifugation, the remaining serum and plasma will be stored in tubes at -80°C.

Analysis methods for analysing stimulus-specific induced immune responses in peripheral blood ex vivo

Appendix B

TruCulture® is a whole blood assay with directly ex vivo human immune stimulation which permits evaluation of individual and stimulus-specific induced immune responses through measurements of released cytokines as a proxy for immune function in vivo against a range of stimuli. TruCulture tubes (Myriad RBM, Austin, TX, USA) are pre-loaded with cell culture media and immune stimulants. In this trial, the tubes are pre-loaded with one of the following immune stimuli: CD3/CD28, R848, lipopolysaccharide (LPS), polyinosinic-polycytidylic acid (polyIC), and a no-stimulus null control.[1]

Peripheral blood is drawn by vein puncture and collected into a Lithium-Heparin tube. The analysis is initiated in the laboratory within 1 hour after sampling. 1 ml is transferred to each TruCulture® tubes and incubated in a dry heat block for 22 (+/- 10 min) hours at 37 °C. Following the 22 hour long immune stimulation, supernatants are collected by inserting a valve separator to separate cells from the culture supernatant as described previously described.[2]

Cytokines will be quantified in TruCulture® tube supernatants using the MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel II (MilliporeSigma, Burlington, Massachusetts) on the Luminex 200 platform following manufacturer protocols.

Ten cytokines will be measured: IFN-α2, IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-12 (p40), IL-13, IL-17, and Monocyte Chemoattractant Protein-1 (MCP-1). The limit of detection (LoD) will be 6,56 pg/ml for IFN-α2, 0,86 pg/ml for IFN-γ, 5.39 pg/ml for TNF-α, 0,28 pg/ml for IL-2, 0.20 pg/ml for IL-4, 0.14 pg/ml for IL-6, 3.24 pg/ml for IL-12 (p40), 2.58 pg/ml for IL-13, and 3.05 pg/ml for MCP-1.

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Intravenous immunoglobulin and prednisolone to women with unexplained recurrent pregnancy loss after assisted reproductive technology treatment: a randomised, double-blind, placebo-controlled trial

STATISTICAL ANALYSIS PLAN

Trial registration numbers:

Clinicaltrials.gov ID: NCT04701034
EudraCT number: 2020-000256-35
Ethical registration number: N-20200066
Project ID at The North Denmark Region: 2020-156
WHO unique trial Number: U1111-1273-8585

Statistical Analysis Plan Version: 1.0

Statistical Analysis Plan Date: 01.05.2022

Number of participants enrolled: 30

This SAP is based on study protocol version 7.0 dated 01.12.2021.

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Contribution:

The SAP was developed and described by CNP and revised by OBC. RGS helped elaborate the SAP description.

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The Statistical Analysis Plan (SAP) for "Intravenous immunoglobulin and prednisolone to women with unexplained recurrent pregnancy loss after assisted reproductive technology treatment: a randomised, double-blind, placebo-controlled trial" describes and expands the statistical information presented in the protocol published in BMJ Open.

Protocol Number Code	CNPOBC2020
Development Phase	Phase II
Products	Prednisolone and Intravenous Immunoglobulin Placebo and Human Albumin
Indication studied	Recurrent pregnancy loss
Clinical Trial Initiation Date	February 6, 2021
Clinical Trial Completion Date	Recruiting
Date of the Analysis Plan	01.05.2022
Version Number	1.0
SAP revisions	N/A

This study is conducted according to the Declaration of Helsinki III, local regulations, and Good Clinical Practice (GCP) guidelines and is monitored by the regional GCP board in Northern Denmark. The protocol, the informed consent form, and the participant written information is approved by the Ethics Committee of North Denmark Region, The National Board of Health, and the Data Protection Agency prior to the inclusion of participants. Informed consent from all participants is signed before enrolment.

This SAP will concern analysis of data that will be shared in more than one manuscript.

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1. Introduction

Recurrent pregnancy loss (RPL) is defined as 2 consecutive pregnancy losses[1] and affects around 5 % of women in reproductive age.[2] It can be divided into primary (pRPL) and secondary RPL (sRPL), and sRPL, incontrast to pRPL, define RPL patients who have had a pregnancy beyond 24 weeks of gestation before the consecutive pregnancy losses (PLs). In Denmark, more than 25 % of all RPL cases happens after fertility treatments (in-vitro fertilization (IVF); intracytoplasmatic sperm injection (ICSI) or frozen embryo/blastocyst transfer (FET)), which in the following jointly are called assisted reproductive technologies (ART). The group of patients with RPL is very heterogenic. The underlying cause of RPL is unknown, probably multifactorial as a series of various risk factors is known and can be identified in less than 50% of patients. These include thrombophilia, and a group of endocrine, chromosomal, and anatomic aberrations.[3] In the remaining unexplained RPL (uRPL) cases, immunological aberrations are thought to be at least partly involved in the pathogenesis. This hypothesis is based on studies finding increased frequency of autoantibodies and specific human leukocyte antigen (HLA) alleles associated with other autoimmune diseases as well as unbalanced distribution of lymphocyte subsets, especially natural killer (NK) cells, T regulatory cells, and T-helper (Th) cells, in uRPL patients. Treatment regimens used in autoimmune diseases have therefore been suggested for uRPL patients, including intravenous immunoglobulin (IVIg) and prednisolone. A meta-analysis of RCTs evaluating the efficacy of IVIg treatment for women with RPL found no significant effect on live birth rate; however, the acquired sample size was not obtained. Since subgroup analyses did show a significant effect in sRPL, the authors suggested that further RCTs were required to obtain sufficient evidence.[4] However, a pilot study suggests that a combination of prednisone and IVIg improves the chance of live birth in women with RPL after ART.[5] Some studies suggest that the immunomodulatory treatment is effective in primarily uRPL patients with aberrant distribution of lymphocyte subsets[6–8]; nonetheless, the data is sparse. It is possible, that the lack of a significant effect of IVIg in RPL patients can be explained by a substantial heterogeneity among enrolled participants in such studies obliterating a possible effect that may present in a certain RPL subgroup. At present, we do not have evidence for an effect of the combination of IVIg and prednisolone on reproductive outcome in RPL patients after ART. Simultaneously, no clear characteristic on patients benefitting from immunomodulatory treatment have been identified. If a blood sample analysis of immune cells can identify a characteristic immune profile in patients who are more likely to respond to treatment and benefit from it (i.e., childbirth), it could help

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clinicians restrict treatment to the right patients while searching for other treatments in the remaining patients, which consequently would shorten the patents' harsh fight to get a child.

Research Hypothesis

The null hypothesis is that there is no difference in normal pregnancy rate after embryo transfer assessed at the nuchal translucency (NT) scan between RPL patients in immunomodulatory treatment and RPL patients taking placebo. The alternative hypothesis is that active treatment is a superior to placebo according to the chance of normal pregnancy at NT scan.

Study Objectives

The primary objective of this study is to determine the effectiveness of prednisolone and IVIg (P&IVIg) compared to placebo for increasing normal pregnancy rate at the NT scan.

The secondary objective is to evaluate if P&IVIg is associated with increased rate of pregnancy complications, negative pregnancy outcome, and perinatal outcomes.

The tertiary objectives are to explore how P&IVIg treatment affects the lymphocyte subset distribution (LSD) in peripheral blood, and whether the LSD can predict which uRPL patients that benefit from P&IVIg.

2. Study Methods

2.1 Trial design

The present study is a randomized 1:1, double-blinded, placebo-controlled, single-center, phase II therapeutic study conducted at Centre for Recurrent Pregnancy loss of Western Denmark, at which the physicians are specialists in treating patients with RPL. Patents from all over Denmark can be referred without costs. Participants are randomised to treatment with P&IVIg or placebo.

2.2 Randomization and Blinding

Participants are randomized in a 1:1 ratio. The participants, investigators, outcome assessor, and care provider are blinded as the Hospital Pharmacy North Denmark Region (THPNDR) perform the randomisation. Randomization will be in blocks of different sizes ensuring an even distribution after half of the number of (n=38) participants have been enrolled at what time a pre-analysis will be conducted. The randomization code will be disclosed when the last participant has completed her treatment and passed NT scan (gestational week (GW) 11-13).

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2.3 Intervention:

The timing for different study interventions including participant enrolment, blood sample collection, and treatment administration is depicted on the flow diagram on Figure 1.

In the menstrual cycle in which the participant expects an embryo transfer (ET) to be carried out, one tablet per day is administered from first day of menstrual bleeding until ET; then, two tablets per day is administered from the day of the ET and until gestational week 8+0, and then gradual discontinuation with one tablet per day from week 8+0 to 8+4. If the participant does not become pregnant after ET or has a pregnancy loss (PL) before week 8+0, gradual discontinuation is initiated right after the negative test or confirmed PL diagnosis.
Intravenous (IV) infusion is administered at the time of the ET (from five working days before to two working days after), and if she has a positive pregnancy test, the IV infusion is repeated in gestational week 5, 6, and 7. Participants with a pre-pregnancy body weight (BW) ≤70 kg will receive 250 ml, participants with BW of 70-85 kg will receive 300 ml, and participants with a BW ≥85 kg will receive 350 ml in each IV infusion.

Active treatment

Prednisolone: 5 mg of prednisolone per tablet. One tablet daily before ET and two tablets daily after ET until GW 8+0.
Intravenous immunoglobulin: Privigen 100 mg/ml (10 %) (CSL Behring) with a dose of approximately 0.4g/kg.

Placebo

Oral placebo: tablets contain 85 mg lactose monohydrate, 86 mg potato starch, 8.1 mg talc, 3 mg gelatine, and 0.9 mg magnesium stearate.
Human albumin: 50 mg/ml (5 %) (CSL Behring) liquid solution for IV infusion.

2.4 Sample size

Based on results from previous studies and a hypothesis that the treatment effect is the same in women with 2 and ≥ 3 consecutive PLs, we expect a minimum live birth rate of 40 % in the P&IVIg group and of 12 % in placebo group.[5,9,10] Based on these expectations, a type I error of 0.05, and type II error of 0.20, the study needs a sample of 74 patients (37 per group) to test if the treatment is effective in uRPL patients after ART.

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A dropout rate <2 % and a rate of protocol deviation <10 % (i.e., no ET) is expected. For each participant excluded from the per-protocol (PP) analysis, an extra participant will be included in order to perform a PP analysis of 74 participants.

Based on the number of new patients with ≥2 PLs after ART admitted to The RPL Centre, an inclusion period of approximately 2 years is expected.

2.5 Data collection

Baseline information will be collected before entry to the study, i.e., during the first appointment at the RPL clinic when the patient is also screened for eligibility.

Information about the primary and secondary outcomes is collected in an online questionnaire sent 13 weeks after ET i.e., after the NT scan (GW 11-13) and an online questionnaire sent after due date. If the participant does not become pregnant after ET or miscarries during the treatment, the outcome will be recorded at the time of her next follow-up, and no questionnaire will be sent. The primary outcome (live fetus at NT scan) will be recorded in the first questionnaire. If the participant does not respond on the questionnaire(s), a reminder will be sent to her, and the final attempt is made by calling the participants.

In all randomized participants, a blood sample will be collected just before first IV infusion and again 3-4 weeks later (i.e., right before third infusion is administered if the participant is pregnant. If non-pregnant, the second blood sample will be collected at the same time as it she became pregnant). Noteworthy; non-pregnant participants have only received one albumin/immunoglobulin IV infusion and have discontinued prednisolone/placebo tablet administration at the time of the second blood sample in contrast to pregnant participants who have received two IV infusions and still administer tablets).

On the day the participant is enrolled, she receives a folder containing a check-list with ticking boxes for reporting daily tablet intake and a diary for reporting adverse events/adverse reactions (AEs/ARs). The folder includes a list of known drug-related ARs according to the Summary of Product Characteristics (SmPC) for each drug, and also an explanation about which information is important to describe in detail if an AE/AR occurs. Participants are encouraged to use this folder in between follow-ups. The folder and remaining tablets should be returned to the RPL Clinic shortly after treatment cessation in order to assess treatment compliance (see section 5.6).

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At each visit, participants are also asked with open-ended questions about AE/ARs by their physician and nurse during and after IV infusion treatment to assess and journalize all AE/ARs and determine whether they are s probably, possibly, or definitively drug-related or not.

For each case, it will be considered whether it is serious, unexpected, and possibly, probably, or definitely related to an intervention based on the Summery of Product Characteristics (SmPC) for each drug and the timing. The definitions of these terms are described in the published protocol and in accordance with the definition by EUR-Lex, CT-3.

The first and second online questionnaire are sent right after her NT scan and right after the due date, respectively, and will collect information on primary and secondary outcomes including the participant´s labour, pregnancy complications, AE/ARs occurring after treatment cessation, medical interventions during pregnancy, and the child's perinatal data. She can also attach medical records on the childbirth in the second questionnaire. Non-pregnant participants are informed to contact the investigators in case of AEs/ARs up to 6 months after treatment cessation. In addition, they are offered to continue as a patient at the RPL Clinic during which follow-up on AE/ARs will be done for 6 months.

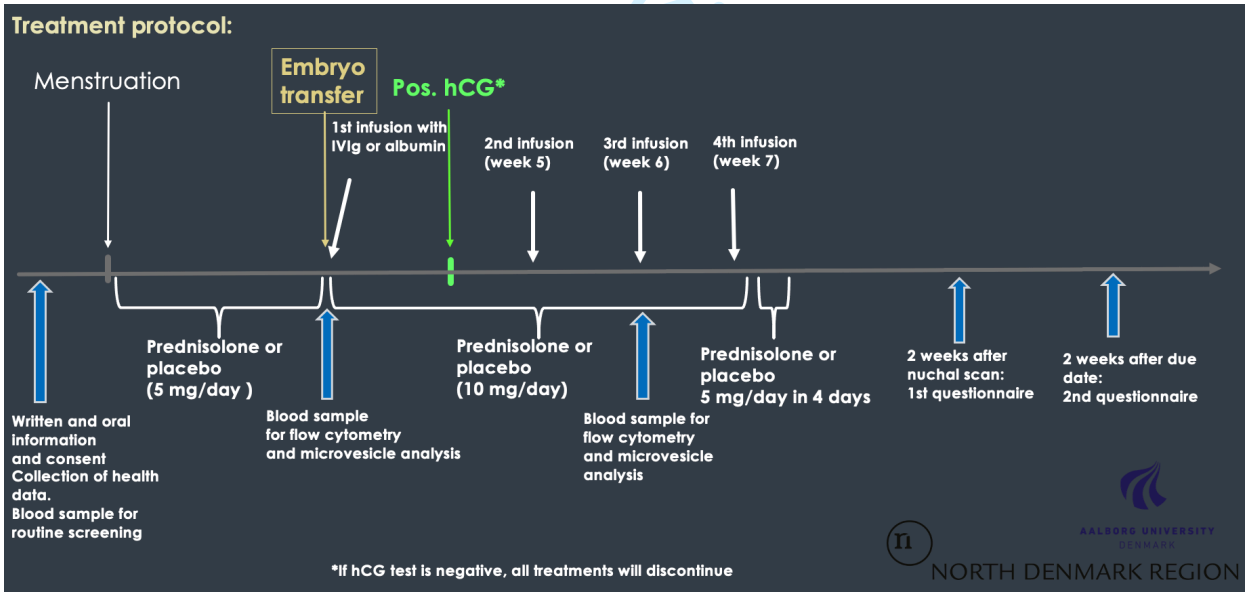

Figure 1. Flow diagram of study interventions.

2.6 Timing of the Interim Analysis and Final Analysis:

A interim analysis will be performed after 38 participants who fulfil criteria for per-protocol analysis have passed their NT scan.

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After termination of the study and before breaking the blind, the final analysis will take place. It will begin with a pre-analysis blind review on data, caried out by the statisticians, to perform data validation, detect outliers, assess distribution of variables, and measure the primary, secondary, and tertiary outcome. Afterwards, the unblinded data analysis will proceed.
The analysis of primary outcome is planned to begin when the last participant has passed NT scan (anticipated to be December 2022) and will be described in one report focusing on treatment effect on reproductive outcome and treatment safety. Publication will therefore await until last pregnant participant has given birth and answered the second questionnaire.
While waiting for the last participants to give birth, the analysis of the tertiary outcomes is planned to be begin, and these results will be described in one or more separate reports focussing on the effect of treatment on LSD and the association between reproductive outcome and LSD.

3. Study Population

3.1 Study Groups

The study will include 74 female patients with uRPL after ART allocated to either active treatment or placebo in a 1:1 ratio.

3.2 Screening Population

All RPL patients admitted to the RPL Center who are also undergoing ART treatment are assessed for eligibility. The number of patients who are assessed for eligibility will be summarised in the manuscript including the total number of screened patients not enrolled divided into the number that did not fulfil criteria after the diagnostic work-up, and the number of patients who did not wish to participate. The number of ineligible patients randomised, if any, will be reported with the reason for ineligibility.

3.3 Study Sample Inclusion and Exclusion Criteria

Inclusion criteria:

- Women with ≥ 2 consecutive pregnancy losses (miscarriages or biochemical pregnancies) before completed gestational week 10 after ART with the same partner or with an egg/semen donor *

Exclusion criteria:

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- BMI ≥35
- Age ≥41 years
- Significant uterine malformation(s)
- Known parental balanced chromosomal translocations
- ≥2 previous pregnancies with fetuses with known abnormal karyotype
- Patients with IgA deficiency, IgA-autoantibodies or hyperprolinaemi
- AMH <4 pmol/l if the next planned IVF/ICSI cycle does not imply the use of donor eggs. If IVF/ICSI with use of donated eggs donation is planned, then a low AMH value is not an exclusion criterion.
- Treatment with medication interacting with prednisolone
    - CYP3A4-inhibitors (for example erythromycin, itraconazol, ritonavir, and lopinavir), CYP3A4- inductors (for example phenobarbital, phenytoin, and rifampicin), loop diuretics, thiazides, amphotericin B, β2-agonists, antidiabetics (Metformin is acceptable), interleukin-2, somatropins, anticholinergics and regular treatment with NSAIDs.
- Patients with moderate/severe hypertension, diabetes mellitus, heart insufficiency, severe mental disorders, Cushing syndrome, myasthenia gravis, ocular herpes simplex, pheochromocytoma, systemic sclerosis, and moderate/severe renal dysfunction.
- Patients with a clinical or biochemical profile indicating need for heparin or levothyroxine treatment during pregnancy **
- Previous treatment with IVIg
- Allergy to prednisolone and/or IVIg

* The GW of the non-induced pregnancy losses will be based on the date of clinical signs of miscarriage or the fetus' crown-rump-length of a missed abortion measured on the ultrasonic scan detecting the pregnancy loss. If the participant plans to have egg donation in the study cycle, the previous two pregnancy losses must also have happened with the use of egg donation; however, it is not required that the same egg donor has been used in all three ETs.

** Indication for levothyroxine decided by the RPL Center is plasma thyroid stimulating hormone (p-TSH) > 3.5 $*10^{-3}$ IU/l together with presence of thyroid peroxidase (TPO) autoantibodies, and indication for heparin is previous clinical thromboses, ≥2 blood samples >3 weeks apart with IgM/IgG anticardiolipin and/or IgM/IgG β2-glycoprotein-1 antibodies > 35 kU/l, presence of lupus anticoagulant, protein S, C or antithrombin III deficiency, or patients being homozygous for the Factor V Leiden or prothrombin G20210A genetic polymorphisms. If treatment with heparin or levothyroxine is prescribed by another physician for other reasons,

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this is respected, and the patient is not eligible for the study. If indication for such treatment occur after cessation of study treatment, i.e., after GW 8+4, the participant is not excluded.

3.4 The CONSORT Flow Diagram

The Consort flow diagram will be included in the manuscript comprising the number of patients who 1) were screened, 2) considered eligible, 3) gave her consent, 4) were randomised, 5) were withdrawn, 5) were not included in PP population due to major protocol deviations, and 6) lost to follow-up. Reason for ineligibility and withdrawal, and also the category of major protocol deviation will be provided. Timing for withdrawal and lost to follow-up will be arranged into four groups: before ET, before cessation of tablet treatment (GW 8), at time for first questionnaire, or at time for second questionnaire. In case of withdrawal, the data collected to date can be used while further data will not be collected.

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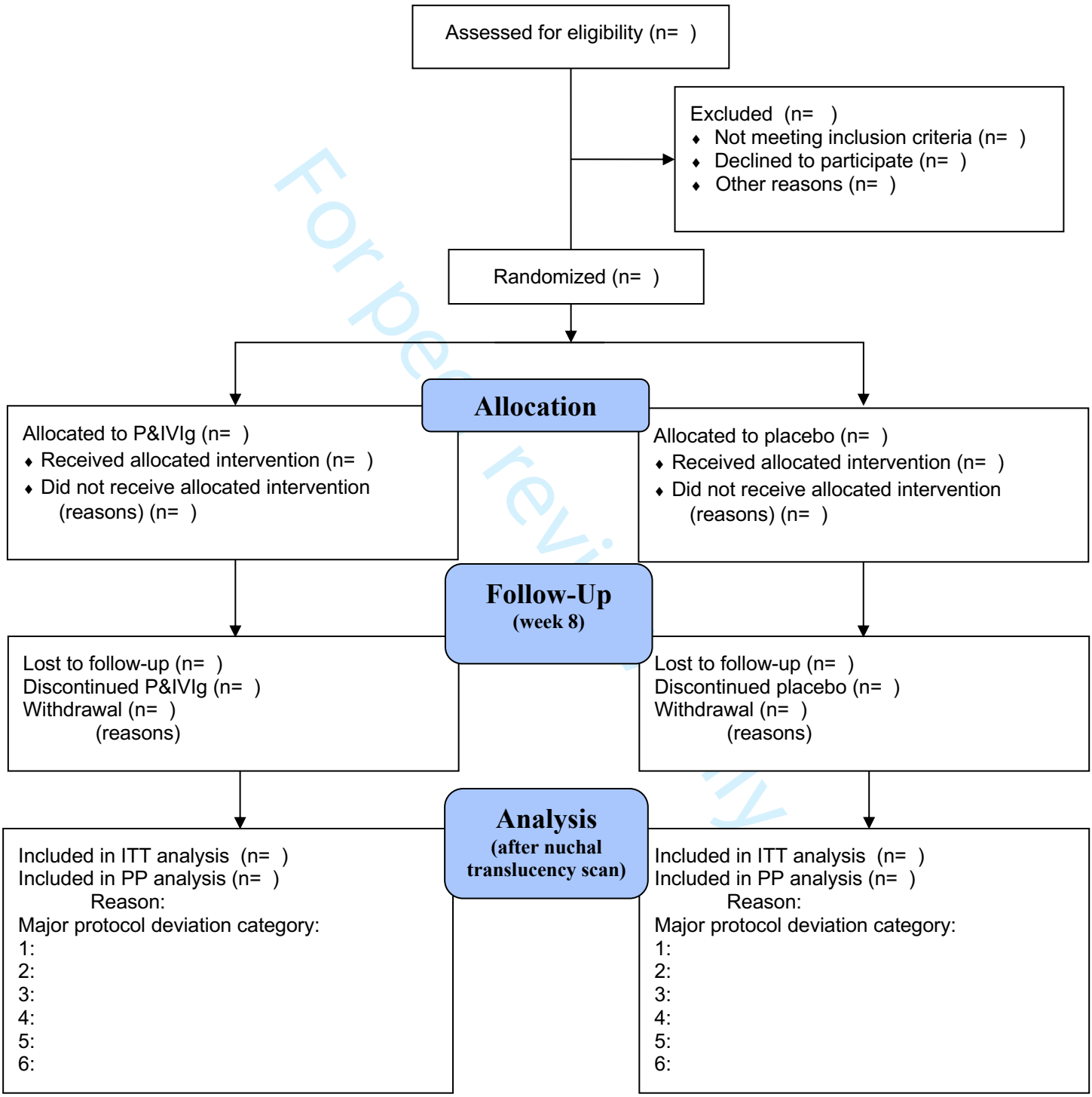
Enrollment Flow Diagram



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3.5 Baseline Characteristics

Each of the following descriptive, baseline and clinical characteristics will be reported for each treatment group in the ITT population. Tests of statistical significance will not be undertaken for baseline characteristics; rather the clinical importance of any imbalance will be noted.

Continuous measures will be summarized by mean and standard deviation (SD) (normally distributed data) or median and interquartile range (non-normally distributed data). Categorical measures will be summarized by frequencies and percentage.

Table I: Baseline characteristics

Characteristic	**Unit**	**Reporting**
Age	Years	Mean (SD)*
BMI	Kg/m^2	Mean (SD)*
Smoking	Yes/No	Frequency (percentage) of participants who smoke
Autoantibody$^+$	Above normal range	Cumulative frequency (percentage) having minimum one autoantibody above normal range
Previous caesarean section	Number of participants	Frequency (percentage)
Prior reproductive outcomes		
Previous birth (after 22 weeks)	Number with: 0 previous birth ≥1 stillbirth ≥1 live birth	Frequency (percentage) of each option
Consecutive PLs	Number of events	Mean (SD)*
Consecutive PLs after ART	Number of events	Mean (SD)* Number (percentage) for each value
Failed ART cycles (no pregnancy after ET)	Number of events	Mean (SD)*
≥1 ART cycle with prednisolone supplement initiated before ET	Number of participants	Frequency (percentage)
Indication for ART	Number with: >12 months unexplained infertility Male factor Tubal factor Endometriosis PCOS Anovulation (non-PCOS)	Frequency (percentage) of each option. Participants, whose indication is a mix of options, are counted in all the related options

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Planned ART treatment in the trial		
ART treatment in study	Number with: IVF ICSI FET *with PGT-A	Frequency (percentage) of each option
Preimplantation stage	Number with: Cleavage stage embryo transfer Blastocyst transfer	Frequency (percentage) of each option
No of embryos transferred	Number	Mean (SD)*
Gamete donation	Number with: No gamete donation Sperm donation Oocyte donation	Frequency (percentage) of each option

PL: pregnancy loss, ART: artificial reproductive technology, ET: embryo transfer, IVF: in-vitro fertilization, ICSI: intracytoplasmatic sperm injection, FET: frozen embryo/blastocyst transfer, PGT-A: pre-implantation genetic testing for aneuploidy

[+] Selected autoantibodies include antinuclear autoantibody *(*ANA), anti-ds-DNA-antibodies, thyroid peroxidase (TPO) antibodies (>60 IU/mL), IgM and IgG β-2-glycoprotein-1 antibodies (20-35 kU/L), and IgM and IgG anti-cardiolipin antibodies (20-35 kU/l). Patients with higher levels of the latter two autoantibodies are not eligible for inclusion in the study.

* The median and interquartile range (IQR) (25^{th} and 75^{th} percentile) for non-normally distributed data

4. Study Objectives, Hypothesis, and Outcomes

4.1. Primary Objective and Outcome

The primary objective of the study is to evaluate the clinical efficacy of treatment with P&IVIg relative to placebo in uRPL patients after ART as assessed by the number (percentage) of participants who are pregnant with minimum one apparently normal fetus alive determined at the time of NT scan in GW 11-13 (referred to as a positive reproductive outcome in the following) in the active treatment group and the placebo group. The study hypothesizes that P&IVIg increases the rate of a positive reproductive outcome compared to placebo. The relative difference between groups will be reported as the relative risk (95% CI) between treatment groups, the absolute risk reduction (95% CI) between treatment groups, and the adjusted relative risk (95% CI) for a positive reproductive outcome. A subgroup analysis will be performed on the primary outcome for pRPL and sRPL participants, separately, based on previous findings suggesting a specific treatment effect in sRPL patients[4]. However, this RCT is underpowered

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to detect statistical significant differences in analyses including pRPL and sRPL patients, separately, if a true subgroup effect do exist.

An additional analysis of the primary outcome will be performed calculating the number (percentage) of participants with minimum one apparently normal fetus alive at NT scan among participants with a positive pregnancy test after ET (plasma hCG>50 IU/L).

These primary outcome analyses will be undertaken as both a PP and ITT analysis.

Based on data from over 400 patients with recurrent early pregnancy losses admitted to our clinic, we expect a low risk of miscarriage after NT scan. Therefore, the primary outcome being assessed at NT scan is expected to represent the difference in live birth rate.

4.2. Secondary Objectives and Outcomes

The secondary objective of the study is to evaluate the safety of P&IVIg relative to placebo when administered in early pregnancy according to both the participant and her offspring as assessed by the incidence of serious AEs and ARs (SAE/SAR), and non-serious AEs and ARs censored up until 6 months after the last IV infusion in non-pregnant participants and until after giving birth in pregnant participants. Also, for the pregnant participants, the safety assessment includes information on pregnancy complications, negative pregnancy and perinatal outcomes, and basic perinatal outcome measures censored right after giving birth. The definitions of an AE, AR, SAE/SAR, and unexpected AE/AR are in accordance with EUR-Lex, CT-3. Specific non-serious AEs occurring in ≥2 (5%) participants and any SAE in each treatment group will be presented.

The secondary outcome analysis will be undertaken as an ITT analysis.

AEs/ARs that are rather a cause of early pregnancy than the study treatment will not be recorded as AEs/ARs, including amenorrhea, vaginal haemorrhage, weight gain, backpain, pelvic pain, drowsiness, nycturia, increased hunger, nausea, and vomiting, unless these AE/ARs are serious.

4.3. Tertiary, Explorative Objectives and Outcomes

The tertiary objective of the study is to explore the impact of P&IVIg on the immune system in peripheral blood and whether the LSD before ET can predict reproductive outcome after ET. Several analyses will be performed immediately on the fresh blood samples collected before first IV infusion and again approximately 4 weeks later (after ET and 1-2 IV infusions), and the excess biological material will be saved for future research in a biobank.

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In this SAP, the statistical analyses for the laboratory analyses performed on fresh blood are described, while future analyses on the biobank material will be described in a separate SAP later.

The tertiary outcome analyses will be undertaken as only PP analyses.

It is important to have an overview of when blood samples are collected in relation to the time when treatment is administered before analysing the tertiary outcomes. Therefore, it is described here in detail:

- Blood sample 1p: Outcomes from the first blood sample collected from a participant in the placebo group will represent the status of the investigated immune biomarkers at the time of ET (five working days before to two working days after ET) after administration of 1 placebo tablet daily for 2-3 weeks.
- Blood sample 2p: Outcomes from the second blood sample collected from a participant 3-4 weeks after ET in the placebo group will represent the status of the immune biomarkers after treatment continuation with a double dose of placebo tablets and 1-2 IV infusions (depending on the reproductive outcome) with human albumin 5% (no immunomodulatory effects expected).
- Blood sample 1a: Outcomes from the first blood sample collected from a participant in the active treatment group will represent the status of the immune biomarkers at the time of ET (five working days before to two working days after ET) after administration of 5 mg prednisolon daily for 2-3 weeks.
- Blood sample 2a: Outcomes from the second blood sample collected from a participant 3-4 weeks after ET in the active treatment group will represent the status of the immune biomarkers after treatment continuation with a double dose prednisolone (10mg from the day of ET and until a negative pregnancy test, early pregnancy loss or pregnancy week 8) and 1-2 IV infusions (depending on the reproductive outcome) with mmunoglobulin 10 % (approximately 0.4g/kg). Thus, participants with sufficiently rising p-hCG receive the second IV infusion, which is one week before blood sample 2 is collected, while participants with a negative pregnancy test, insufficient p-hCG increase, or a p-hCG decrease, receive only one IV infusion (i.e., before ET = 3-4 weeks before blood sample 2 is collected) and have ceased prednisolone intake 1-2 weeks before the blood sample 2 is collected.

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So, when comparing 1p and 1a, the difference will represent the effect of low dose of prednisolone. When comparing 2p and 2a, the difference will represent the effect of double dose prednisolone and IVIg. The difference between blood samples 1 and 2 will represent the effect of IV infusion(s) and natural physiologic changes during a menstrual cycle and/or early pregnancy. Noteworthy, the participants' latest administration of study drugs before the second blood sample will vary depending on their reproductive outcome and therefore, a sensitivity analysis will decide how subgroups (based on reproductive outcome) should be divided, see section 4.4.3.1.

4.3.1 Flow cytometry:

Cell concentrations and fractions

This RCT aims to determine how P&IVIg affects the distribution of leukocyte subsets in peripheral blood and whether a difference in distribution of cells between participants with different reproductive outcomes exists.

A difference in distribution of leukocyte subsets between treatment groups will describe how P&IVIg affects the composition of the cellular immune system. By use of the current knowledge of how the different leukocyte subsets function, the results can also to some degree be used to infer the functional effects of P&IVIg.

According to the results from small observational studies, this study expects a reduction of the NK cell, Th1 cell, and Th17 cell population, and an increase of the Th2 cell and Treg cell population after P&IVIg therapy.[8,11–13] The study has no a-priori defined hypothesis on changes in monocytes, neutrophils, $CD19^+$ B cells, and $CD8^+$ T cells after P&IVIg, as this is sparsely explored in RPL patients.

The distribution of leukocyte subsets in participants with different reproductive outcomes will be compared. Although the subgroups within each treatment group will be small, these comparisons may elaborate if a specific immune profile (i.e., distribution of (certain) leukocytes in peripheral blood) is important for a successful implantation. Thus, the RCT is not powered to find statistically significant differences in leukocyte subset distribution between subgroups with different reproductive outcomes, and therefore, only trends for such associations can be expected to be found although a true difference may exist. Nonetheless, if a clear trend or significant difference is observed between such subgroups, it may help characterize the immune profile in peripheral blood associated with a positive reproductive outcome after ET that can be

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used in the clinic to identify uRPL patients with an immunological genesis expected to benefit from immunomodulatory P&IVIg treatment.

Since a previous study found that the impact of IVIg on Th17 and Treg cell was only significant in patients with a high Th17 and/or low Treg cell fraction before treatment[14], this RCT will evaluate if the effect of IVIg on a given lymphocyte subset is more pronounced when the level before IVIg is high or low for the given lymphocyte subset (percentile subgroup analyses).

These analyses combined may help define which immune cell profile(s) that characterizes uRPL patients in whom the P&IVIg treatment increases the reproductive prognosis.

The outcomes include

- The difference in total white blood cell (WBC) count and fractions of leukocyte subsets between treatment groups, respectively, and the change from blood sample 1 to blood sample 2 in each treatment group.
- The Th1/Th2 cell ratio and the Th17/Treg cell ratio. These cell ratios have been reported as markers to identify patients who might benefit from IVIg treatment previously.[8,15]
- The $CD56^{bright}$ NK/$CD56^{dim}$ NK cell ratio. This cell ratio has been reported to be lower in RPL patients than healthy controls.[16] As the $CD56^{bright}$ NK cell type is predominant in the uterus and has immunoregulatory phenotype, and the $CD56^{dim}$ NK cell type is predominant in peripheral blood and has a cytotoxic phenotype[17], the relative NK cell subset distribution and the treatment effects on this cell ratio may be important for implantation.
- The neutrophil-to-lymphocyte ratio (NLR), monocyte-to-lymphocyte ratio (MLR), and $CD4^+$/$CD8^+$ T cell ratio. These ratios are generally used as biomarkers reflecting the balance between acute and chronic inflammation (neutrophils or monocytes) and immunity (lymphocytes). To the investigators' knowledge, they have not been reported in uRPL patients before, but the ratios are used in several diseases as a surrogate for disease activity or as a prognostic factor which could possibly also be the case for uRPL. [18–20]

Receiver operating characteristic curve analyses

In normal, uncomplicated pregnancies, studies have found that Th2 and Treg cell fractions increases while Th1 and Th17 cell fractions decrease, which paradigm is often interrupted in uRPL patients.[21–23] Previous small, observational studies have suggested that an elevated

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Th1/Th2 ratio predicts a beneficial effect of IVIg on reproductive outcome in uRPL patients; however, these studies used Th1/Th2 cytokine ratios[24,25], which cannot be directedly converted to cell ratios that will be measured in this RCT. Other studies have focused on Th17 and Treg, and they found that uRPL patients with a high Th17/Treg cell ratio before IVIg more often responded to treatment by decreasing the cell ratio and increasing the successful pregnancy rate compared to patients with normal Th17/Treg cell ratio.[14]
Instead, this RCT will determine the optimal threshold for Th1/Th2 ratio, Th17/Treg ratio, and NK cell fraction at the time for ET to determine if a specific immune profile can help clinicians identify, which patients benefit from immunomodulatory treatment.

In previous studies[6, 17, 24,26,27], an elevated NK cell fraction has been defined as >12% and it predicted a beneficial effect of IVIg on reproductive outcome in uRPL patients. Therefore, this RCT will also examine this specific threshold in a separate analysis; that will evaluate if participants in the active treatment group who have an NK cell fraction ≥12% in blood sample 1 have a higher frequency of a normal viable pregnancy censored at NT scan than participants with a NK cell fraction <12%. The same analysis will be performed in participants in the placebo group to evaluate if participants with a high NK cell fraction not receiving immunomodulatory treatment have a worse prognosis than similar patients in the active treatment group.

4.3.2 TruCulture analysis (pilot study)

A small sample of 25 participants from the RCT population will be used in a pilot study, which aims to explore if a stimulus-specific immune response is affected by immunomodulatory treatment. The analysis will be performed in the middle one-third of participants enrolled; thus, in 25 consecutively, randomized participants. The stimulus-specific immune response will be assessed as the cytokine concentration after exposure to different immune stimuli and compared within and between treatment groups. Despite a small sample size, the outcomes will also be reported for treatment subgroups separating participants into pregnant and non-pregnant patients in GW 8, to test if such subgroups of patients with different reproductive outcomes differ in response to the specific stimuli.

No prior study has reported how the stimulus-specific immune response is affected by immunomodulatory treatment in early pregnancy using the highly standardized TruCulture®

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analysis. As it is an expensive analysis with unknown relevance in this context, it will be only performed on both blood samples from 25 participants.

The outcome from the TruCulture® analysis include the concentration of ten different cytokines measured after the participant's whole blood will be exposed to one of four different immune stimuli or no stimuli. These immune stimuli include:

- CD3/CD8: mimicking T cell activation by antigen presenting cells (APC).
- R848: mimicking single stranded RNA viral activation of toll like receptor (TLR) 7/8
- Polyinosinic-polycytidylic acid (polyIC): mimicking double stranded (ds) RNA virus activation of TLR3
- Lipopolysaccharide (LPS): mimicking bacterial (E.coli**)** activation of TLR4
- No-stimulus null control: as a proxy for in vivo activation and baseline circulating cytokine concentration.

4.4. Specification of Endpoints

4.4.1. Primary Endpoint

The primary endpoint is the number (percentage) of participants who are pregnant with ≥1 apparently normal, viable fetus censored at NT scan (GW 11-13) by a sonographer in the ITT and PP population. The relative risk (95% CI) and absolute risk reduction (95% CI) between treatment groups will be reported.

In addition, four other endpoints will be reported:

- The number (percentage) of participants who are pregnant with ≥1 apparently normal, viable fetus censored at NT scan among women with a positive p-hCG after ET.
- The number (percentage) of participants who are pregnant with ≥1 apparently normal, viable fetus censored at NT scan among all women and among women with a positive p-hCG after ET excluding pregnancies with a fetus with confirmed aneuploidy, ectopic pregnancies and partial/complete mola pregnancies.
- The adjusted relative risk (95% CI) of being pregnant with ≥1 normal, viable fetus censored at NT scan for participants allocated to active treatment compared to the placebo group. The confounding variables included are smoking (binary), BMI, and age (continuous).

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- Subgroup analysis: The primary endpoint in subgroups based on diagnosis of pRPL and sRPL, respectively, i.e., no prior birth or a history of ≥1 previous birth after 22 GW.

These analyses will be performed as an ITT and PP analysis. Criteria for ITT and PP analysis are described in the section about Major Protocol Deviations.

4.4.2. Secondary Endpoints

The secondary endpoints are the frequency (percentage) of the conditions or adverse events in the ITT population stratified by treatment listed in table II.

Table II: Secondary endpoints

Secondary Endpoints		
Non-serious AEs and ARs	AEs/ARs (non-specific)	The frequency of participants with ≥1 AEs/ARs (non-specific)
	Specific AEs/ARs	The frequency of patients having a specific AE/AR - only for those AEs/ARs that occur in ≥2 (5%) of participants
	Withdrawal	The frequency of withdrawals due to AE/AR
Serious AEs and ARs	SAEs/SARs (non-specific)	The frequency of participants with ≥1 SAEs/SARs (non-specific)
	Specific SAEs/SARs	The frequency of participants with each type of SAE/SAR occurring
	Withdrawal	The frequency of withdrawals due to SAE/SAR
Pregnancy complications	Preeclampsia	Incidence Defined as gestational hypertension and

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		proteinuria (urine albumin/creatinine ratio >300mg/g or urine albumin >3g/day or >1+ on urine stix) presenting after GW 20+0.
	Gestational hypertension	Incidence Defined as blood pressure >140 mmHg systolic or >90 mmHg diastolic presenting after GW 20+0
	Gestational diabetes mellitus	Incidence Defined as an oral glucose challenge test (OGCT) with >9.0 mmol/l 2 hours after oral intake of 75g glucose solution
	Instrumental delivery	Incidence of: Elective caesarean section Emergency caesarean section
Negative pregnancy outcomes	Negative pregnancy test	Number of participants with a negative pregnancy test after ET among all participants
	Pregnancy loss	After a hCG measure >50 IU/L Specific measures of pregnancy loss: The frequency of pregnancy losses with unknown or normal karyotype among all participants and all participants with a positive pregnancy test The frequency of pregnancy losses with known aneuploidy among all pregnancy losses tested for aneuploidy The frequency of biochemical pregnancy

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		losses among all pregnancy losses The frequency (percentage) of clinical miscarriages among all pregnancy losses The frequency of late miscarriages (week 12+1-21+6) among all pregnancy losses
	Stillbirth	The frequency of participants with a birth after GW 24 which died in utero or within one week after birth
Perinatal outcomes	Congenital deformity	The frequency of a congenital deformity identified during pregnancy or within 1 week after birth
	Abnormal karyotype	The frequency of an abnormal karyotype identified during pregnancy or within 1 week after birth
	Prematurity	The frequency of a birth before 37 weeks of gestation
	Small for gestational age (SGA)	The frequency of a birthweight <10th percentile
	Low birth weight	The frequency of a birth weight <2500g
	Admission to neonatal care unit	The frequency of an admission to the neonatal care unit in >24 hours
	Sex ratio	male:female ratio
	Birth weight of singletons	Mean birth weight (g), SD Continuous variable
	Gestational length	Mean gestational age at birth (days), SD Continuous variable

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(All secondary outcome variables are binary variables unless otherwise stated)

Results on binary variables except from perinatal outcome variables will be presented in a dot plot displaying the frequency, the relative risk (95% CI), and the percentage of participants in each group.[28] The perinatal outcomes will be presented in a separate table.

4.4.3. Tertiary Endpoints

The tertiary endpoints are measured in the PP population. The endpoints will be reported for each two blood samples separately, in the active treatment group and in the placebo group. For further evaluation of treatment on leukocyte subset distribution, subgroup analysis will be performed, in which the participants in each treatment group will be separated into smaller subgroups based on reproductive outcome after ET or WBC percentiles.

Subgroups of participants based on reproductive outcome

If each treatment group has <5 participants with no pregnancy (NP) and an early pregnancy loss (EPL) after ET, respectively, the two treatment groups will be divided based on a negative (NP+EPL) and positive (ongoing pregnancy (OP) at NT scan) reproductive outcome. If each treatment group has ≥5 participants with NP, EPL, and OP, respectively, a sensitivity analyses will be performed to check for differences in total WBC count and total lymphocyte count:

1) Between participants with no pregnancy (NP) and early pregnancy loss (EPL) after ET

And subsequently,

2) Between participants receiving one and two IVIg infusions before collecting the second blood sample, i.e., a comparison of participants with NP or an EPL, who received only one IV infusion, with participants with an EPL who received two IV infusions.

If the sensitivity analysis finds no significant differences, these participants will be unified in one group representing those with a negative reproductive outcome. If the first sensitivity analysis finds a significant difference, the subgroups will be divided into three groups: NP, EPL, and ongoing pregnancy (OP) at NT scan. If the second sensitivity analysis finds a significant difference, the subgroups will be divided into the following three groups: 1) participants with NP or EPL receiving one IV infusions, 2) participants with an EPL (GA week 5-12) receiving two infusions, and 3) participants with an ongoing pregnancy (OP) at NT scan. Thus, depending on the sensitivity analyses, the subsequent analyses stratified for reproductive outcome will be in two or three subgroups.

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Flow cytometry: cell concentration and cell fraction

Flow cytometry endpoints include those listed in table III.

Table III: Endpoints from flow cytometry analyses

White blood cell count and differential	Mean total WBC count (x10^9/L) (SD) (continuous) Mean concentration (x10^9/L) (SD) and relative percentage (SD) of the following WBC differential (continuous): 1) Monocytes 2) Neutrophils 3) Lymphocytes
Lymphocyte subsets	The mean percentage (SD) of the following lymphocytes subsets in the total lymphocyte population (continuous). 1) $CD19^+$ B cells 2) $CD8^+$ Tc cells 3) $CD3^+CD4^+$ $CCR4^-CCR10$ Th1 cells 4) $CD3^+CD4^+$ $CCR4^+CCR10^-CCR6^-$ Th2 cells 5) $CD3^+CD4^+$ $CCR4^+CCR10^-CCR6^+$ Th17 cells 6) $CD3^+CD4^+$ $CD25^+$ $CD127^{low}$ Treg cells 7) $CD3^-CD56^+$ NK Cell (total) 8) $CD16^+CD56^{dim}$ NK cells 9) $CD16^-CD56^{bright}$ NK cells.
Cell ratios	Mean cell ratios (SD) (continuous), calculated based on cell fractions, include the following: 1) Neutrophil-to-lymphocyte ratio (NLR) 2) Monocyte-to-lymphocyte ratio (MLR) 3) $CD8^+$Tc/$CD4^+$Th ratio 4) Th1/Th2 ratio 5) Th17/Treg ratio 6) $CD56^{bright}$ NK/$CD56^{dim}$ NK ratio

If the variable is not normally distributed, the median and 25-75% interquartile range (IQR) will be reported.

These endpoints will be reported for both treatment groups, separately, and subsequently for subgroups based on the reproductive outcome.

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The mean change of leukocyte fractions (absolute concentration (x10^9/L) and/or percentage point) from blood sample 1 to 2 will be reported in each treatment group based on the formula:

$\Delta Leukocyte = Fraction_{before} - Fraction_{after}$

Subgroup analysis of participants based on cell level percentiles

Participants in the active treatment group will be divided into three subgroups based on ascending order of the cell fraction level of each lymphocyte subset: the lowest percentile subgroup (PS1) counting 1/3 of participants with the lowest cell fraction levels; the medium percentile subgroup (PS2) counting participants with the cell fraction levels between the 33th and 66th percentile; and the highest percentile subgroup (PS3) counting participants with the highest cell fraction levels above 66th percentile.

The mean (SD) cell fraction in blood sample 1 and 2, respectively, will be presented in each percentile subgroup (PS) and the fraction in blood sample 1 will be compared to the fraction in blood sample 2. Subsequently, for each lymphocyte subset, the change (percentage points (SD)) in each PS will be compared.

Receiver operating characteristic curve analysis

Endpoints from the receiver operating characteristic (ROC) curve analyses include:

- The optimal threshold for Th1/Th2 cell ratio, Th17/Treg cell ratio, and NK cell fraction in the active treatment group for identification of uRPL patients at high chance of an ongoing pregnancy at NT scan.
- In active treatment group, the number (percentage) of participants with 0 or ≥1 abnormal value (i.e., abnormal immune profile), respectively, based on the three optimal thresholds calculated using data from the active treatment group.
- The number (percentage) of participants with ongoing pregnancy at NT scan in the group with 0 and with ≥1 abnormal value before ET, respectively, in each treatment group, separately.

The same endpoints will be provided only using NK cell fraction >12 % (and not using any Th1/Th2 or Th17/Treg ratio) to divide participants in each treatment group into those with or without an abnormal immune profile.

TruCulture

The mean (SD) or median (25-75 IQR) concentration (pg/mL) (continuous) of IFN-α2, IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-12 (p40), IL-13, IL-17, and Monocyte Chemoattractant Protein-1 (MCP-1) will be reported for each of the four different immune stimuli and the non-stimulation analysis in each blood sample, separately.

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These endpoints will be illustrated in boxplots for the active treatment group relative to placebo group. If ≥5 participants in active treatment group have a positive reproductive outcome, subgroup analysis for these endpoints in participants with OP relative to NP&EPL will be performed.

5. Statistical Analysis

5.1 Statistical Principles

All the applicable statistical tests used will be two-sided and performed using a 5% significance level unless otherwise stated.

Baseline characteristics will for continuous variables be described with mean and standard deviation (SD) (normally distributed data) or median and the interquartile range (25^{th} and 75^{th} percentile) (non-normally distributed data). For categorical variables, the frequency and percentage will be provided. Data distribution of continuous variables will be determined using QQ-plots and histograms.

5.2 The interim analysis

When 38 patients, who fulfil criteria for entering the PP-analysis, have been included in the study, an interim analysis will be conducted by independent statisticians in order to remain all investigators blinded. Blinded results will be shared with investigators. Differences between treatment groups regarding the primary outcome as well as the number of women with SARs/SAEs and SUSARs in the two groups (denoted A and B) will be analysed with the objective of early evaluation of unacceptable side effects of the treatments to continue the study.

The interim analysis will analyse the primary outcome and compare the incidence of SARs/SAEs and SUSARs between groups. A between group difference of ≥3 SUSARs or SARs/SAEs will lead to early termination of the trial. The alpha spending method used for adjusting significance levels for the analysis of SARs/SAEs and SUSARs between groups will be explicitly defined.[29] Analysis of the primary outcome will not lead to early termination in case of futility nor any modifications to trial procedures. The sponsor has the ultimate authority to terminate the trial.

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A correlation between occurrence of SARs/SAEs and SUSARs and the primary outcome may exist, however, one such correlation will be discussed for clinical relevance and no adjustments of significance levels on the analysis of the primary outcome will be made.

5.3 Analysis Methods for Primary Endpoint

In two separate analyses in all participants in the ITT and PP population, respectively, the number (percentage) of participants in each treatment group who had an apparently normal viable fetus at NT scan will be compared using Fisher's Exact Test (0=no pregnancy or early pregnancy loss, 1= normal pregnancy at NT scan). Also, the corresponding relative risk (95% CI) and absolute risk reduction (95% CI) will be reported.

Additional analyses on the primary outcome include:

1) The number (percentage) of participants who had an apparently normal viable fetus at NT scan among all participants with a positive pregnancy test (p-hCG >50) in each treatment group will be compared using Fisher's Exact Test (0= pregnancy loss before NT scan; 1= normal viable fetus at NT scan).
2) The number (percentage) of participants who had an apparently normal viable fetus at NT scan among a) all participants and b) all participants with a positive pregnancy test (p-hCG >50) in each treatment group after exclusion of pregnancies with a fetus with confirmed aneuploidy, ectopic pregnancies and partial/complete mola pregnancies, will be compared using Fisher's Exact Test ($0_{a)}$ = no pregnancy or pregnancy loss before NT scan; $0_{b)}$ = pregnancy loss before NT scan; 1= normal viable fetus at NT scan).
3) A modified poisson regression with robust variance will estimate the adjusted relative risk (RR) (95% CI) for an apparently normal viable fetus at NT scan adjusted for baseline characteristics including age at enrolment (*continuous*), BMI at enrolment (*continuous*), and smoking habits at enrolment (*binary).* No alternative method will be applied if assumptions are not completely fulfilled, since the modified poisson regression uses a robust error variance, which is robust to violations of model assumptions.

   A sensitivity analysis will be conducted with the following covariates included: low plasma mannose binding lectin (p-MBL) level (*binary),* previous pregnancy > 22 weeks (*binary),* and presence of minimum one of the measured autoantibodies (*binary)*.
4) The primary outcome will be analysed in subgroups based on whether the participants were diagnosed with pRPL or sRPL, i.e., had no previous birth after 22 GW or had ≥1

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previous birth after 22 GW, respectively. The relative risk (95% CI) between treatment groups will be provided.

Table IV: Definition of binary covariates

Definition of binary variables used as covariates in regression analyses:		
	0	1
Smoking	No smoking within the last 3 months before enrolment	Smoking minimum 1 cigarette daily during the last 3 months before enrolment.
Low MBL level	>500 ug/l	≤500 ug/l
Previous liveborn	0 liveborn (=pRPL)	≥1 pregnancy >22 weeks (=sRPL).
Autoantibody	No autoantibody	Minimum 1 of the following autoantibodies above normal range: TPO-Ab (>60kU/l), anti-ds-DNA-Ab (>10kIU/l), ANA (≥1), anti-cardiolipin and/or anti-β2-glycoprotein antibody (20-35 kU/l in two measurements with >3 weeks interval).

5.4. Analysis Methods for Secondary Endpoints

The secondary endpoints including all binary outcome variables will be compared between active treatment group and placebo group using Fisher's Exact Test. Furthermore, the relative risk (95% CI) will be reported. The continuous outcome variables will be compared using unpaired t-test for parametric variables and unpaired Mann-Whitney U Test for non-parametric variables.

5.5 Analysis Methods for Tertiary outcomes

5.5.1 Flow cytometry: cell concentration and fraction

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The mean absolute cell count (ACC) (SD) of WBC differentials, and the mean percentage (SD) of lymphocyte subsets of the total lymphocyte population in peripheral blood in blood sample 1 and 2, separately, will be reported. These endpoints will be used in the following statistical analyses.

Treatment effects on Lymphocytes Subsets

Tertiary endpoints from flow cytometry analyses will be reported in a contingency table or bar/dot plot (example in Table V). A comparison within the treatment group, i.e., comparing the endpoints in blood sample 1 (before) with blood sample 2 (after IV infusion), will be performed using paired t-test for parametric variables and paired Wilcoxon signed-rank test for non-parametric variables. Between-group comparisons, i.e., comparing endpoints from each two blood samples between the two treatment groups, will be performed using unpaired t-test for parametric variables and unpaired Mann-Whitney U Test for non-parametric variables. The mean change will be calculated by subtracting the value in blood sample 1 (before IV infusion) with the value blood sample 2 (after IV infusion), and the mean change will be compared between treatment groups using the same unpaired statistical tests.

Table V: Results from immunological analyses on blood samples collected before first IV infusion (blood sample 1) and after IV infusion(s) (blood sample 2) in uRPL patients. The table will contain a row for each the leukocyte subsets and the cell ratios described in the section on "Tertiary Endpoints". P[1]: comparison of blood sample 1 and 2 within the treatment group. P[2]: comparison of endpoints from either 1) before IV infusion or 2) after IV infusion or 3) the mean change between the sample collected before and after IV infusion within a treatment group, respectively, between the two treatment groups. ACC: absolute cell count. SD: standard deviation

Leukocyte subset distribution	Placebo				Active treatment				Comparisons between treatment groups		
	Before IV	After IV	Mean change	P[1]	Before IV	After IV	Mean change	P[1]	P[2] (Before)	P[2] (After)	P[2] (Mean change)

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Total WBC count	ACC, Mean (SD) Percentage, Mean (SD)											
Lymphocyte subset	Percentage, Mean (SD)											

Subgroup analysis: Association between the lymphocyte subset distribution and the reproductive outcome

The participants in each two treatment groups will be divided into subgroups based on their reproductive outcome. The number of groups depend on the sensitivity analysis.

The sensitivity analysis made before dividing participants into subgroups based on reproductive outcome will use an unpaired t-test or Mann-Whitney U test to test for differences in total WBC count and total lymphocyte count when ≥5 participants are expected in each group, i.e., NP and EPL group.

Flow cytometry endpoints will be reported in a contingency table (See table II). To test for differences in ACC and/or percentage of each lymphocyte subset between subgroups based on reproductive outcome, an unpaired t-test (two subgroups) or One-Way Anova analysis (three subgroups) will be used if the variable of interest is normally distributed, and an unpaired Mann-Whitney U Test or Kruskal-Wallis One-Way ANOVA will be used if the variable of interest is skewed. To test if the ACC and/or percentages differ between participants with the same reproductive outcome but in different treatment groups, an unpaired t-test or Mann-Whitney U Test will be used depending on the data distribution of the variable of interest.

Table VI: Immune cell levels in patients with different treatments and reproductive outcomes. The table will contain a row for each of the leukocyte subsets measured as well as the cell ratios in blood sample 1 and 2, respectively. The cell types and ratios are described in the section on "Tertiary Endpoints". There will be one column for each subgroup based on the reproductive outcome in each treatment group (NP: no pregnancy, EPL: early pregnancy loss, OP: ongoing pregnancy at NT scan). P^1: comparison of NP&EPL with OP. P^2: comparison of subgroups with the same reproductive outcome between treatment groups.

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Cell type		Placebo			Active treatment			Between treatment groups	
		NP&EPL	OP	P[1]	NP&EPL	OP	P[1]	P[2] (NP&EPL)	P[2] (OP)
Total Leukocyte count ACC, Mean (SD)	Before IV After IV Mean change								

If no differences are observed between treatment groups with the same reproductive outcome in either blood sample 1 or 2, the flow cytometry endpoints will be reported for all participants only stratified for the reproductive outcome (but not stratified for treatment) (NP and EPL combined or separated according to the sensitivity analysis for reproductive treatment subgroups) and compared (See table VII). The tests used for comparing data will be same as described for results reported in table VI.

Table VII*: Immune cell levels in patients with different reproductive outcomes. The table will contain a row for each of the leukocyte subsets measured as well as the cell ratios in blood sample 1 and 2, respectively. The cell types and ratios are described in the section on "Tertiary Endpoints". There will be one column for each subgroup based on the reproductive outcome (NP: no pregnancy, EPL: early pregnancy loss, OP: ongoing pregnancy at NT scan).*

		All participants		
		NP&EPL combined	Ongoing pregnancy	P
Th1 cells, mean percentage (SD) $CD3^+CD4^+ CCR4^-CCR10^-$	Before IV After IV Mean change			

Percentile subgroup analysis: Association between the immune cell level before IV infusion and the treatment response

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Three percentile subgroups (PS) will be made based on ascending leukocyte cell level in each leukocyte subset. Thus, when comparing e.g., Th1 cell fraction, participants will be divided into three groups based on the 33rd and 67th percentile of Th1 cell fraction. The same way is PSs made for each leukocyte subset (see Table VIII).

The cell fraction level in the blood sample 1 will be compared with blood sample 2 in each PS, respectively, using paired t-test for parametric variables and paired Wilcoxon signed-rank test for non-parametric variables in active treatment group. The change in cell fraction (ACC or percentage point) observed in each three PS will be compared using One-Way ANOVA for parametric variables and Kruskal-Wallis One-Way ANOVA for non-parametric variables. These analyses will be performed for Th1, Th2, Th17, Treg, $CD8^+$ T cell, B cells, total NK cells, and the two NK subsets.

Table VIII*: Lymphocyte subset level in percentile subgroups (PS) before and after intravenous (IV) infusion (blood sample 1 and 2). The level before is compared with the level after IV. The mean change in each PS is compared. The will one row for each lymphocyte subset described in section "Tertiary endpoints".*

	PS1			PS2			PS3		
	Before IV	After IV	Mean change	Before IV	After IV	Mean change	Before IV	After IV	Mean change
Th1 cells, mean percentage (SD) $CD3^+CD4^+$ $CCR4^-$ $CCR10^-$									

Receiver operating characteristic curve analyses

ROC curve analyses will be performed to determine the performance of Th1/Th2 ratio, Th17/Treg ratio, and the NK cell fraction in blood sample 1 to predict a positive reproductive outcome (i.e., pregnancy at NT scan) in the active treatment group. The optimal cut-off for each of the three immune markers will be defined as the value with maximum sensitivity and specificity in the ROC curve analysis.

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The AUC value and the Youden's index (J_{max}) together with the corresponding sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) will be reported for each ROC curve analysis.

Noteworthy, the sample size in this RCT has not been calculated based on this analysis and with only this small sample available, no post validation cohort will be separated from the treatment group before the ROC curve analysis will be performed. Bootstrapping with 1000 bootstrap samples will be applied to estimate 95% confidence limits on the estimates.

Using the three thresholds, the number of participants in active treatment group with ≥1 "abnormal" value will be determined. An abnormal value will be defined as a value higher than the threshold for Th1/Th2 ratio, Th17/Treg ratio, and the NK cell fraction. The number of participants with an ongoing pregnancy at NT scan will be compared between participants with 0 and with ≥1 abnormal value in the treatment groups, separately, using χ^2 Test or Fisher's Exact Test.

The number (percentage) of participants with a total NK cell fraction >12% (including both $CD3^-CD16^+CD56^{dim}$ and $CD3^-CD16^-CD56^{bright}$) of the total lymphocyte population in the sample before and after IV infusion, respectively, will be reported. The number of participants with elevated NK cell fraction before IV infusion will be compared with the number after IV infusion using χ^2 Test or Fisher's Exact Test.

The number of participants with an ongoing pregnancy at NT scan among all participants with NK cell fraction ≥12% and <12%, respectively, will be compared using χ^2 Test or Fisher's Exact Test in each treatment group, respectively.

5.5.2 TruCulture pilot study: Immune response ex vivo after induced stimulation

The concentration of cytokines before and after IV infusion will be compared in each treatment group, separately, using unpaired t-test or Mann-Whitney U Test depending on the distribution of the variable of interest. Results will be presented in boxplots for each of the four stimuli-specific protocols and for the no-stimuli protocol. The boxplot will include the information described in Table IX.

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Table IX: For each stimulator, mean cytokine concentration (pg/ml) will be presented in a box plot containing data stratified and compared as described in this table, evaluating the treatment effect on the stimulus-specific immune cell response by in-group and in-between group comparisons. Information on each cytokine will be reported. P^1: comparison of blood sample 1 and 2 within the treatment group. P^2: comparison of endpoints from either 1) before IV infusion or 2) after IV infusion or 3) the mean change between the sample collected before and after IV infusion within a treatment group, respectively, between the two treatment groups.

Stimulator	Placebo				Active treatment				Comparison between groups		
	Before IV	After IV	Mean change	P^1	Before IV	After IV	Mean change	P^1	P^2 (Before)	P^2 (After)	P^2 (Mean change)
IFN-α2											

5.6. Major Protocol Deviations

The predefined major protocol violations are divided into six categories described in Figure 2

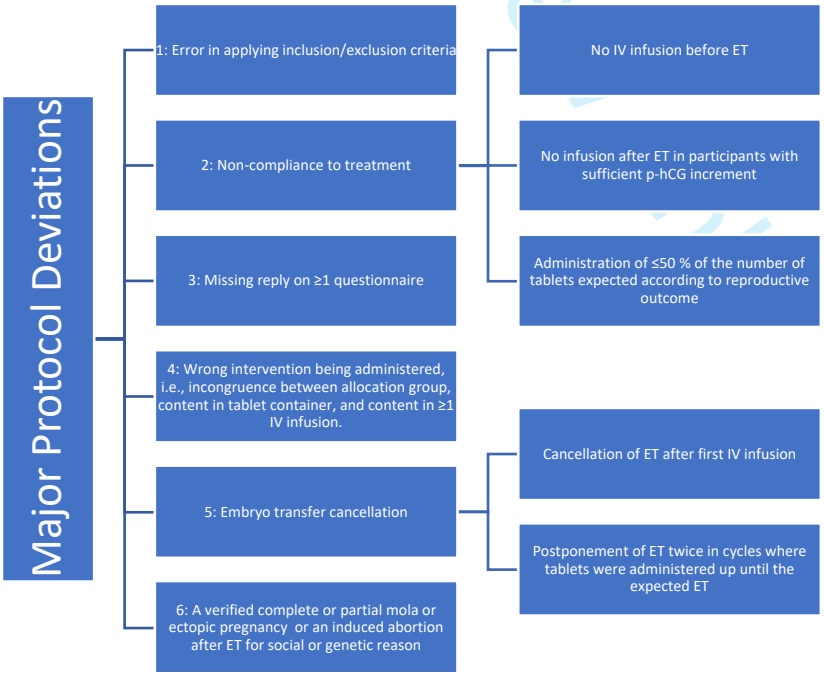

Figure 2: description of major protocol deviations excluding participants from per-protocol population.

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All randomised participants will be included in ITT analysis population. Participants who completed the trial with no major protocol deviations will be included in the PP analysis. The reason for not fulfilling eligibility criteria will be reported. In case of category 4 violations, the participant will be counted in according to the treatment she was randomised to receive, All protocol deviations will be recorded in the eCRF and summarised by the treatment group and the category with frequency and percentage of all included participants.

5.7 Missing data

To prevent missing data, the eCRF has a record status dashboard with an overview of instruments to be filled in after follow-up appointments including instruments for the investigator to fill in and questionnaires for the participant to fill in. When the instrument is changed from incomplete to complete, i.e., all data items are filled in, the instrument on the record status dashboard changes colour from red to green. Also, key data items on primary and secondary outcomes collected at follow-ups before gestational week 9 are described in the participant's medical records. In addition, AEs and treatment compliance are described in the information folder returned to the RPL Clinic after treatment cessation. Thus, these key data items are readily obtainable if missing in the eCRF by the end of the study. For this reason, the degree of missing data on key data items is expected to be minor or not existing. However, in case of missing data, the number missing will be reported.

For the analysis of the primary and secondary outcome, complete case analysis will be performed. In analysis tertiary outcomes, the management of missing data will follow the guideline presented by Jakobsen et al. and include complete case analysis and single or multiple imputation.[30] A sensitivity analysis will be performed if MI or other methods to account for missing data has been used in accordance with the description by Jakobsen et al.[30]

6. Data Handling and implementation of Statistical Analysis Plan

All data regarding primary and secondary (except data on blood samples) outcome will be collected and stored in an electronic case report form (e-CRF) in a REDCap database (Vanderbilt University, Nashville, TN, USA) which only the sponsor and chief investigator have full access to. GCP board members have rights to "see only". Information will be obtained from the participant's electronic medical records, information folder, and directly from the participant. REDCap makes audit trail logging and back-ups. The eCRF will be used to collect

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all clinical data about the participant, and also to automatically send out and collect questionnaires replies after NT scan and after due date. Personal identifiers are marked, and they will not be exported from the database when the study terminates. Data will be exported to Microsoft Excel (Microsoft Corporation, Washington, USA) and prepared for analyses performed in Stata/MP 15.0 (TX, US) for Mac.

Data regarding tertiary outcomes will be collected in a secured database at the Department of Clinical Immunology (DCI) until all participants have been enrolled and the last pregnant participant has passed her NT scan. At this time, blinded data will be shared with the sponsor and chief investigator at the RPL clinic and not at any time before, since sharing results from these analyses at any time before may reveal enough information for investigators to speculate and predict treatment allocation. Thus, in order to remain fully blinded, data on peripheral blood immunological analyses will only be handled by the DCI personnel, who have no contact to study participants, until all data has been collected.

When the last pregnant patient has passed her NT scan, we will start data analysis. Before data is unblinded, a blind review pre-analysis on data will be caried out to check for outliers in continuous outcome variables, distribution of continuous and ordered data (using Q-Q plots and histograms to assess data normality), missing data points (and if possible, collecting these missing data points) and important potential covariates identified in other recent research may be added to statistical models. This final pre-analysis will be handled by statisticians who had not been involved in the study. They will present the investigators for any uncertainties as well as blinded interpretations of the primary endpoint results. The pre-analysis may add modifications to the SAP, which will all be described in an amendment to the SAP before performing unblinded analyses. After data validation is complete, unblinded data analysis will proceed. Unblinded analyses will be conducted by the chief investigator supervised by statisticians.

This SAP will be used by all statistician and investigators analysing study data. Analysis of primary, secondary, and tertiary endpoints will be carried out in continuous order in the pre-analysis.

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study and will not have any role during its execution, analyses, interpretation of the data, or decision to submit results. The investigators also thank the DCI for performing all immunological analyses and preparing infusion medicine for all participants.

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10. Abbreviations

AaUH: Aalborg University Hospital

ACC: Absolute cell count

AE: adverse events

ANA: antinuclear autoantibody

AR: adverse reactions

ART: assisted reproductive technologies

BMI: body mass index

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DCI: the Department of Clinical Immunology

e-CRF: electronic case report form

EPL: early pregnancy loss

FET: frozen embryo/blastocyst transfer

GCP: Good Clinical Practice

HLA: Human leukocyte antigen

ICSI: intracytoplasmatic sperm injection

Ig: Immunoglobulin

ITT: Intention to treat

IVF: in-vitro fertilization

IVIG: intravenous immunoglobulin

J_{max}: Youden index

LR: likelihood ratio

NK: natural killer

NK: natural killer

NP: no pregnancy

NPV: negative predictive value

OGCT: oral glucose challenge test

OP: ongoing pregnancy

OR: odds ratio

p-MBL: plasma mannose binding lectin

PGT-A: pre-implantation genetic testing for aneuploidy

PP: Per protocol

PPV: positive predictive value

pRPL: primary recurrent pregnancy loss

PS: percentile subgroup

ROC: receiver operating characteristic

RPL: recurrent pregnancy loss

RR: relative risk

SAE: serious adverse event

SAP: statistical analysis plan

SAR: serious adverse reaction

SD: standard deviation

SGA: small for gestational age

sRPL: secondary recurrent pregnancy loss

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SUSAR: suspected unexpected serious adverse reaction

Tc: cytotoxic T

Th: T-helper

TPO: thyroid peroxidase

Treg: regulatory T

uRPL. Unexplained recurrent pregnancy loss

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SPIRIT
STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	2, 15
	2b	All items from the World Health Organization Trial Registration Data Set	Appendix
Protocol version	3	Date and version identifier	2
Funding	4	Sources and types of financial, material, and other support	16
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	16
	5b	Name and contact information for the trial sponsor	16
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	17
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	N/A

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Introduction

Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	3
	6b	Explanation for choice of comparators	7
Objectives	7	Specific objectives or hypotheses	4
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	4

Methods: Participants, interventions, and outcomes

Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	4
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	4
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	7
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	7
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	6
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	4, 9
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	11
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	7

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Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	12
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	4
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	6
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	6
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	6
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	6
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	6
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	9
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	10-11

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Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	9
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	13-14, and SAP in appendix
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	13-14, and SAP in appendix
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	13-14
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	14
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	13
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	11-12
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	14
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	15
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	15

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Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	6
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	15
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	10
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	16
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	16
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	8, 15
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	15-16
	31b	Authorship eligibility guidelines and any intended use of professional writers	16
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	16
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Appendix
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	Appendix

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

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BMJ Open

Intravenous immunoglobulin and prednisolone to women with unexplained recurrent pregnancy loss after assisted reproductive technology treatment: a protocol for a randomised, double-blind, placebo-controlled trial

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Intravenous immunoglobulin and prednisolone to women with unexplained recurrent pregnancy loss after assisted reproductive technology treatment: a protocol for a randomised, double-blind, placebo-controlled trial

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Key words: recurrent pregnancy loss, habitual abortion, reproductive immunology, immunotherapy, artificial reproductive technology

Word count: from introduction to end of discussion: 4475 + strengths and limitation box: 145

Abstract:

Introduction: Recurrent pregnancy loss (RPL), defined as two or more consecutive pregnancy losses in the first trimester, affects around 5 % of fertile women. The underlying causes remain unknown in up to 60% of patients; however, most studies point at an immunological pathology in unexplained RPL, and therefore, an effective treatment may be immunomodulatory. This study aims to evaluate the effect of intravenous immunoglobulin (IVIg) and prednisolone on reproductive outcome and the immune system in women with unexplained RPL undergoing assisted reproductive technology treatment.

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Methods and analysis: This randomized, placebo-controlled trial with double-blinded randomization to two parallel arms evaluate if immunomodulatory (active) treatment is superior to placebo in increasing the chance of ongoing pregnancy assessed at nuchal translucency scan in gestational week 11-13 after embryo transfer (ET) in 74 RPL patients with ≥2 pregnancy losses as its primary objective. The active treatment consists of IVIg (one infusion preferably 1-5 days before ET and in gestational week 5, 6, and 7) and prednisolone (5 mg/day from first day of menstrual bleeding until ET and 10 mg/day from ET to gestational week 8+0) while the comparator consists of intravenous human albumin (5%) and placebo tablets. Allocation is concealed for participants, caregivers, and investigators until trial termination and is performed in a 1:1 ratio. The secondary objective is to evaluate treatment safety, and the tertiary objective is exploration of the association between treatment, reproductive outcome after ET, and the lymphocyte subset distribution in peripheral blood collected before and after intravenous infusion(s). Excess biological material is stored in a biobank for future research.

Ethics and dissemination: The North Denmark Region Committee on Health Research Ethics (N-20200066) approved this trial. The results will be published in peer-reviewed scientific journals and presented to relevant patient associations, at relevant academic conferences and to key stakeholders.

Trial registration: Clinicaltrials.gov ID: NCT04701034. WHO unique trial Number: U1111-1273-8585.

Protocol version: this article was written per the study protocol version 8, dated 01.06.2022.

Strengths and limitations of this study

- The pragmatic, randomized, double-blinded study design reflecting the contemporary practice strengthens the study results' external validity.
- The combination of clinical and immunochemical outcome measures evaluated by blinded clinicians and the randomised study-settings strengthen the current research that evaluate how the immune system is changed during early pregnancy and whether it is related to the reproductive outcome.
- The combination of two active drugs in the active treatment group does not allow differentiation of whether one of the active drugs or only the combination possesses the effect on reproductive outcome observed in the study.

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- Participants are not selected based on a biomarker associated with an aberrant immune function, which was done in some of the previous studies reporting a significant effect of IVIg on uRPL; however, since the evidence of such an association is still sparse, further elaboration on this topic is needed.

INTRODUCTION

Background

Recurrent pregnancy loss (RPL) defined as ≥2 consecutive pregnancy losses (PLs)[1] affects around 5 % of women in reproductive age.[2] In Denmark, >25 % of all RPL cases happens after assisted reproductive technologies (ART) including in-vitro fertilization (IVF), intracytoplasmatic sperm injection (ICSI), and frozen embryo/blastocyst transfer (FET), and >10 % children born after 2019 in Denmark are conceived using ART. In up to 60 % of RPL patients, no risk factor is found, and for unexplained RPL (uRPL) no treatment with proven benefit has been found yet.[1] Among women with uRPL, immunological aberrations are thought to be at least partly responsible. For that reason, many treatment regimens used in autoimmune diseases have been tested on uRPL patients. A meta-analysis of RCTs evaluating the efficacy of IVIg treatment for women with RPL found no overall significant effect on live birth rate; however, the acquired sample size was not obtained. Since subgroup analyses did show a significant effect in secondary RPL, the authors suggested that further RCTs were required to obtain sufficient evidence.[3] A pilot study suggested that a combination of prednisone (PRS) and IVIg starting before pregnancy improves the chance of live birth in women with RPL after ART.[4,5] Additionally, previous studies of PRS supplement to ART treatment have shown improved pregnancy rate, especially for women with immune aberrations.[6–9] The underlying mechanism for an improved ART outcome after immunomodulatory treatment may be the regulation of immunological processes occurring particularly in early pregnancy. Some prior studies have suggested different biomarkers for an aberrant immune system in the uterus[10–14] and peripheral blood [15–19] in uRPL patients, which have been associated with unsuccessful pregnancy outcome in some small studies[11,15,20] but not in others.[12]

Previous studies have observed a counterbalanced lymphocyte distribution in uRPL patients treated with PRS; thus, the treatment re-established the immune system presumed to be beneficial for embryo implantation.[21–24] The same effects have been observed in uRPL patients treated with IVIg.[22,25–27] For that reason, treatment before pregnancy with PRS and IVIg (PRS&IVIg) is

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expected to contribute to a favourable immunological environment in peripheral blood and the uterus which is expected to increase the chance of an uncomplicated pregnancy.

STUDY OBJECTIVES

Primary objective

- To investigate whether treatment with PRS&IVIg before and in early pregnancy improves the reproductive outcome in RPL patients undergoing ART treatment.

Secondary objective

- To evaluate if PRS&IVIg treatment is associated with decreased incidence of pregnancy complications, negative pregnancy outcome, and negative perinatal outcomes.

Tertiary explorative objective

- To investigate how PRS&IVIg affect the leukocyte subset distribution (LSD) in peripheral blood and whether a specific LSD can predict which uRPL patients benefit from PRS&IVIg

METHODS

Study design

In a randomized 1:1 double-blinded, placebo-controlled trial of uRPL patients undergoing ART, the effect of PRS&IVIg treatment is compared to treatment with placebo (placebo tablets and human albumin). The study also possesses an explorative capacity by examining the association between treatment, reproductive outcome, and LSD.

Population

The population consist of RPL patients with ≥2 consecutive PLs after ART referred to The Center for Recurrent Pregnancy Loss of Western Denmark (RPL Center) at Aalborg University Hospital (AaUH). In Danish public clinics, the costs for a couple's first child are covered by tax-financed public health insurance. Also, all Danish citizens with RPL can be referred to The RPL Center without costs.

The public and private fertility clinics in Denmark have received a detailed description of the RCT to inform their personnel and ask for referral of potentially eligible patients with ≥2 PLs to the RPL Center. All referred patients undergoing ART are screened for eligibility.

Inclusion criteria:

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1. Women with ≥2 consecutive PLs (biochemical pregnancies or clinical miscarriages) before completed GW 10 after ART with the same partner or with an egg/semen donor*

Exclusion criteria:

1. BMI ≥35
2. Age ≥41 years
3. Significant uterine malformation(s)
4. Known parental balanced chromosomal translocations
5. ≥2 previous pregnancies with fetuses with known abnormal karyotype
6. Patients with IgA deficiency, IgA-autoantibodies or hyperprolinaemia
7. Anti-Müllerian hormone (AMH) <4 pmol/l *
8. Treatment with medication interacting with PRS:
    - CYP3A4-inhibitors (e.g., erythromycin, itraconazole, ritonavir, and lopinavir), CYP3A4-inductors (e.g., phenobarbital, phenytoin, and rifampicin), loop diuretics, thiazides, amphotericin B, β2-agonists, antidiabetics (metformin is acceptable), interleukin-2, somatropins, anticholinergics, and regular treatment with NSAIDs.
9. Patients with moderate/severe hypertension, diabetes mellitus, heart insufficiency, severe mental disorders, Cushing syndrome, myasthenia gravis, ocular herpes simplex, pheochromocytoma, systemic sclerosis, and moderate/severe renal dysfunction.
10. Patients with a clinical or biochemical profile indicating need for heparin or levothyroxine treatment during pregnancy
11. Previous treatment with IVIg
12. Allergy to PRS and/or IVIg

* for women using egg donation two exceptions occur: First, if the participant plans to use egg donation in the study cycle, the previous two PLs must also have happened with the use of egg donation; however, it is not required to use the same egg donor in all three embryo transfers (ET). Second, a low AMH value is not an exclusion criterion for patients using egg donation.

Allocation, randomization, and blinding

Investigators at the RPL Center screen for inclusion and exclusion criteria, deliver the written and oral study information, and obtain a signed written consent form (see supplemental material 1). The recruited participant is assigned an ID-number, which is given in continuous order, and she receives

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a tablet container with treatment corresponding to the randomisation list and labelled with the matching ID-number.

Also, a written information folder is given after randomisation with a summary of known adverse reactions (AR) for study drugs, boxes in which the participant can describe her ARs and adverse events (AE), a description on how to respond to such ARs/AEs, and a telephone number available 24/7. It also contains a check-list with ticking boxes to tick off each day the participant takes her tablet(s). It describes when to change the dose and serves as a reminder improving treatment compliance. The folder is examined at every appointment to aid high compliance and safety.

To ensure blinding of participants, investigators, outcome assessors, and care providers, a computer-generated simple randomisation list is created by the Hospital Pharmacy North Denmark Region (HPNDR) and kept confidential. The list contains 74 ID-numbers with an allocation ratio 1:1. It is made before the first patient is included and located at the HPNDR. Only the personnel at the Department of Clinical Immunology (DCI) preparing intravenous (IV) infusion medicine have a copy. No personnel with access to the randomisation list will be in contact with participants nor be involved in data collection. The allocation sequence is concealed as ID-numbers are assigned to participants in consecutive order by the blinded chief investigator.

Randomisation is in blocks of different sizes arranged to ensure a 1:1 ratio when conducting the interim analysis. For every participant who does not meet the criteria for per-protocol (PP) analysis, an extra patient will be included. The additional ID-numbers on the randomisation list will be generated by the HPNDR in correspondence with the number of participants missing in each treatment group.

The investigator will authorize the pharmacist to break the code in order to reveal the participant's allocation group in case of serious illness and the code will only be broken if it is of substantial importance to a participant´s health or functional capacity and only the chief investigator and sponsor can make this decision.

The randomisation code will be disclosed when the primary outcome is collected on 74 participants with no protocol deviations and a pre-analysis blind review on all outcomes described in the SAP has been performed which is expected to happen in November 2023.

Study medicine preparation

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PRS and the placebo comparator are in tablet form with identical appearance and prepacked for each participant by the HPNDR in identical containers labelled with ID-numbers corresponding to the randomisation list. The chief investigator informs the DCI personnel about when and to whom they should prepare IV infusion medicine for. Within 4hours before administration, the relevant volume and content is transferred into a transparent, yellow (UV-protected) ethylene vinyl acetate (EVA) bag labelled with volume and ID-number. Human albumin is chosen as IVIg comparator due to the similar physical form and appearance e.g., both drugs form foam. The yellow EVA bags and IV sets make visual distinction even more difficult.

Intervention

Within the first three days of the participant's menstrual cycle, she starts administration of one tablet daily (5 mg of prednisolone or placebo) until ET on which day the dose increases to two tablets daily. At the time of the ET (from five working days before to two working days after), the participant receives the first IV infusion of study medicine and have the first study-specific blood sample taken. Participants with a pre-pregnancy body weight (BW) $\leq$ 70 kg will receive 250 ml, participants with BW 70-85 kg will receive 300 ml, and participants with a BW $\geq$85 kg will receive 350 ml in each infusion. Around 14 days after ET, plasma-hCG is measured twice with 24-48hrs interval. If plasma-hCG increases sufficiently (here defined as $\geq$30 % per 24hrs), the treatment continues (see Figure 1).

IV infusions containing the same volume is repeated in gestational week (GW) 5, 6, and 7, respectively; however, no IV infusion is administered after a negative pregnancy test or a PL. The tablet treatment discontinues gradually over four days when the participant has a negative pregnancy test, a PL, or when she reaches GW 8+0, whichever comes first.

Before each infusion, a plasma-hCG measurement or ultrasound scan is performed to confirm that the pregnancy continues as expected. Initial infusion rate of 0.3 ml/kg BW/hr and if well-tolerated, the infusion rate may gradually increase. Blood pressure and pulse are monitored before, during, and after the treatment. In case of anaphylaxis, the treatment is discontinued, adrenaline (0.1 %) is administered, and the participant is excluded.

On the day of the first IV infusion and again about four weeks later (corresponding to GW 6, i.e., the day of her third IV infusion), a blood sample is taken.

After the last infusion, routine monitoring at the RPL Center or at her local hospital is offered. A NT scan is offered in GW 11-13. The participant receives an e-mail with a unique link to a

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questionnaire two weeks after the NT scan and two weeks after her due date. After GW 8, the participant is offered the same treatment and follow-up as any other Danish pregnant women except from two additional fetal growth scans in the third trimester due to her history of RPL.

Active treatment

Prednisolone: 5 mg of prednisolone per tablet. One tablets daily rising to two tablets daily after ET.

Intravenous immunoglobulin (IVIg): Privigen 100 mg/ml (10 %) (CSL Behring). The three optional volumes to be administered depend on BW as previously described; thus, the dose corresponds to approximately 0.4 g IVIg per kg BW.

Placebo

Prednisolone comparator: Placebo tablets contain 85 mg lactose monohydrate, 86 mg potato starch, 8.1 mg talc, 3 mg gelatine, and 0.9 mg magnesium stearate. One tablets daily rising to two tablets daily after ET.

IVIg comparator: Human albumin 50 mg/ml (5 %) (CSL Behring).

Other medication:

The study medicine does not affect the participant's ART treatment decided solely by her fertility clinic; however, no immunotherapy is accepted. Thus, hormone-replacement-therapy and natural cycles with fresh or frozen embryos and blastocyst transfers are accepted.

Data collection and management

Study-relevant data is recorded in an electronic case report form (eCRF) managed in REDCap; an electronic data capture tool hosted at AaUH. The information is obtained from electronic medical records, the information folder, and directly from the participant. Demographic and medical baseline data is collected before randomisation during the diagnostic workup. After randomisation, data related to the index ART cycle is collected including information about fertilisation method, use of fresh or frozen embryos, transfer of cleavage stage embryos or blastocyst and possible performance of PGT-A. When the treatment is terminated, remaining tablets and the information folder are returned which enables the investigators to assess compliance and drug safety. The remaining tablets from each participant are counted by the unblinded DCI personnel. If a

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participant experiences a missed abortion, the investigators will apply for a uterine evacuation with chromosomal examination of fetal/placental tissue.
The two questionnaires collect data after NT scan and birth, respectively, about pregnancy complications, AEs/ARs, the participant's and her fetus/baby's wellbeing, and perinatal outcome. The second questionnaire also requests a copy of the hospital´s delivery record.

The chief investigator is the primary data manager responsible for the validity of data in the eCRF. An audit trail ensures data traceability and validity to maintain transparency and accountability. During the study period, continuous review and evaluation of the audit trail and the data accuracy are performed by the chief investigator and the GCP board. The eCRF have range checks, description of codes, and valid options of choices where applicable. After one week, it automatically sends a reminder to participants who have not replied to the questionnaires and notifies the investigators. Personal identifier variables are marked which allows anonymous data export. The same data is collected in participants with and without major protocol deviations, and the reason for any protocol deviation is described. Data is protected according to the GDPR (the General Data Protection Regulation), the Danish Data Protection Act, and the Danish Health Act. Specific authorized persons from The North Denmark Region Committee on Health Research Ethics or the Danish Medicines Agency have unrestricted access throughout the trial to monitor, audit, and inspect the source data regarding the study participants.

Blood sample analysis

The study-specific blood sample analyses are performed by the DCI, and the results will be shared with the investigators after the randomisation code is disclosed. In each fresh EDTA blood sample, the total leucocyte cell count, and the fractions of T helper cells (Th) 1, Th2, Th17, cytotoxic T cells (Tc), regulatory T cells (Tregs), B cells, and natural killer (NK) cells ($CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16$) are measured with flow cytometry. A TruCulture analysis is performed on both blood samples from only 25 participants as this RCT serve as a pilot study exploring the relevance of measuring stimuli-specific cytokine production in RPL patients treated with immunomodulatory medicine. The appendices contain a detailed description of the methods of the flow cytometric and TruCulture analyses used (see Appendix A and B in supplemental material 2).

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Remaining biological material is saved in a research biobank for later analysis of small extracellular vesicles (sEV) with different phenotypes and for other future studies on immunological risk factors to RPL. The biological material is stored at -80^{0}C and consists of three 6 ml collection tubes containing serum, EDTA plasma, and citrate plasma, respectively.

Endpoints

The primary endpoint is the percentage of participants with ≥1 normal, viable fetus at NT scan in GW 11-13 among all participants in each treatment group, and subsequently among participants pregnant after ET. Also, the percentage participants with ≥1 normal, viable fetus at NT scan among all participants except those pregnant with a fetus having a confirmed chromosomal abnormality will be reported. Furthermore, the relative risk, absolute risk reduction, and an adjusted risk ratio for this primary outcome will describe the primary outcome. The primary endpoint is also measured in subgroups based on diagnosis of primary and secondary RPL, respectively, i.e., no prior birth or a history of ≥1 previous birth after 22 GW. The secondary endpoints are the incidence of AE/ARs and pregnancy complications (miscarriage rate, negative pregnancy rate, preeclampsia, gestational hypertension, gestational diabetes mellitus, acute instrumental delivery), and the perinatal outcomes (congenital deformations, prematurity, small for gestational age, low birth weight, admission to neonatal care unit, sex, birth weight and gestational age), while the tertiary endpoints are the distribution of leukocyte subsets. A thoroughly description can be found in the statistical analysis plan (SAP) (see supplemental material 3).

Safety considerations, safety monitoring, and reporting

An AE is defined as any untoward medical occurrence (including any unfavourable or unintended sign, symptom, or disease) in a participant during the study (from the day of randomization until childbirth or 6 months after last IV infusion) and which does not necessarily have a causal relationship with the treatment in contrast to an AR. A serious AE (SAE) is defined as an AE that results in death, is life-threatening, requires hospitalization or prolongation of existing hospitalisation, results in persistent or significant disability or incapacity, or is a congenital anomaly or birth defect. Some medical events may jeopardize the subject or may require an intervention to prevent one of the above characteristics/consequences. Such events will also be considered as serious ARs (SARs). A suspected unexpected serious adverse reaction (SUSAR) is defined as an adverse reaction, the nature or severity of which is not consistent with the applicable product

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information (EUR-Lex, CT-3). For each case, it is considered whether it is serious, unexpected, and possibly, probably, or definitely related to an intervention based on the Summery of Product Characteristics (SmPC) and timing.

Participants is encouraged to describe all AEs in their information folder, and they are followed-up on each appointment at the RPL Center. The questionnaires follow-up on AEs in the participants and their foetuses/children after treatment cessation. If a new AE is described in the questionnaire, an investigator contacts the participant to assess the AE's character and whether follow-up consultations or therapy is required. Non-pregnant participants are informed to contact the investigators in case of AEs after treatment cessation. All AEs are reported from the investigator to the sponsor immediately (<24hours) and described in detail in a follow-up report to the sponsor. The sponsor reports SUSARs to The National Board of Health and The North Denmark Region Committee on Health Research Ethics during the entire study period within seven days. In addition, an annual report on SAE will be sent to these authorities. Thus, the study follows the EudraLex Clinical Trials Guidelines Chapter II - safety reporting. Some AEs linked to pregnancy, ART, or RPL are expected and includes amenorrhea, vaginal haemorrhage, weight gain, backpain, pelvic pain, drowsiness, nycturia, increased hunger, nausea, and vomiting. These AEs are not recorded unless they are serious; likewise, hospitalisation due to symptoms of a threatened miscarriage, induced abortion, or surgical abortion (evacuation), is not assessed as a SAR/SAE.

Sample size

Based on previous studies of similar patients [4,5] reporting a LBR of 36.5% and 34.2% after IVIg and prednisolone treatment, respectively, and an expectation of similar treatment effects in women with 2 and ≥3 PLs, a minimum LBR of 40 % in the active treatment group is anticipated. In the RCT by Stephenson and Fluker [28], a LBR of 12% in IVF/ICSI patients with a mean of 3.2 previous failed embryo transfers receiving placebo treatment was reported. The patients admitted to the RPL Clinic within the last 3 years, who would have been eligible for the study, have had a mean of 6.4 previous failed embryo transfers, and we therefore think that a reasonable estimate of the LBR in those of our patients allocated to placebo will be similar to the observed 12%. Based on these expectations, a type I error of 0.05, and a type II error of 0.20, the study will need a sample of 74 participants. Based on experience from previous trials, <2 % dropouts, and <10 % protocol deviations (see Figure 2) are expected.

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Statistical analysis plan

The statistical analyses will be carried out by the investigators in collaboration with professional statisticians. A $p \leq 0.05$ is considered statistically significant. All randomized participants will be included in the intention-to-treat (ITT) population, while the PP population will only include participants with no major protocol deviations listed on Figure 2.

When the primary outcome is collected in 38 participants, who fulfil criteria for the PP analysis, an interim analysis will be conducted by an independent group of statisticians who get access to data on the primary outcome; SARs and SUSARs; and a simple-coded (A/B) randomisation list. The statisticians will not get data on ARs/AEs, since it will increase the risk of breaking the code. The interim analysis will provide information of differences in efficacy and safety between groups. If a difference of ≥3 SUSARs or SARs between groups is observed, the study will discontinue. The study will not terminate in case of futility, and it will not implement any modifications to trial procedures. Therefore, no correction of the reported P value will be performed in the final analysis. The sponsor has the ultimate authority to stop the study.

After completion of the study inclusion and before breaking the blind, a pre-analysis blind review on data will be caried out by the statisticians to perform data validation, detect outliers, assess distribution of variables, and measure the outcomes. Afterwards, the unblinded data analysis will proceed.

The analyses on primary outcome will be performed as an ITT and a PP analysis, while the secondary outcomes are performed on the ITT population, and the tertiary outcomes are performed on the PP population. A $\chi 2$ test will compare the primary, secondary, and tertiary categorical outcome variables between treatment groups. A Fisher's Exact Test will be used when less than five participants are expected in one group. An adjusted relative risk for a normal viable pregnancy at NT scan between the two treatment groups is estimated using Poisson regression and is adjusted for relevant confounders including BMI, smoking, and age.

For the analysis of the tertiary outcomes, including the concentration and fraction (continuous) of leukocyte subsets, an unpaired two-sample Mann-Whitney U test or t-test will be used for between group comparisons depending on data distribution while paired Wilcoxon signed-rank test or paired t-test will be used for within group comparisons. When comparing more than two groups, i.e.,

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subgroups according to reproductive outcome after ET or percentile subgroups, an ANOVA or Kruskal-Wallis test will be used.

The analyses will compare

1. The outcomes from blood sample 1 with blood sample 2 within each two treatment groups, respectively.
2. The outcomes from each two blood samples, respectively, between treatment groups
3. The outcome changes from blood sample 1 to 2 between treatment groups and between subgroups based on pre-treatment cell fraction (3 percentile subgroups).

Also, receiver operation characteristic curve analyses will determine the optimal cut-off values for the Th1/Th2 ratio, Th17/Treg ratio, and the NK cell fraction in blood sample 1 to identify participants with a positive reproductive outcome in the active treatment group.

Further information on the statistical analysis is described in detail in the SAP in supplemental material.

Data quality and monitoring

The study protocol, trial master file (TMF), and eCRF have been reviewed by the regional GCP-board. The GCP-board comprises experts in clinical trials who are not involved in other aspects of the study and have no competing interests. They make systematic inspections including examinations of the TMF, study procedures, safety, and data collection, accuracy, completeness, and validity to ensure it is performed in accordance with the protocol and comply with the Danish legislation and GCP guidelines. The trial will also be open for inspection from the Danish Health Authority.

Patient and public involvement:

The active treatment was given to ten RPL patients according to a preliminary protocol proposal as well as presented to stakeholders in the field of ART. In a post-treatment semi-structured interview with the patients, proposals for optimizations according to treatment plan and compliance as well as motivational factors and barriers were performed. Following this, stakeholders submitted their proposals for optimisation of the feasibility of the treatment protocol, which is highly dependent on cooperation with fertility clinics and their treatments. These proposals were all used to revise the protocol.

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DISCUSSION

Ethics and dissemination

The study protocol, participant material, and informed consent (see supplemental material) are approved by The North Denmark Region Committee on Health Research Ethics(N-20200066), The National Board of Health (EudraCT number: 2020-000256-35), and the Data Protection Agency (2020-156). It was registered at ClinicalTrials.gov before the study was initiated (NCT04701034). In case of protocol modifications, a formal amendment will be approved by these authorities before implementation and when it is approved, it will be sent by e-mail to all collaborating parties in the study. The study adheres to the Declaration of Helsinki II, local regulations, and GCP guidelines.

Participation is voluntary, and the written informed consent can be withdrawn at any time without consequences for the subsequent treatment at the RPL Center. Future studies on the research biobank material not already described will require a new approval from ethical committee and if required, an additional consent provision from the participants. The study is covered by the national patient health insurance. Study drugs are given to participants without expenses. None of the study investigators will get any economic or professional advantages before, during, or after the study. The findings will be submitted for publication in internationally acknowledged peer-reviewed journals in obstetrics and gynaecology or general medicine and will be described in at least two scientific papers: one manuscript focusing on the reproductive outcome and minimum one manuscript focusing on changes of immune biomarkers according to treatment and the reproductive outcome. Data will also be presented at national and international congresses. Future research based on the biobank will subsequently be performed and shared in scientific papers. Authorship eligibility will be based on the International Committee of Medical Journal Editors (ICMJE) recommendations 2018. An anonymous dataset may be shared upon reasonable request after agreement and approval by the sponsor and chief investigator. Study results and the allocation group of each participant will be shared with the participants who requested this information after the complete data analysis. We will publish both positive, negative, and inconclusive results. No publication restrictions are imposed.

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Competing interest statement

USK received consulting fees from Merck for Update of patient information leaflet and from IBSA Nordic for Clinical instruction and support for attending meeting/congress from Merck.
All other authors have no conflicts of interest to declare.

Author contributorship statement

OBC is sponsor of the study. CNP and OBC made the study design, and planned, conceived, initiated, and conducted the study and the clinical implementation. They are both grant holders. CNP is the chief investigator and wrote the protocol, made the statistical analysis plan, and made the eCRF and arranged all data collection, data management, data reporting, data analysis. OBC and USK made critical protocol revisions. KRN, LE, MMJ, and RS provided detailed information on immunological analyses on blood samples and planned and performed all laboratory analyses on participants' blood samples. USK provided information on ART and supported CNP and OBC in patient and stakeholder involvement. CNP decided to submit protocol for publication. CNP, USK, and OBC will interpret data after final data analysis. All authors contributed to refinement of the study protocol and approved the final manuscript.

Abbreviations:

AaUH: Aalborg University Hospital
AE: adverse event
AMH: Anti-Müllerian hormone
AR: adverse reaction
ART: artificial reproductive technology
BMI: body mass index
BW: body weight
DCI: department of clinical immunology
eCRF: electronic case report form

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ET: embryo transfer

EVA: ethylene vinyl acetate

FET: frozen embryo/blastocyst transfer

GCP: good clinical practice

GDPR: General Data Protection Regulation

GW: gestational week

ICMJE: International Committee of Medical Journal Editors

ICSI: intracytoplasmatic sperm injection

ITT: intention-to-treat

PP: per-protocol

HPNDR: The Hospital Pharmacy North Denmark Region

IV: intravenous

IVF: in intro fertilisation

IVIg: intravenous Immunoglobulin

LBR: live birth rate

LSD: lymphocyte subset distribution

NK: natural killer

NT: nuchal translucency

PL: pregnancy loss

PRS&IVIg: prednisolone and intravenous Immunoglobulin

RCT: randomised controlled trial

RPL: recurrent pregnancy loss

SAR: serious adverse reaction

SmPC: Summery of Product Characteristics

SUSAR: suspected unexpected serious adverse reaction

Tc: T cytotoxic

Th: T helper

TMF. Trial master file

Treg: T regulatory

uRPL: unexplained recurrent pregnancy loss

Appendices and supplemental material:

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- Appendix A: Blood sample analysis in flow cytometry
- Appendix B: Blood sample analysis in TruCulture
- SAP
- Information folder
- Consent form

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Figures:

Figure 1: A flow-diagram on the treatment for each participant.

Figure 2: Diagram over the criteria for major protocol deviations.

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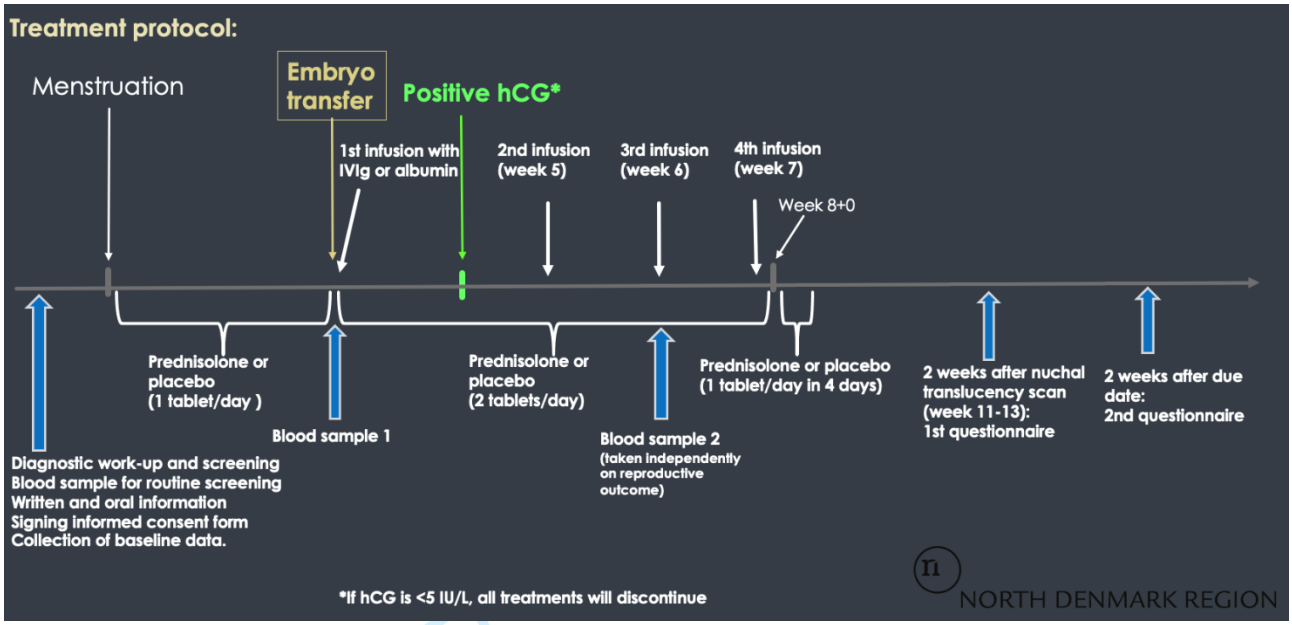

Figure 1: A flow-diagram on the treatment for each participant.

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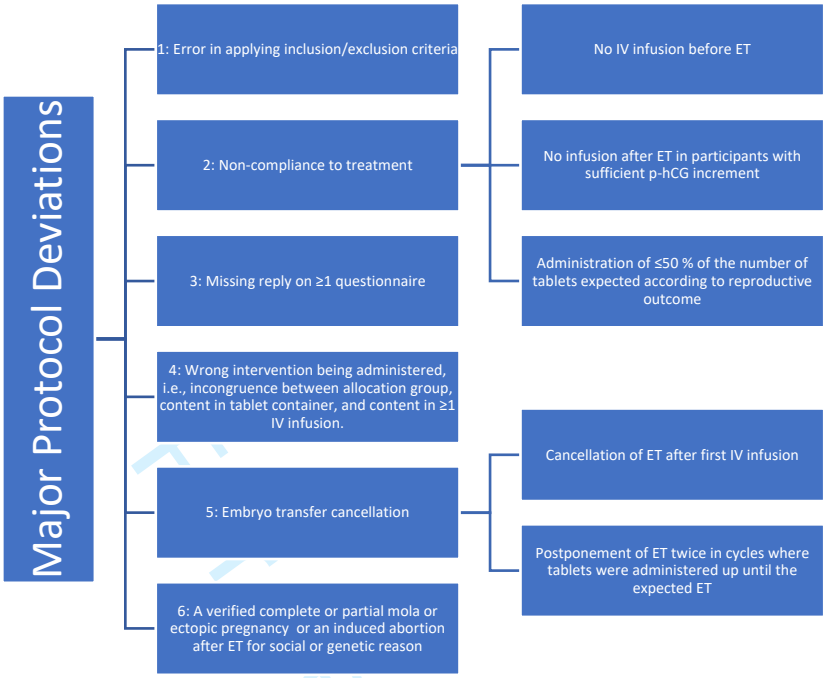

Figure 2: Diagram over the criteria for major protocol deviations.

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(S3)

Informeret samtykke til deltagelse i et sundhedsvidenskabeligt forskningsprojekt.

Forskningsprojektets titel: Et randomiseret placebo-kontrolleret studie af intravenøs immunglobulin og prednisolon i behandlingen af gentagne graviditetstab efter IVF/ICSI

Erklæring fra forsøgspersonen:

Jeg har fået skriftlig og mundtlig information og jeg ved nok om formål, metode, fordele og ulemper til at sige ja til at deltage.

Jeg ved, at det er frivilligt at deltage, og at jeg altid kan trække mit samtykke tilbage uden at miste mine nuværende eller fremtidige rettigheder til behandling.

Jeg giver samtykke til at deltage i forskningsprojektet og til, at mit biologiske materiale udtages med henblik på opbevaring i en forskningsbiobank. Jeg giver samtykke til at måtte blive kontaktet i fremtiden med henblik på indhentning af samtykke til fremtidig forskning i biobanken. Jeg har fået en kopi af dette samtykkeark samt
en kopi af den skriftlige information om projektet til eget brug.

Forsøgspersonens navn: _____

Dato: _____ Underskrift: _____

Ønsker du at blive informeret om forskningsprojektets resultat samt eventuelle konsekvenser for dig?:

Ja _____ (sæt x) Nej _____ (sæt x)

Erklæring fra den, der afgiver information:

Jeg erklærer, at forsøgspersonen har modtaget mundtlig og skriftlig information om forsøget.

Efter min overbevisning er der givet tilstrækkelig information til, at der kan træffes beslutning om deltagelse i forsøget.

Navnet på den, der afgiver information:

Dato: _____ Underskrift: _____

Protokol kode: CNPOBC2020
Version nummer: 8
Dato: 01.06.2021
EudraCT nummer: 2020-000256-35

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Analysis method for the analysis of the leukocyte subset distribution in peripheral blood

Appendix A

The participant's blood sample 1 and 2, which are collected before and after intravenous treatment with albumin or immunoglobulin, respectively, are analysed in the exact same manner.
First, a total leucocyte count (TLC) will be measured using the Sysmex XN-1000 blood analyser. The Sysmex provides absolute count for white blood cells (WBC), lymphocytes, monocytes, and neutrophil granulocytes.

The EDTA blood sample collected from the participant will be prepared for flow cytometric analysis using two different protocols. These flow cytometer analyses will be performed throughout the study and the analyses are initiated within 1 hour from blood samples were collected. The gating will be checked in the end of the study on all analyses.

The first flow cytometric protocol will provide the concentration of T helper cells (Th), cytotoxic T cells (Tc), B cells, and natural killer (NK) cells, and the fraction of each subgroup of the total lymphocyte population using two different antibody panels. These two panels contain 10 µL of fluorochrome-conjugated-antibodies against CD3, CD4, CD8, and CD45 (BD Bioscience) (tube 1) or CD3, CD16, CD56, and CD45 (BD Bioscience) and CD19 (BD Bioscience) (tube 2) which will be added to FACS tube 1 and 2, respectively, followed by 50 µL EDTA blood from the participant. Then the tubes will incubate in the dark in room temperature 15 minutes before a lysing solution (BD Biosciences) will be added to the tubes removing the red blood cells (RBC). The samples are run through the Novocyte 3000 flow cytometer (Acea Biosciences, Inc., San Diego, USA).
The collected data includes all WBC. Thus, to analyse only the lymphocytes, the $CD45^{+}SSC^{low}$ cells will be gated. In the tube 1, the $CD3^{+}$ T cells will further be divided into $CD3^{+}CD8^{+}$ Tc-cells and $CD3^{+}CD4^{+}$ Th-cells. In the tube 2, $CD3^{-}CD56^{+}CD16^{+}$ NK cells and $CD3^{-}CD19^{+}$ B cells are gated.

The second protocol will provide the percentage of Th1-, Th2-, Th17-cells, T regulatory (Treg) cells, and $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{-}$ NK cells of the total peripheral blood mononuclear cell (PBMC) population using two different antibody panels.
The first panel includes fluorochrome-conjugated-antibodies against CD3, CD4, CD16, CD25, CD56, and CD127 (all from BD Bioscience). These markers can differentiate $CD56^{dim}CD16^{+}$ NK cells from $CD56^{bright}CD16^{-}$ NK cells. This antibody panel have CD16 and CD56 conjugated to different fluorochromes to be able to distinguish the NK subgroups, while the panel used in tube 2 in the first flow cytometric protocol contained anti-CD16 and anti-CD56 antibodies conjugated to the same fluorochrome to provide the total NK cell count. Anti-CD25 and anti-CD127 antibodies will be used to gate Treg cells ($CD25^{+}CD127^{low}$).
The second panel will differentiate Th1, Th2 and Th17 cells. These groups will be distinguished using the CCR4 (Biolegend), CCR6(BD Bioscience), CCR10(BD Bioscience) and CXCR3(BD Bioscience) chemokine receptors in combination with the anti-CD3 and anti-CD4 antibodies. This analysis will provide the percentage of each Th-cell subgroup.
All antibody concentrations will be optimized for this second protocol before the antibodies will be included in this project. The optimization is lot number specific, which means different dilution factors are

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used throughout the project. Of the optimized antibody concentration, we will use 5µL of all antibodies except CD25, of which we will use 20 µL.

300 µL EDTA blood will be added to each FACS tube labelled full panel 1, full panel 2 or necessary controls for each panel. The blood will be lysed and washed to remove RBCs. Then each antibody panel will be added to different tubes and the samples will incubate in the dark in room temperature 15 minutes followed by two washes. The samples will be resuspended in 300µL wash buffer containing phosphate buffered saline (PBS) (VWR Chemicals) + 1% FCS (Gibco) before running the samples on the flow cytometer within 6 hours after the blood sample was taken.

The remaining of the blood sample will be frozen and saved for future analysis. This research biobank for future research will consist of three 6 ml collection tubes containing serum, EDTA plasma, and citrate plasma, respectively. After immediate centrifugation, the remaining serum and plasma will be stored in tubes at -80°C.

Analysis methods for analysing stimulus-specific induced immune responses in peripheral blood ex vivo

Appendix B

TruCulture® is a whole blood assay with directly ex vivo human immune stimulation which permits evaluation of individual and stimulus-specific induced immune responses through measurements of released cytokines as a proxy for immune function in vivo against a range of stimuli. TruCulture tubes (Myriad RBM, Austin, TX, USA) are pre-loaded with cell culture media and immune stimulants. In this trial, the tubes are pre-loaded with one of the following immune stimuli: CD3/CD28, R848, lipopolysaccharide (LPS), polyinosinic-polycytidylic acid (polyIC), and a no-stimulus null control.[1]

Peripheral blood is drawn by vein puncture and collected into a Lithium-Heparin tube. The analysis is initiated in the laboratory within 1 hour after sampling. 1 ml is transferred to each TruCulture® tubes and incubated in a dry heat block for 22 (+/- 10 min) hours at 37 °C. Following the 22 hour long immune stimulation, supernatants are collected by inserting a valve separator to separate cells from the culture supernatant as described previously described.[2]

Cytokines will be quantified in TruCulture® tube supernatants using the MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel II (MilliporeSigma, Burlington, Massachusetts) on the Luminex 200 platform following manufacturer protocols.

Ten cytokines will be measured: IFN-α2, IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-12 (p40), IL-13, IL-17, and Monocyte Chemoattractant Protein-1 (MCP-1). The limit of detection (LoD) will be 6,56 pg/ml for IFN-α2, 0,86 pg/ml for IFN-γ, 5.39 pg/ml for TNF-α, 0,28 pg/ml for IL-2, 0.20 pg/ml for IL-4, 0.14 pg/ml for IL-6, 3.24 pg/ml for IL-12 (p40), 2.58 pg/ml for IL-13, and 3.05 pg/ml for MCP-1.

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Intravenous immunoglobulin and prednisolone to women with unexplained recurrent pregnancy loss after assisted reproductive technology treatment: a randomised, double-blind, placebo-controlled trial

STATISTICAL ANALYSIS PLAN

Trial registration numbers:

Clinicaltrials.gov ID: NCT04701034
EudraCT number: 2020-000256-35
Ethical registration number: N-20200066
Project ID at The North Denmark Region: 2020-156
WHO unique trial Number: U1111-1273-8585

Statistical Analysis Plan Version: 1.0

Statistical Analysis Plan Date: 01.06.2022

Number of participants enrolled: 30

This SAP is based on study protocol version 8.0 dated 01.06.2022.

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The Statistical Analysis Plan (SAP) for "Intravenous immunoglobulin and prednisolone to women with unexplained recurrent pregnancy loss after assisted reproductive technology treatment: a randomised, double-blind, placebo-controlled trial" describes and expands the statistical information presented in the protocol published in BMJ Open.

Protocol Number Code	CNPOBC2020
Development Phase	Phase II
Products	Prednisolone and Intravenous Immunoglobulin Placebo and Human Albumin
Indication studied	Recurrent pregnancy loss
Clinical Trial Initiation Date	February 6, 2021
Clinical Trial Completion Date	Recruiting
Date of the Analysis Plan	01.06.2022
Version Number	1.0
SAP revisions	N/A

This study is conducted according to the Declaration of Helsinki III, local regulations, and Good Clinical Practice (GCP) guidelines and is monitored by the regional GCP board in Northern Denmark. The protocol, the informed consent form, and the participant written information is approved by the Ethics Committee of North Denmark Region, The National Board of Health, and the Data Protection Agency prior to the inclusion of participants. Informed consent from all participants is signed before enrolment.

This SAP will concern analysis of data that will be shared in more than one manuscript.

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1. Introduction

Recurrent pregnancy loss (RPL) is defined as 2 consecutive pregnancy losses[1] and affects around 5 % of women in reproductive age.[2] It can be divided into primary (pRPL) and secondary RPL (sRPL), and sRPL, incontrast to pRPL, define RPL patients who have had a pregnancy beyond 24 weeks of gestation before the consecutive pregnancy losses (PLs). In Denmark, more than 25 % of all RPL cases happens after fertility treatments (in-vitro fertilization (IVF); intracytoplasmatic sperm injection (ICSI) or frozen embryo/blastocyst transfer (FET)), which in the following jointly are called assisted reproductive technologies (ART). The group of patients with RPL is very heterogenic. The underlying cause of RPL is unknown, probably multifactorial as a series of various risk factors is known and can be identified in less than 50% of patients. These include thrombophilia, and a group of endocrine, chromosomal, and anatomic aberrations.[3] In the remaining unexplained RPL (uRPL) cases, immunological aberrations are thought to be at least partly involved in the pathogenesis. This hypothesis is based on studies finding increased frequency of autoantibodies and specific human leukocyte antigen (HLA) alleles associated with other autoimmune diseases as well as unbalanced distribution of lymphocyte subsets, especially natural killer (NK) cells, T regulatory cells, and T-helper (Th) cells, in uRPL patients. Treatment regimens used in autoimmune diseases have therefore been suggested for uRPL patients, including intravenous immunoglobulin (IVIg) and prednisolone. A meta-analysis of RCTs evaluating the efficacy of IVIg treatment for women with RPL found no significant effect on live birth rate; however, the acquired sample size was not obtained. Since subgroup analyses did show a significant effect in sRPL, the authors suggested that further RCTs were required to obtain sufficient evidence.[4] However, a pilot study suggests that a combination of prednisone and IVIg improves the chance of live birth in women with RPL after ART.[5] Some studies suggest that the immunomodulatory treatment is effective in primarily uRPL patients with aberrant distribution of lymphocyte subsets[6–8]; nonetheless, the data is sparse. It is possible, that the lack of a significant effect of IVIg in RPL patients can be explained by a substantial heterogeneity among enrolled participants in such studies obliterating a possible effect that may present in a certain RPL subgroup. At present, we do not have evidence for an effect of the combination of IVIg and prednisolone on reproductive outcome in RPL patients after ART. Simultaneously, no clear characteristic on patients benefitting from immunomodulatory treatment have been identified. If a blood sample analysis of immune cells can identify a characteristic immune profile in patients who are more likely to respond to treatment and benefit from it (i.e., childbirth), it could help

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clinicians restrict treatment to the right patients while searching for other treatments in the remaining patients, which consequently would shorten the patents' harsh fight to get a child.

Research Hypothesis

The null hypothesis is that there is no difference in normal pregnancy rate after embryo transfer assessed at the nuchal translucency (NT) scan between RPL patients in immunomodulatory treatment and RPL patients taking placebo. The alternative hypothesis is that active treatment is a superior to placebo according to the chance of normal pregnancy at NT scan.

Study Objectives

The primary objective of this study is to determine the effectiveness of prednisolone and IVIg (P&IVIg) compared to placebo for increasing normal pregnancy rate at the NT scan.

The secondary objective is to evaluate if P&IVIg is associated with increased rate of pregnancy complications, negative pregnancy outcome, and perinatal outcomes.

The tertiary objectives are to explore how P&IVIg treatment affects the lymphocyte subset distribution (LSD) in peripheral blood, and whether the LSD can predict which uRPL patients that benefit from P&IVIg.

2. Study Methods

2.1 Trial design

The present study is a randomized 1:1, double-blinded, placebo-controlled, single-center, phase II therapeutic study conducted at Centre for Recurrent Pregnancy loss of Western Denmark, at which the physicians are specialists in treating patients with RPL. Patents from all over Denmark can be referred without costs. Participants are randomised to treatment with P&IVIg or placebo.

2.2 Randomization and Blinding

Participants are randomized in a 1:1 ratio. The participants, investigators, outcome assessor, and care provider are blinded as the Hospital Pharmacy North Denmark Region (THPNDR) perform the randomisation. Randomization will be in blocks of different sizes ensuring an even distribution after half of the number of (n=38) participants have been enrolled at what time a pre-analysis will be conducted. The randomization code will be disclosed when the last participant has completed her treatment and passed NT scan (gestational week (GW) 11-13).

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2.3 Intervention:

The timing for different study interventions including participant enrolment, blood sample collection, and treatment administration is depicted on the flow diagram on Figure 1.

In the menstrual cycle in which the participant expects an embryo transfer (ET) to be carried out, one tablet per day is administered from first day of menstrual bleeding until ET; then, two tablets per day is administered from the day of the ET and until gestational week 8+0, and then gradual discontinuation with one tablet per day from week 8+0 to 8+4. If the participant does not become pregnant after ET or has a pregnancy loss (PL) before week 8+0, gradual discontinuation is initiated right after the negative test or confirmed PL diagnosis.
Intravenous (IV) infusion is administered at the time of the ET (from five working days before to two working days after), and if she has a positive pregnancy test, the IV infusion is repeated in gestational week 5, 6, and 7. Participants with a pre-pregnancy body weight (BW) ≤70 kg will receive 250 ml, participants with BW of 70-85 kg will receive 300 ml, and participants with a BW ≥85 kg will receive 350 ml in each IV infusion.

Active treatment

Prednisolone: 5 mg of prednisolone per tablet. One tablet daily before ET and two tablets daily after ET until GW 8+0.
Intravenous immunoglobulin: Privigen 100 mg/ml (10 %) (CSL Behring) with a dose of approximately 0.4g/kg.

Placebo

Oral placebo: tablets contain 85 mg lactose monohydrate, 86 mg potato starch, 8.1 mg talc, 3 mg gelatine, and 0.9 mg magnesium stearate.
Human albumin: 50 mg/ml (5 %) (CSL Behring) liquid solution for IV infusion.

2.4 Sample size

Based on results from previous studies and a hypothesis that the treatment effect is the same in women with 2 and ≥ 3 consecutive PLs, we expect a minimum live birth rate of 40 % in the P&IVIg group and of 12 % in placebo group.[5,9,10] Based on these expectations, a type I error of 0.05, and type II error of 0.20, the study needs a sample of 74 patients (37 per group) to test if the treatment is effective in uRPL patients after ART.

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A dropout rate <2 % and a rate of protocol deviation <10 % (i.e., no ET) is expected. For each participant excluded from the per-protocol (PP) analysis, an extra participant will be included in order to perform a PP analysis of 74 participants.

Based on the number of new patients with ≥2 PLs after ART admitted to The RPL Centre, an inclusion period of approximately 2 years is expected.

2.5 Data collection

Baseline information will be collected before entry to the study, i.e., during the first appointment at the RPL clinic when the patient is also screened for eligibility.

Information about the primary and secondary outcomes is collected in an online questionnaire sent 13 weeks after ET i.e., after the NT scan (GW 11-13) and an online questionnaire sent after due date. If the participant does not become pregnant after ET or miscarries during the treatment, the outcome will be recorded at the time of her next follow-up, and no questionnaire will be sent. The primary outcome (live fetus at NT scan) will be recorded in the first questionnaire. If the participant does not respond on the questionnaire(s), a reminder will be sent to her, and the final attempt is made by calling the participants.

In all randomized participants, a blood sample will be collected just before first IV infusion and again 3-4 weeks later (i.e., right before third infusion is administered if the participant is pregnant. If non-pregnant, the second blood sample will be collected at the same time as it she became pregnant). Noteworthy; non-pregnant participants have only received one albumin/immunoglobulin IV infusion and have discontinued prednisolone/placebo tablet administration at the time of the second blood sample in contrast to pregnant participants who have received two IV infusions and still administer tablets).

On the day the participant is enrolled, she receives a folder containing a check-list with ticking boxes for reporting daily tablet intake and a diary for reporting adverse events/adverse reactions (AEs/ARs). The folder includes a list of known drug-related ARs according to the Summary of Product Characteristics (SmPC) for each drug, and also an explanation about which information is important to describe in detail if an AE/AR occurs. Participants are encouraged to use this folder in between follow-ups. The folder and remaining tablets should be returned to the RPL Clinic shortly after treatment cessation in order to assess treatment compliance (see section 5.6).

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At each visit, participants are also asked with open-ended questions about AE/ARs by their physician and nurse during and after IV infusion treatment to assess and journalize all AE/ARs and determine whether they are s probably, possibly, or definitively drug-related or not.

For each case, it will be considered whether it is serious, unexpected, and possibly, probably, or definitely related to an intervention based on the Summery of Product Characteristics (SmPC) for each drug and the timing. The definitions of these terms are described in the published protocol and in accordance with the definition by EUR-Lex, CT-3.

The first and second online questionnaire are sent right after her NT scan and right after the due date, respectively, and will collect information on primary and secondary outcomes including the participant´s labour, pregnancy complications, AE/ARs occurring after treatment cessation, medical interventions during pregnancy, and the child's perinatal data. She can also attach medical records on the childbirth in the second questionnaire. Non-pregnant participants are informed to contact the investigators in case of AEs/ARs up to 6 months after treatment cessation. In addition, they are offered to continue as a patient at the RPL Clinic during which follow-up on AE/ARs will be done for 6 months.

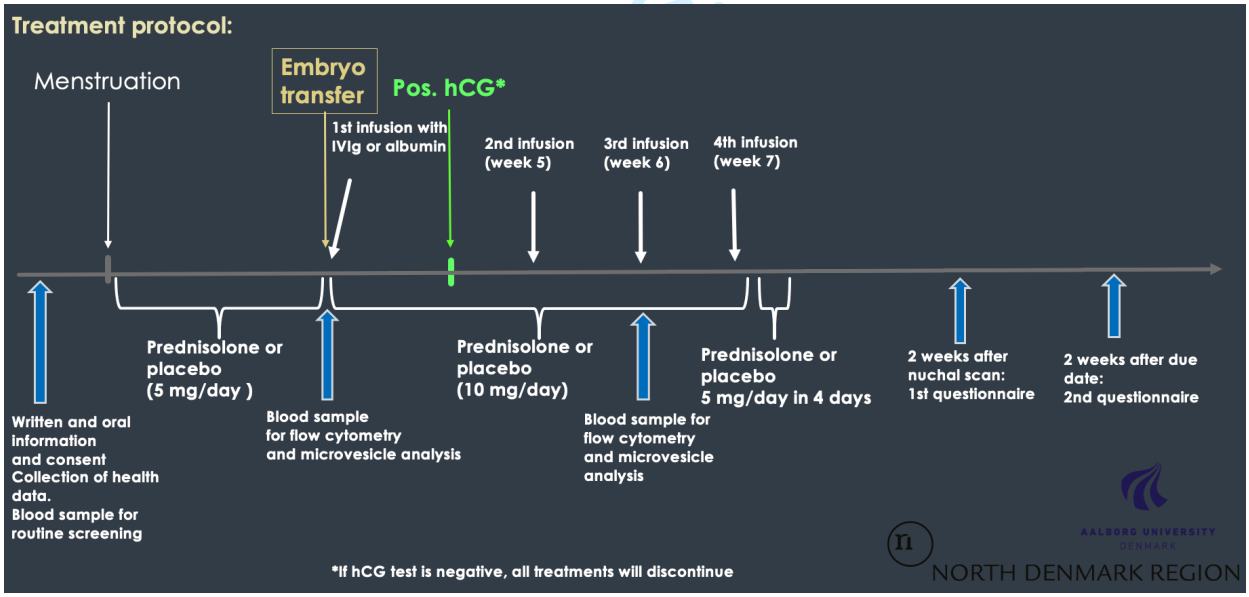

Figure 1. Flow diagram of study interventions.

2.6 Timing of the Interim Analysis and Final Analysis:

A interim analysis will be performed after 38 participants who fulfil criteria for per-protocol analysis have passed their NT scan.

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After termination of the study and before breaking the blind, the final analysis will take place. It will begin with a pre-analysis blind review on data, caried out by the statisticians, to perform data validation, detect outliers, assess distribution of variables, and measure the primary, secondary, and tertiary outcome. Afterwards, the unblinded data analysis will proceed.
The analysis of primary outcome is planned to begin when the last participant has passed NT scan (anticipated to be November 2023) and will be described in one report focusing on treatment effect on reproductive outcome and treatment safety. Publication will therefore await until last pregnant participant has given birth and answered the second questionnaire.
While waiting for the last participants to give birth, the analysis of the tertiary outcomes is planned to be begin, and these results will be described in one or more separate reports focussing on the effect of treatment on LSD and the association between reproductive outcome and LSD.

3. Study Population

3.1 Study Groups

The study will include 74 female patients with uRPL after ART allocated to either active treatment or placebo in a 1:1 ratio.

3.2 Screening Population

All RPL patients admitted to the RPL Center who are also undergoing ART treatment are assessed for eligibility. The number of patients who are assessed for eligibility will be summarised in the manuscript including the total number of screened patients not enrolled divided into the number that did not fulfil criteria after the diagnostic work-up, and the number of patients who did not wish to participate. The number of ineligible patients randomised, if any, will be reported with the reason for ineligibility.

3.3 Study Sample Inclusion and Exclusion Criteria

Inclusion criteria:

- Women with ≥ 2 consecutive pregnancy losses (miscarriages or biochemical pregnancies) before completed gestational week 10 after ART with the same partner or with an egg/semen donor *

Exclusion criteria:

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- BMI ≥35
- Age ≥41 years
- Significant uterine malformation(s)
- Known parental balanced chromosomal translocations
- ≥2 previous pregnancies with fetuses with known abnormal karyotype
- Patients with IgA deficiency, IgA-autoantibodies or hyperprolinaemi
- AMH <4 pmol/l if the next planned IVF/ICSI cycle does not imply the use of donor eggs. If IVF/ICSI with use of donated eggs donation is planned, then a low AMH value is not an exclusion criterion.
- Treatment with medication interacting with prednisolone
    - CYP3A4-inhibitors (for example erythromycin, itraconazol, ritonavir, and lopinavir), CYP3A4- inductors (for example phenobarbital, phenytoin, and rifampicin), loop diuretics, thiazides, amphotericin B, β2-agonists, antidiabetics (Metformin is acceptable), interleukin-2, somatropins, anticholinergics and regular treatment with NSAIDs.
- Patients with moderate/severe hypertension, diabetes mellitus, heart insufficiency, severe mental disorders, Cushing syndrome, myasthenia gravis, ocular herpes simplex, pheochromocytoma, systemic sclerosis, and moderate/severe renal dysfunction.
- Patients with a clinical or biochemical profile indicating need for heparin or levothyroxine treatment during pregnancy **
- Previous treatment with IVIg
- Allergy to prednisolone and/or IVIg

* The GW of the non-induced pregnancy losses will be based on the date of clinical signs of miscarriage or the fetus' crown-rump-length of a missed abortion measured on the ultrasonic scan detecting the pregnancy loss. If the participant plans to have egg donation in the study cycle, the previous two pregnancy losses must also have happened with the use of egg donation; however, it is not required that the same egg donor has been used in all three ETs.

** Indication for levothyroxine decided by the RPL Center is plasma thyroid stimulating hormone (p-TSH) > 3.5 $*10^{-3}$ IU/l together with presence of thyroid peroxidase (TPO) autoantibodies, and indication for heparin is previous clinical thromboses, ≥2 blood samples >3 weeks apart with IgM/IgG anticardiolipin and/or IgM/IgG β2-glycoprotein-1 antibodies > 35 kU/l, presence of lupus anticoagulant, protein S, C or antithrombin III deficiency, or patients being homozygous for the Factor V Leiden or prothrombin G20210A genetic polymorphisms. If treatment with heparin or levothyroxine is prescribed by another physician for other reasons,

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this is respected, and the patient is not eligible for the study. If indication for such treatment occur after cessation of study treatment, i.e., after GW 8+4, the participant is not excluded.

3.4 The CONSORT Flow Diagram

The Consort flow diagram will be included in the manuscript comprising the number of patients who 1) were screened, 2) considered eligible, 3) gave her consent, 4) were randomised, 5) were withdrawn, 5) were not included in PP population due to major protocol deviations, and 6) lost to follow-up. Reason for ineligibility and withdrawal, and also the category of major protocol deviation will be provided. Timing for withdrawal and lost to follow-up will be arranged into four groups: before ET, before cessation of tablet treatment (GW 8), at time for first questionnaire, or at time for second questionnaire. In case of withdrawal, the data collected to date can be used while further data will not be collected.

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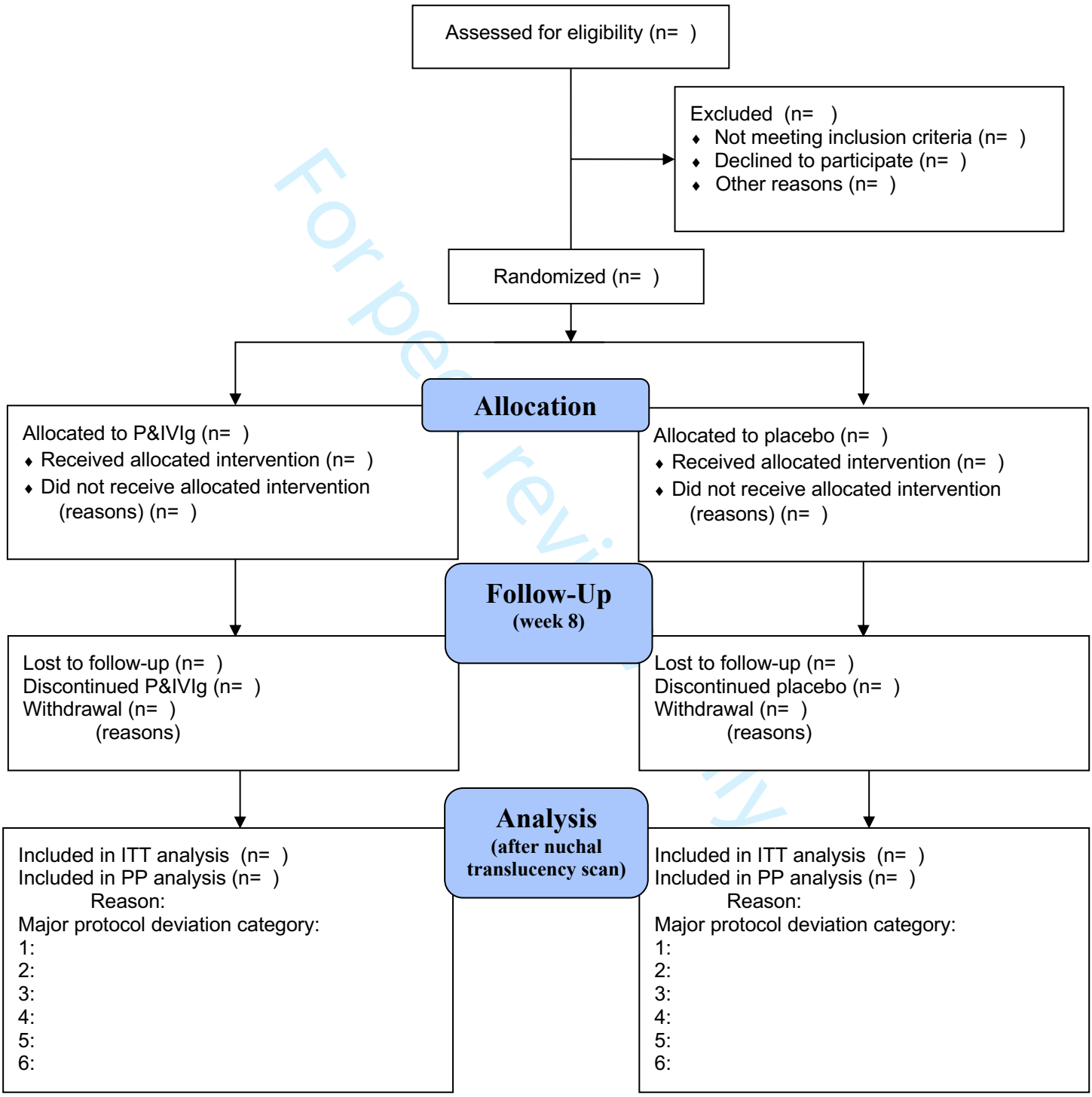
Enrollment Flow Diagram



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3.5 Baseline Characteristics

Each of the following descriptive, baseline and clinical characteristics will be reported for each treatment group in the ITT population. Tests of statistical significance will not be undertaken for baseline characteristics; rather the clinical importance of any imbalance will be noted.

Continuous measures will be summarized by mean and standard deviation (SD) (normally distributed data) or median and interquartile range (non-normally distributed data). Categorical measures will be summarized by frequencies and percentage.

Table I: Baseline characteristics

Characteristic	**Unit**	**Reporting**
Age	Years	Mean (SD)*
BMI	Kg/m^2	Mean (SD)*
Smoking	Yes/No	Frequency (percentage) of participants who smoke
Autoantibody$^+$	Above normal range	Cumulative frequency (percentage) having minimum one autoantibody above normal range
Previous caesarean section	Number of participants	Frequency (percentage)
Prior reproductive outcomes		
Previous birth (after 22 weeks)	Number with: 0 previous birth ≥1 stillbirth ≥1 live birth	Frequency (percentage) of each option
Consecutive PLs	Number of events	Mean (SD)*
Consecutive PLs after ART	Number of events	Mean (SD)* Number (percentage) for each value
Failed ART cycles (no pregnancy after ET)	Number of events	Mean (SD)*
≥1 ART cycle with prednisolone supplement initiated before ET	Number of participants	Frequency (percentage)
Indication for ART	Number with: >12 months unexplained infertility Male factor Tubal factor Endometriosis PCOS Anovulation (non-PCOS)	Frequency (percentage) of each option. Participants, whose indication is a mix of options, are counted in all the related options

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Planned ART treatment in the trial		
ART treatment in study	Number with: IVF ICSI FET *with PGT-A	Frequency (percentage) of each option
Preimplantation stage	Number with: Cleavage stage embryo transfer Blastocyst transfer	Frequency (percentage) of each option
No of embryos transferred	Number	Mean (SD)*
Gamete donation	Number with: No gamete donation Sperm donation Oocyte donation	Frequency (percentage) of each option

PL: pregnancy loss, ART: artificial reproductive technology, ET: embryo transfer, IVF: in-vitro fertilization, ICSI: intracytoplasmatic sperm injection, FET: frozen embryo/blastocyst transfer, PGT-A: pre-implantation genetic testing for aneuploidy

[+] Selected autoantibodies include antinuclear autoantibody *(*ANA), anti-ds-DNA-antibodies, thyroid peroxidase (TPO) antibodies (>60 IU/mL), IgM and IgG β-2-glycoprotein-1 antibodies (20-35 kU/L), and IgM and IgG anti-cardiolipin antibodies (20-35 kU/l). Patients with higher levels of the latter two autoantibodies are not eligible for inclusion in the study.

* The median and interquartile range (IQR) (25^{th} and 75^{th} percentile) for non-normally distributed data

4. Study Objectives, Hypothesis, and Outcomes

4.1. Primary Objective and Outcome

The primary objective of the study is to evaluate the clinical efficacy of treatment with P&IVIg relative to placebo in uRPL patients after ART as assessed by the number (percentage) of participants who are pregnant with minimum one apparently normal fetus alive determined at the time of NT scan in GW 11-13 (referred to as a positive reproductive outcome in the following) in the active treatment group and the placebo group. The study hypothesizes that P&IVIg increases the rate of a positive reproductive outcome compared to placebo. The relative difference between groups will be reported as the relative risk (95% CI) between treatment groups, the absolute risk reduction (95% CI) between treatment groups, and the adjusted relative risk (95% CI) for a positive reproductive outcome. A subgroup analysis will be performed on the primary outcome for pRPL and sRPL participants, separately, based on previous findings suggesting a specific treatment effect in sRPL patients[4]. However, this RCT is underpowered

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to detect statistical significant differences in analyses including pRPL and sRPL patients, separately, if a true subgroup effect do exist.

An additional analysis of the primary outcome will be performed calculating the number (percentage) of participants with minimum one apparently normal fetus alive at NT scan among participants with a positive pregnancy test after ET (plasma hCG>50 IU/L).

These primary outcome analyses will be undertaken as both a PP and ITT analysis.

Based on data from over 400 patients with recurrent early pregnancy losses admitted to our clinic, we expect a low risk of miscarriage after NT scan. Therefore, the primary outcome being assessed at NT scan is expected to represent the difference in live birth rate.

4.2. Secondary Objectives and Outcomes

The secondary objective of the study is to evaluate the safety of P&IVIg relative to placebo when administered in early pregnancy according to both the participant and her offspring as assessed by the incidence of serious AEs and ARs (SAE/SAR), and non-serious AEs and ARs censored up until 6 months after the last IV infusion in non-pregnant participants and until after giving birth in pregnant participants. Also, for the pregnant participants, the safety assessment includes information on pregnancy complications, negative pregnancy and perinatal outcomes, and basic perinatal outcome measures censored right after giving birth. The definitions of an AE, AR, SAE/SAR, and unexpected AE/AR are in accordance with EUR-Lex, CT-3. Specific non-serious AEs occurring in ≥2 (5%) participants and any SAE in each treatment group will be presented.

The secondary outcome analysis will be undertaken as an ITT analysis.

AEs/ARs that are rather a cause of early pregnancy than the study treatment will not be recorded as AEs/ARs, including amenorrhea, vaginal haemorrhage, weight gain, backpain, pelvic pain, drowsiness, nycturia, increased hunger, nausea, and vomiting, unless these AE/ARs are serious.

4.3. Tertiary, Explorative Objectives and Outcomes

The tertiary objective of the study is to explore the impact of P&IVIg on the immune system in peripheral blood and whether the LSD before ET can predict reproductive outcome after ET. Several analyses will be performed immediately on the fresh blood samples collected before first IV infusion and again approximately 4 weeks later (after ET and 1-2 IV infusions), and the excess biological material will be saved for future research in a biobank.

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In this SAP, the statistical analyses for the laboratory analyses performed on fresh blood are described, while future analyses on the biobank material will be described in a separate SAP later.

The tertiary outcome analyses will be undertaken as only PP analyses.

It is important to have an overview of when blood samples are collected in relation to the time when treatment is administered before analysing the tertiary outcomes. Therefore, it is described here in detail:

- Blood sample 1p: Outcomes from the first blood sample collected from a participant in the placebo group will represent the status of the investigated immune biomarkers at the time of ET (five working days before to two working days after ET) after administration of 1 placebo tablet daily for 2-3 weeks.
- Blood sample 2p: Outcomes from the second blood sample collected from a participant 3-4 weeks after ET in the placebo group will represent the status of the immune biomarkers after treatment continuation with a double dose of placebo tablets and 1-2 IV infusions (depending on the reproductive outcome) with human albumin 5% (no immunomodulatory effects expected).
- Blood sample 1a: Outcomes from the first blood sample collected from a participant in the active treatment group will represent the status of the immune biomarkers at the time of ET (five working days before to two working days after ET) after administration of 5 mg prednisolon daily for 2-3 weeks.
- Blood sample 2a: Outcomes from the second blood sample collected from a participant 3-4 weeks after ET in the active treatment group will represent the status of the immune biomarkers after treatment continuation with a double dose prednisolone (10mg from the day of ET and until a negative pregnancy test, early pregnancy loss or pregnancy week 8) and 1-2 IV infusions (depending on the reproductive outcome) with mmunoglobulin 10 % (approximately 0.4g/kg). Thus, participants with sufficiently rising p-hCG receive the second IV infusion, which is one week before blood sample 2 is collected, while participants with a negative pregnancy test, insufficient p-hCG increase, or a p-hCG decrease, receive only one IV infusion (i.e., before ET = 3-4 weeks before blood sample 2 is collected) and have ceased prednisolone intake 1-2 weeks before the blood sample 2 is collected.

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So, when comparing 1p and 1a, the difference will represent the effect of low dose of prednisolone. When comparing 2p and 2a, the difference will represent the effect of double dose prednisolone and IVIg. The difference between blood samples 1 and 2 will represent the effect of IV infusion(s) and natural physiologic changes during a menstrual cycle and/or early pregnancy. Noteworthy, the participants' latest administration of study drugs before the second blood sample will vary depending on their reproductive outcome and therefore, a sensitivity analysis will decide how subgroups (based on reproductive outcome) should be divided, see section 4.4.3.1.

4.3.1 Flow cytometry:

Cell concentrations and fractions

This RCT aims to determine how P&IVIg affects the distribution of leukocyte subsets in peripheral blood and whether a difference in distribution of cells between participants with different reproductive outcomes exists.

A difference in distribution of leukocyte subsets between treatment groups will describe how P&IVIg affects the composition of the cellular immune system. By use of the current knowledge of how the different leukocyte subsets function, the results can also to some degree be used to infer the functional effects of P&IVIg.

According to the results from small observational studies, this study expects a reduction of the NK cell, Th1 cell, and Th17 cell population, and an increase of the Th2 cell and Treg cell population after P&IVIg therapy.[8,11–13] The study has no a-priori defined hypothesis on changes in monocytes, neutrophils, $CD19^+$ B cells, and $CD8^+$ T cells after P&IVIg, as this is sparsely explored in RPL patients.

The distribution of leukocyte subsets in participants with different reproductive outcomes will be compared. Although the subgroups within each treatment group will be small, these comparisons may elaborate if a specific immune profile (i.e., distribution of (certain) leukocytes in peripheral blood) is important for a successful implantation. Thus, the RCT is not powered to find statistically significant differences in leukocyte subset distribution between subgroups with different reproductive outcomes, and therefore, only trends for such associations can be expected to be found although a true difference may exist. Nonetheless, if a clear trend or significant difference is observed between such subgroups, it may help characterize the immune profile in peripheral blood associated with a positive reproductive outcome after ET that can be

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used in the clinic to identify uRPL patients with an immunological genesis expected to benefit from immunomodulatory P&IVIg treatment.

Since a previous study found that the impact of IVIg on Th17 and Treg cell was only significant in patients with a high Th17 and/or low Treg cell fraction before treatment[14], this RCT will evaluate if the effect of IVIg on a given lymphocyte subset is more pronounced when the level before IVIg is high or low for the given lymphocyte subset (percentile subgroup analyses).

These analyses combined may help define which immune cell profile(s) that characterizes uRPL patients in whom the P&IVIg treatment increases the reproductive prognosis.

The outcomes include

- The difference in total white blood cell (WBC) count and fractions of leukocyte subsets between treatment groups, respectively, and the change from blood sample 1 to blood sample 2 in each treatment group.
- The Th1/Th2 cell ratio and the Th17/Treg cell ratio. These cell ratios have been reported as markers to identify patients who might benefit from IVIg treatment previously.[8,15]
- The $CD56^{bright}$ NK/$CD56^{dim}$ NK cell ratio. This cell ratio has been reported to be lower in RPL patients than healthy controls.[16] As the $CD56^{bright}$ NK cell type is predominant in the uterus and has immunoregulatory phenotype, and the $CD56^{dim}$ NK cell type is predominant in peripheral blood and has a cytotoxic phenotype[17], the relative NK cell subset distribution and the treatment effects on this cell ratio may be important for implantation.
- The neutrophil-to-lymphocyte ratio (NLR), monocyte-to-lymphocyte ratio (MLR), and $CD4^+$/$CD8^+$ T cell ratio. These ratios are generally used as biomarkers reflecting the balance between acute and chronic inflammation (neutrophils or monocytes) and immunity (lymphocytes). To the investigators' knowledge, they have not been reported in uRPL patients before, but the ratios are used in several diseases as a surrogate for disease activity or as a prognostic factor which could possibly also be the case for uRPL. [18–20]

Receiver operating characteristic curve analyses

In normal, uncomplicated pregnancies, studies have found that Th2 and Treg cell fractions increases while Th1 and Th17 cell fractions decrease, which paradigm is often interrupted in uRPL patients.[21–23] Previous small, observational studies have suggested that an elevated

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Th1/Th2 ratio predicts a beneficial effect of IVIg on reproductive outcome in uRPL patients; however, these studies used Th1/Th2 cytokine ratios[24,25], which cannot be directedly converted to cell ratios that will be measured in this RCT. Other studies have focused on Th17 and Treg, and they found that uRPL patients with a high Th17/Treg cell ratio before IVIg more often responded to treatment by decreasing the cell ratio and increasing the successful pregnancy rate compared to patients with normal Th17/Treg cell ratio.[14]
Instead, this RCT will determine the optimal threshold for Th1/Th2 ratio, Th17/Treg ratio, and NK cell fraction at the time for ET to determine if a specific immune profile can help clinicians identify, which patients benefit from immunomodulatory treatment.

In previous studies[6, 17, 24,26,27], an elevated NK cell fraction has been defined as >12% and it predicted a beneficial effect of IVIg on reproductive outcome in uRPL patients. Therefore, this RCT will also examine this specific threshold in a separate analysis; that will evaluate if participants in the active treatment group who have an NK cell fraction ≥12% in blood sample 1 have a higher frequency of a normal viable pregnancy censored at NT scan than participants with a NK cell fraction <12%. The same analysis will be performed in participants in the placebo group to evaluate if participants with a high NK cell fraction not receiving immunomodulatory treatment have a worse prognosis than similar patients in the active treatment group.

4.3.2 TruCulture analysis (pilot study)

A small sample of 25 participants from the RCT population will be used in a pilot study, which aims to explore if a stimulus-specific immune response is affected by immunomodulatory treatment. The analysis will be performed in the middle one-third of participants enrolled; thus, in 25 consecutively, randomized participants. The stimulus-specific immune response will be assessed as the cytokine concentration after exposure to different immune stimuli and compared within and between treatment groups. Despite a small sample size, the outcomes will also be reported for treatment subgroups separating participants into pregnant and non-pregnant patients in GW 8, to test if such subgroups of patients with different reproductive outcomes differ in response to the specific stimuli.

No prior study has reported how the stimulus-specific immune response is affected by immunomodulatory treatment in early pregnancy using the highly standardized TruCulture®

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analysis. As it is an expensive analysis with unknown relevance in this context, it will be only performed on both blood samples from 25 participants.

The outcome from the TruCulture® analysis include the concentration of ten different cytokines measured after the participant's whole blood will be exposed to one of four different immune stimuli or no stimuli. These immune stimuli include:

- CD3/CD8: mimicking T cell activation by antigen presenting cells (APC).
- R848: mimicking single stranded RNA viral activation of toll like receptor (TLR) 7/8
- Polyinosinic-polycytidylic acid (polyIC): mimicking double stranded (ds) RNA virus activation of TLR3
- Lipopolysaccharide (LPS): mimicking bacterial (E.coli**)** activation of TLR4
- No-stimulus null control: as a proxy for in vivo activation and baseline circulating cytokine concentration.

4.4. Specification of Endpoints

4.4.1. Primary Endpoint

The primary endpoint is the number (percentage) of participants who are pregnant with ≥1 apparently normal, viable fetus censored at NT scan (GW 11-13) by a sonographer in the ITT and PP population. The relative risk (95% CI) and absolute risk reduction (95% CI) between treatment groups will be reported.

In addition, four other endpoints will be reported:

- The number (percentage) of participants who are pregnant with ≥1 apparently normal, viable fetus censored at NT scan among women with a positive p-hCG after ET.
- The number (percentage) of participants who are pregnant with ≥1 apparently normal, viable fetus censored at NT scan among all women and among women with a positive p-hCG after ET excluding pregnancies with a fetus with confirmed aneuploidy, ectopic pregnancies and partial/complete mola pregnancies.
- The adjusted relative risk (95% CI) of being pregnant with ≥1 normal, viable fetus censored at NT scan for participants allocated to active treatment compared to the placebo group. The confounding variables included are smoking (binary), BMI, and age (continuous).

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- Subgroup analysis: The primary endpoint in subgroups based on diagnosis of pRPL and sRPL, respectively, i.e., no prior birth or a history of ≥1 previous birth after 22 GW.

These analyses will be performed as an ITT and PP analysis. Criteria for ITT and PP analysis are described in the section about Major Protocol Deviations.

4.4.2. Secondary Endpoints

The secondary endpoints are the frequency (percentage) of the conditions or adverse events in the ITT population stratified by treatment listed in table II.

Table II: Secondary endpoints

Secondary Endpoints		
Non-serious AEs and ARs	AEs/ARs (non-specific)	The frequency of participants with ≥1 AEs/ARs (non-specific)
	Specific AEs/ARs	The frequency of patients having a specific AE/AR - only for those AEs/ARs that occur in ≥2 (5%) of participants
	Withdrawal	The frequency of withdrawals due to AE/AR
Serious AEs and ARs	SAEs/SARs (non-specific)	The frequency of participants with ≥1 SAEs/SARs (non-specific)
	Specific SAEs/SARs	The frequency of participants with each type of SAE/SAR occurring
	Withdrawal	The frequency of withdrawals due to SAE/SAR
Pregnancy complications	Preeclampsia	Incidence Defined as gestational hypertension and

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		proteinuria (urine albumin/creatinine ratio >300mg/g or urine albumin >3g/day or >1+ on urine stix) presenting after GW 20+0.
	Gestational hypertension	Incidence Defined as blood pressure >140 mmHg systolic or >90 mmHg diastolic presenting after GW 20+0
	Gestational diabetes mellitus	Incidence Defined as an oral glucose challenge test (OGCT) with >9.0 mmol/l 2 hours after oral intake of 75g glucose solution
	Instrumental delivery	Incidence of: Elective caesarean section Emergency caesarean section
Negative pregnancy outcomes	Negative pregnancy test	Number of participants with a negative pregnancy test after ET among all participants
	Pregnancy loss	After a hCG measure >50 IU/L Specific measures of pregnancy loss: The frequency of pregnancy losses with unknown or normal karyotype among all participants and all participants with a positive pregnancy test The frequency of pregnancy losses with known aneuploidy among all pregnancy losses tested for aneuploidy

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		The frequency of biochemical pregnancy losses among all pregnancy losses The frequency (percentage) of clinical miscarriages among all pregnancy losses The frequency of late miscarriages (week 12+1-21+6) among all pregnancy losses
	Stillbirth	The frequency of participants with a birth after GW 24 which died in utero or within one week after birth
Perinatal outcomes	Congenital deformity	The frequency of a congenital deformity identified during pregnancy or within 1 week after birth
	Abnormal karyotype	The frequency of an abnormal karyotype identified during pregnancy or within 1 week after birth
	Prematurity	The frequency of a birth before 37 weeks of gestation
	Small for gestational age (SGA)	The frequency of a birthweight <10th percentile
	Low birth weight	The frequency of a birth weight <2500g
	Admission to neonatal care unit	The frequency of an admission to the neonatal care unit in >24 hours
	Sex ratio	male:female ratio
	Birth weight of singletons	Mean birth weight (g), SD Continuous variable
	Gestational length	Mean gestational age at birth (days), SD

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		Continuous variable

(All secondary outcome variables are binary variables unless otherwise stated)

Results on binary variables except from perinatal outcome variables will be presented in a dot plot displaying the frequency, the relative risk (95% CI), and the percentage of participants in each group.[28] The perinatal outcomes will be presented in a separate table.

4.4.3. Tertiary Endpoints

The tertiary endpoints are measured in the PP population. The endpoints will be reported for each two blood samples separately, in the active treatment group and in the placebo group. For further evaluation of treatment on leukocyte subset distribution, subgroup analysis will be performed, in which the participants in each treatment group will be separated into smaller subgroups based on reproductive outcome after ET or WBC percentiles.

Subgroups of participants based on reproductive outcome

If each treatment group has <5 participants with no pregnancy (NP) and an early pregnancy loss (EPL) after ET, respectively, the two treatment groups will be divided based on a negative (NP+EPL) and positive (ongoing pregnancy (OP) at NT scan) reproductive outcome. If each treatment group has ≥5 participants with NP, EPL, and OP, respectively, a sensitivity analyses will be performed to check for differences in total WBC count and total lymphocyte count:

1) Between participants with no pregnancy (NP) and early pregnancy loss (EPL) after ET

And subsequently,

2) Between participants receiving one and two IVIg infusions before collecting the second blood sample, i.e., a comparison of participants with NP or an EPL, who received only one IV infusion, with participants with an EPL who received two IV infusions.

If the sensitivity analysis finds no significant differences, these participants will be unified in one group representing those with a negative reproductive outcome. If the first sensitivity analysis finds a significant difference, the subgroups will be divided into three groups: NP, EPL, and ongoing pregnancy (OP) at NT scan. If the second sensitivity analysis finds a significant difference, the subgroups will be divided into the following three groups: 1) participants with NP or EPL receiving one IV infusions, 2) participants with an EPL (GA week 5-12) receiving two infusions, and 3) participants with an ongoing pregnancy (OP) at NT scan. Thus, depending on the sensitivity analyses, the subsequent analyses stratified for reproductive outcome will be in two or three subgroups.

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Flow cytometry: cell concentration and cell fraction

Flow cytometry endpoints include those listed in table III.

Table III: Endpoints from flow cytometry analyses

White blood cell count and differential	Mean total WBC count ($x10^9$/L) (SD) (continuous) Mean concentration ($x10^9$/L) (SD) and relative percentage (SD) of the following WBC differential (continuous): 1) Monocytes 2) Neutrophils 3) Lymphocytes
Lymphocyte subsets	The mean percentage (SD) of the following lymphocytes subsets in the total lymphocyte population (continuous). 1) $CD19^+$ B cells 2) $CD8^+$ Tc cells 3) $CD3^+CD4^+$ $CCR4^-CCR10$ Th1 cells 4) $CD3^+CD4^+$ $CCR4^+CCR10^-CCR6^-$ Th2 cells 5) $CD3^+CD4^+$ $CCR4^+CCR10^-CCR6^+$ Th17 cells 6) $CD3^+CD4^+$ $CD25^+$ $CD127^{low}$ Treg cells 7) $CD3^-CD56^+$ NK Cell (total) 8) $CD16^+CD56^{dim}$ NK cells 9) $CD16^-CD56^{bright}$ NK cells.
Cell ratios	Mean cell ratios (SD) (continuous), calculated based on cell fractions, include the following: 1) Neutrophil-to-lymphocyte ratio (NLR) 2) Monocyte-to-lymphocyte ratio (MLR) 3) $CD8^+$Tc/$CD4^+$Th ratio 4) Th1/Th2 ratio 5) Th17/Treg ratio 6) $CD56^{bright}$ NK/$CD56^{dim}$ NK ratio

If the variable is not normally distributed, the median and 25-75% interquartile range (IQR) will be reported.

These endpoints will be reported for both treatment groups, separately, and subsequently for subgroups based on the reproductive outcome.

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The mean change of leukocyte fractions (absolute concentration (x10^9/L) and/or percentage point) from blood sample 1 to 2 will be reported in each treatment group based on the formula:

$\Delta Leukocyte = Fraction_{before} - Fraction_{after}$

Subgroup analysis of participants based on cell level percentiles

Participants in the active treatment group will be divided into three subgroups based on ascending order of the cell fraction level of each lymphocyte subset: the lowest percentile subgroup (PS1) counting 1/3 of participants with the lowest cell fraction levels; the medium percentile subgroup (PS2) counting participants with the cell fraction levels between the 33th and 66th percentile; and the highest percentile subgroup (PS3) counting participants with the highest cell fraction levels above 66th percentile.

The mean (SD) cell fraction in blood sample 1 and 2, respectively, will be presented in each percentile subgroup (PS) and the fraction in blood sample 1 will be compared to the fraction in blood sample 2. Subsequently, for each lymphocyte subset, the change (percentage points (SD)) in each PS will be compared.

Receiver operating characteristic curve analysis

Endpoints from the receiver operating characteristic (ROC) curve analyses include:

- The optimal threshold for Th1/Th2 cell ratio, Th17/Treg cell ratio, and NK cell fraction in the active treatment group for identification of uRPL patients at high chance of an ongoing pregnancy at NT scan.
- In active treatment group, the number (percentage) of participants with 0 or ≥1 abnormal value (i.e., abnormal immune profile), respectively, based on the three optimal thresholds calculated using data from the active treatment group.
- The number (percentage) of participants with ongoing pregnancy at NT scan in the group with 0 and with ≥1 abnormal value before ET, respectively, in each treatment group, separately.

The same endpoints will be provided only using NK cell fraction >12 % (and not using any Th1/Th2 or Th17/Treg ratio) to divide participants in each treatment group into those with or without an abnormal immune profile.

TruCulture

The mean (SD) or median (25-75 IQR) concentration (pg/mL) (continuous) of IFN-α2, IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-12 (p40), IL-13, IL-17, and Monocyte Chemoattractant Protein-1 (MCP-1) will be reported for each of the four different immune stimuli and the non-stimulation analysis in each blood sample, separately.

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These endpoints will be illustrated in boxplots for the active treatment group relative to placebo group. If ≥5 participants in active treatment group have a positive reproductive outcome, subgroup analysis for these endpoints in participants with OP relative to NP&EPL will be performed.

5. Statistical Analysis

5.1 Statistical Principles

All the applicable statistical tests used will be two-sided and performed using a 5% significance level unless otherwise stated.

Baseline characteristics will for continuous variables be described with mean and standard deviation (SD) (normally distributed data) or median and the interquartile range (25^{th} and 75^{th} percentile) (non-normally distributed data). For categorical variables, the frequency and percentage will be provided. Data distribution of continuous variables will be determined using QQ-plots and histograms.

5.2 The interim analysis

When 38 patients, who fulfil criteria for entering the PP-analysis, have been included in the study, an interim analysis will be conducted by independent statisticians in order to remain all investigators blinded. Blinded results will be shared with investigators. Differences between treatment groups regarding the primary outcome as well as the number of women with SARs/SAEs and SUSARs in the two groups (denoted A and B) will be analysed with the objective of early evaluation of unacceptable side effects of the treatments to continue the study.

The interim analysis will analyse the primary outcome and compare the incidence of SARs/SAEs and SUSARs between groups. A between group difference of ≥3 SUSARs or SARs/SAEs will lead to early termination of the trial. The alpha spending method used for adjusting significance levels for the analysis of SARs/SAEs and SUSARs between groups will be explicitly defined.[29] Analysis of the primary outcome will not lead to early termination in case of futility nor any modifications to trial procedures. The sponsor has the ultimate authority to terminate the trial.

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A correlation between occurrence of SARs/SAEs and SUSARs and the primary outcome may exist, however, one such correlation will be discussed for clinical relevance and no adjustments of significance levels on the analysis of the primary outcome will be made.

5.3 Analysis Methods for Primary Endpoint

In two separate analyses in all participants in the ITT and PP population, respectively, the number (percentage) of participants in each treatment group who had an apparently normal viable fetus at NT scan will be compared using Fisher's Exact Test (0=no pregnancy or early pregnancy loss, 1= normal pregnancy at NT scan). Also, the corresponding relative risk (95% CI) and absolute risk reduction (95% CI) will be reported.

Additional analyses on the primary outcome include:

1) The number (percentage) of participants who had an apparently normal viable fetus at NT scan among all participants with a positive pregnancy test (p-hCG >50) in each treatment group will be compared using Fisher's Exact Test (0= pregnancy loss before NT scan; 1= normal viable fetus at NT scan).
2) The number (percentage) of participants who had an apparently normal viable fetus at NT scan among a) all participants and b) all participants with a positive pregnancy test (p-hCG >50) in each treatment group after exclusion of pregnancies with a fetus with confirmed aneuploidy, ectopic pregnancies and partial/complete mola pregnancies, will be compared using Fisher's Exact Test ($0_{a)}$ = no pregnancy or pregnancy loss before NT scan; $0_{b)}$ = pregnancy loss before NT scan; 1= normal viable fetus at NT scan).
3) A modified poisson regression with robust variance will estimate the adjusted relative risk (RR) (95% CI) for an apparently normal viable fetus at NT scan adjusted for baseline characteristics including age at enrolment (*continuous*), BMI at enrolment (*continuous*), and smoking habits at enrolment (*binary).* No alternative method will be applied if assumptions are not completely fulfilled, since the modified poisson regression uses a robust error variance, which is robust to violations of model assumptions.

   A sensitivity analysis will be conducted with the following covariates included: low plasma mannose binding lectin (p-MBL) level (*binary),* previous pregnancy > 22 weeks (*binary),* and presence of minimum one of the measured autoantibodies (*binary)*.
4) The primary outcome will be analysed in subgroups based on whether the participants were diagnosed with pRPL or sRPL, i.e., had no previous birth after 22 GW or had ≥1

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previous birth after 22 GW, respectively. The relative risk (95% CI) between treatment groups will be provided.

Table IV: Definition of binary covariates

Definition of binary variables used as covariates in regression analyses:		
	0	1
Smoking	No smoking within the last 3 months before enrolment	Smoking minimum 1 cigarette daily during the last 3 months before enrolment.
Low MBL level	>500 ug/l	≤500 ug/l
Previous liveborn	0 liveborn (=pRPL)	≥1 pregnancy >22 weeks (=sRPL).
Autoantibody	No autoantibody	Minimum 1 of the following autoantibodies above normal range: TPO-Ab (>60kU/l), anti-ds-DNA-Ab (>10kIU/l), ANA (≥1), anti-cardiolipin and/or anti-β2-glycoprotein antibody (20-35 kU/l in two measurements with >3 weeks interval).

5.4. Analysis Methods for Secondary Endpoints

The secondary endpoints including all binary outcome variables will be compared between active treatment group and placebo group using Fisher's Exact Test. Furthermore, the relative risk (95% CI) will be reported. The continuous outcome variables will be compared using unpaired t-test for parametric variables and unpaired Mann-Whitney U Test for non-parametric variables.

5.5 Analysis Methods for Tertiary outcomes

5.5.1 Flow cytometry: cell concentration and fraction

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The mean absolute cell count (ACC) (SD) of WBC differentials, and the mean percentage (SD) of lymphocyte subsets of the total lymphocyte population in peripheral blood in blood sample 1 and 2, separately, will be reported. These endpoints will be used in the following statistical analyses.

Treatment effects on Lymphocytes Subsets

Tertiary endpoints from flow cytometry analyses will be reported in a contingency table or bar/dot plot (example in Table V). A comparison within the treatment group, i.e., comparing the endpoints in blood sample 1 (before) with blood sample 2 (after IV infusion), will be performed using paired t-test for parametric variables and paired Wilcoxon signed-rank test for non-parametric variables. Between-group comparisons, i.e., comparing endpoints from each two blood samples between the two treatment groups, will be performed using unpaired t-test for parametric variables and unpaired Mann-Whitney U Test for non-parametric variables. The mean change will be calculated by subtracting the value in blood sample 1 (before IV infusion) with the value blood sample 2 (after IV infusion), and the mean change will be compared between treatment groups using the same unpaired statistical tests.

Table V: Results from immunological analyses on blood samples collected before first IV infusion (blood sample 1) and after IV infusion(s) (blood sample 2) in uRPL patients. The table will contain a row for each the leukocyte subsets and the cell ratios described in the section on "Tertiary Endpoints". P^1: comparison of blood sample 1 and 2 within the treatment group. P^2: comparison of endpoints from either 1) before IV infusion or 2) after IV infusion or 3) the mean change between the sample collected before and after IV infusion within a treatment group, respectively, between the two treatment groups. ACC: absolute cell count. SD: standard deviation

Leukocyte subset distribution	Placebo				Active treatment				Comparisons between treatment groups		
	Before IV	After IV	Mean change	P^1	Before IV	After IV	Mean change	P^1	P^2 (Before)	P^2 (After)	P^2 (Mean change)

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Total WBC count	ACC, Mean (SD) Percentage, Mean (SD)											
Lymphocyte subset	Percentage, Mean (SD)											

Subgroup analysis: Association between the lymphocyte subset distribution and the reproductive outcome

The participants in each two treatment groups will be divided into subgroups based on their reproductive outcome. The number of groups depend on the sensitivity analysis.

The sensitivity analysis made before dividing participants into subgroups based on reproductive outcome will use an unpaired t-test or Mann-Whitney U test to test for differences in total WBC count and total lymphocyte count when ≥5 participants are expected in each group, i.e., NP and EPL group.

Flow cytometry endpoints will be reported in a contingency table (See table II). To test for differences in ACC and/or percentage of each lymphocyte subset between subgroups based on reproductive outcome, an unpaired t-test (two subgroups) or One-Way Anova analysis (three subgroups) will be used if the variable of interest is normally distributed, and an unpaired Mann-Whitney U Test or Kruskal-Wallis One-Way ANOVA will be used if the variable of interest is skewed. To test if the ACC and/or percentages differ between participants with the same reproductive outcome but in different treatment groups, an unpaired t-test or Mann-Whitney U Test will be used depending on the data distribution of the variable of interest.

Table VI: Immune cell levels in patients with different treatments and reproductive outcomes. The table will contain a row for each of the leukocyte subsets measured as well as the cell ratios in blood sample 1 and 2, respectively. The cell types and ratios are described in the section on "Tertiary Endpoints". There will be one column for each subgroup based on the reproductive outcome in each treatment group (NP: no pregnancy, EPL: early pregnancy loss, OP: ongoing pregnancy at NT scan). P^1: comparison of NP&EPL with OP. P^2: comparison of subgroups with the same reproductive outcome between treatment groups.

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Cell type		Placebo			Active treatment			Between treatment groups	
		NP&EPL	OP	P[1]	NP&EPL	OP	P[1]	P[2] (NP&EPL)	P[2] (OP)
Total Leukocyte count ACC, Mean (SD)	Before IV After IV Mean change								

If no differences are observed between treatment groups with the same reproductive outcome in either blood sample 1 or 2, the flow cytometry endpoints will be reported for all participants only stratified for the reproductive outcome (but not stratified for treatment) (NP and EPL combined or separated according to the sensitivity analysis for reproductive treatment subgroups) and compared (See table VII). The tests used for comparing data will be same as described for results reported in table VI.

Table VII*: Immune cell levels in patients with different reproductive outcomes. The table will contain a row for each of the leukocyte subsets measured as well as the cell ratios in blood sample 1 and 2, respectively. The cell types and ratios are described in the section on "Tertiary Endpoints". There will be one column for each subgroup based on the reproductive outcome (NP: no pregnancy, EPL: early pregnancy loss, OP: ongoing pregnancy at NT scan).*

		All participants		
		NP&EPL combined	Ongoing pregnancy	P
Th1 cells, mean percentage (SD) $CD3^+CD4^+ CCR4^-CCR10^-$	Before IV After IV Mean change			

Percentile subgroup analysis: Association between the immune cell level before IV infusion and the treatment response

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Three percentile subgroups (PS) will be made based on ascending leukocyte cell level in each leukocyte subset. Thus, when comparing e.g., Th1 cell fraction, participants will be divided into three groups based on the 33rd and 67th percentile of Th1 cell fraction. The same way is PSs made for each leukocyte subset (see Table VIII).

The cell fraction level in the blood sample 1 will be compared with blood sample 2 in each PS, respectively, using paired t-test for parametric variables and paired Wilcoxon signed-rank test for non-parametric variables in active treatment group. The change in cell fraction (ACC or percentage point) observed in each three PS will be compared using One-Way ANOVA for parametric variables and Kruskal-Wallis One-Way ANOVA for non-parametric variables. These analyses will be performed for Th1, Th2, Th17, Treg, $CD8^+$ T cell, B cells, total NK cells, and the two NK subsets.

Table VIII*: Lymphocyte subset level in percentile subgroups (PS) before and after intravenous (IV) infusion (blood sample 1 and 2). The level before is compared with the level after IV. The mean change in each PS is compared. The will one row for each lymphocyte subset described in section "Tertiary endpoints".*

	PS1			PS2			PS3		
	Before IV	After IV	Mean change	Before IV	After IV	Mean change	Before IV	After IV	Mean change
Th1 cells, mean percentage (SD) $CD3^+CD4^+$ $CCR4^-$ $CCR10^-$									

Receiver operating characteristic curve analyses

ROC curve analyses will be performed to determine the performance of Th1/Th2 ratio, Th17/Treg ratio, and the NK cell fraction in blood sample 1 to predict a positive reproductive outcome (i.e., pregnancy at NT scan) in the active treatment group. The optimal cut-off for each of the three immune markers will be defined as the value with maximum sensitivity and specificity in the ROC curve analysis.

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The AUC value and the Youden's index (J_{max}) together with the corresponding sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) will be reported for each ROC curve analysis.

Noteworthy, the sample size in this RCT has not been calculated based on this analysis and with only this small sample available, no post validation cohort will be separated from the treatment group before the ROC curve analysis will be performed. Bootstrapping with 1000 bootstrap samples will be applied to estimate 95% confidence limits on the estimates.

Using the three thresholds, the number of participants in active treatment group with ≥1 "abnormal" value will be determined. An abnormal value will be defined as a value higher than the threshold for Th1/Th2 ratio, Th17/Treg ratio, and the NK cell fraction. The number of participants with an ongoing pregnancy at NT scan will be compared between participants with 0 and with ≥1 abnormal value in the treatment groups, separately, using χ^2 Test or Fisher's Exact Test.

The number (percentage) of participants with a total NK cell fraction >12% (including both $CD3^-CD16^+CD56^{dim}$ and $CD3^-CD16^-CD56^{bright}$) of the total lymphocyte population in the sample before and after IV infusion, respectively, will be reported. The number of participants with elevated NK cell fraction before IV infusion will be compared with the number after IV infusion using χ^2 Test or Fisher's Exact Test.

The number of participants with an ongoing pregnancy at NT scan among all participants with NK cell fraction ≥12% and <12%, respectively, will be compared using χ^2 Test or Fisher's Exact Test in each treatment group, respectively.

5.5.2 TruCulture pilot study: Immune response ex vivo after induced stimulation

The concentration of cytokines before and after IV infusion will be compared in each treatment group, separately, using unpaired t-test or Mann-Whitney U Test depending on the distribution of the variable of interest. Results will be presented in boxplots for each of the four stimuli-specific protocols and for the no-stimuli protocol. The boxplot will include the information described in Table IX.

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Table IX: For each stimulator, mean cytokine concentration (pg/ml) will be presented in a box plot containing data stratified and compared as described in this table, evaluating the treatment effect on the stimulus-specific immune cell response by in-group and in-between group comparisons. Information on each cytokine will be reported. P^1: comparison of blood sample 1 and 2 within the treatment group. P^2: comparison of endpoints from either 1) before IV infusion or 2) after IV infusion or 3) the mean change between the sample collected before and after IV infusion within a treatment group, respectively, between the two treatment groups.

Stimulator	Placebo				Active treatment				Comparison between groups		
	Before IV	After IV	Mean change	P^1	Before IV	After IV	Mean change	P^1	P^2 (Before)	P^2 (After)	P^2 (Mean change)
IFN-α2											

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5.6. Major Protocol Deviations

The predefined major protocol violations are divided into six categories described in Figure 2

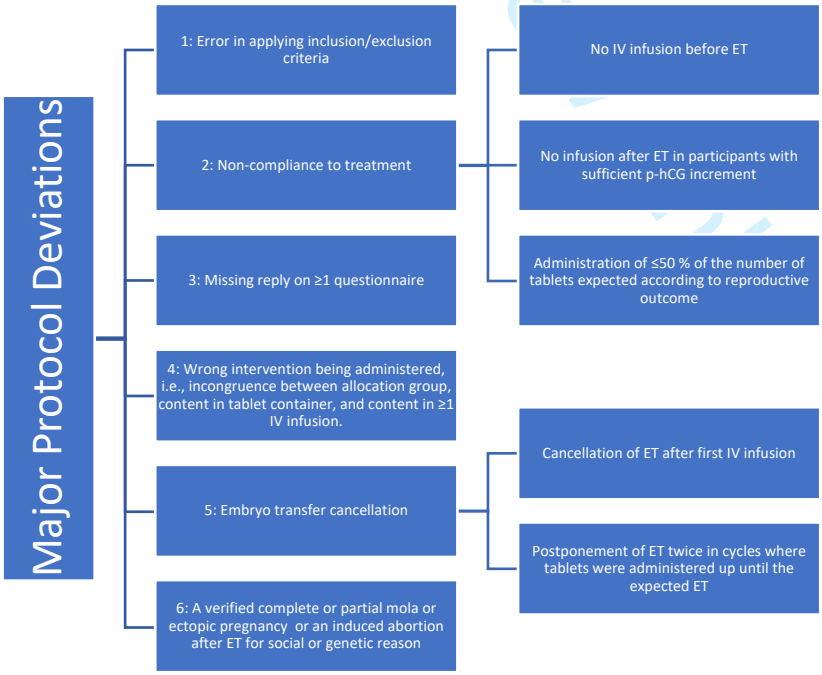

Figure 2: description of major protocol deviations excluding participants from per-protocol population.

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All randomised participants will be included in ITT analysis population. Participants who completed the trial with no major protocol deviations will be included in the PP analysis. The reason for not fulfilling eligibility criteria will be reported. In case of category 4 violations, the participant will be counted in according to the treatment she was randomised to receive, All protocol deviations will be recorded in the eCRF and summarised by the treatment group and the category with frequency and percentage of all included participants.

5.7 Missing data

To prevent missing data, the eCRF has a record status dashboard with an overview of instruments to be filled in after follow-up appointments including instruments for the investigator to fill in and questionnaires for the participant to fill in. When the instrument is changed from incomplete to complete, i.e., all data items are filled in, the instrument on the record status dashboard changes colour from red to green. Also, key data items on primary and secondary outcomes collected at follow-ups before gestational week 9 are described in the participant's medical records. In addition, AEs and treatment compliance are described in the information folder returned to the RPL Clinic after treatment cessation. Thus, these key data items are readily obtainable if missing in the eCRF by the end of the study. For this reason, the degree of missing data on key data items is expected to be minor or not existing. However, in case of missing data, the number missing will be reported.

For the analysis of the primary and secondary outcome, complete case analysis will be performed. In analysis tertiary outcomes, the management of missing data will follow the guideline presented by Jakobsen et al. and include complete case analysis and single or multiple imputation.[30] A sensitivity analysis will be performed if MI or other methods to account for missing data has been used in accordance with the description by Jakobsen et al.[30]

6. Data Handling and implementation of Statistical Analysis Plan

All data regarding primary and secondary (except data on blood samples) outcome will be collected and stored in an electronic case report form (e-CRF) in a REDCap database (Vanderbilt University, Nashville, TN, USA) which only the sponsor and chief investigator have full access to. GCP board members have rights to "see only". Information will be obtained from the participant's electronic medical records, information folder, and directly from the participant. REDCap makes audit trail logging and back-ups. The eCRF will be used to collect

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all clinical data about the participant, and also to automatically send out and collect questionnaires replies after NT scan and after due date. Personal identifiers are marked, and they will not be exported from the database when the study terminates. Data will be exported to Microsoft Excel (Microsoft Corporation, Washington, USA) and prepared for analyses performed in Stata/MP 15.0 (TX, US) for Mac.

Data regarding tertiary outcomes will be collected in a secured database at the Department of Clinical Immunology (DCI) until all participants have been enrolled and the last pregnant participant has passed her NT scan. At this time, blinded data will be shared with the sponsor and chief investigator at the RPL clinic and not at any time before, since sharing results from these analyses at any time before may reveal enough information for investigators to speculate and predict treatment allocation. Thus, in order to remain fully blinded, data on peripheral blood immunological analyses will only be handled by the DCI personnel, who have no contact to study participants, until all data has been collected.

When the last pregnant patient has passed her NT scan, we will start data analysis. Before data is unblinded, a blind review pre-analysis on data will be caried out to check for outliers in continuous outcome variables, distribution of continuous and ordered data (using Q-Q plots and histograms to assess data normality), missing data points (and if possible, collecting these missing data points) and important potential covariates identified in other recent research may be added to statistical models. This final pre-analysis will be handled by statisticians who had not been involved in the study. They will present the investigators for any uncertainties as well as blinded interpretations of the primary endpoint results. The pre-analysis may add modifications to the SAP, which will all be described in an amendment to the SAP before performing unblinded analyses. After data validation is complete, unblinded data analysis will proceed. Unblinded analyses will be conducted by the chief investigator supervised by statisticians.

This SAP will be used by all statistician and investigators analysing study data. Analysis of primary, secondary, and tertiary endpoints will be carried out in continuous order in the pre-analysis.

7. Finances

The investigators gratefully acknowledge The Svend Andersen Fund for funds to help defray medicinal expenditure, “Grosserer L.F. Foght’s” fund and “Beckett-Fonden” for funds for expenditure for immunological analyses. The funding sources had no role in the design of the

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study and will not have any role during its execution, analyses, interpretation of the data, or decision to submit results. The investigators also thank the DCI for performing all immunological analyses and preparing infusion medicine for all participants.

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10. Abbreviations

AaUH: Aalborg University Hospital

ACC: Absolute cell count

AE: adverse events

ANA: antinuclear autoantibody

AR: adverse reactions

ART: assisted reproductive technologies

BMI: body mass index

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DCI: the Department of Clinical Immunology

e-CRF: electronic case report form

EPL: early pregnancy loss

FET: frozen embryo/blastocyst transfer

GCP: Good Clinical Practice

HLA: Human leukocyte antigen

ICSI: intracytoplasmatic sperm injection

Ig: Immunoglobulin

ITT: Intention to treat

IVF: in-vitro fertilization

IVIG: intravenous immunoglobulin

J_{max}: Youden index

LR: likelihood ratio

NK: natural killer

NK: natural killer

NP: no pregnancy

NPV: negative predictive value

OGCT: oral glucose challenge test

OP: ongoing pregnancy

OR: odds ratio

p-MBL: plasma mannose binding lectin

PGT-A: pre-implantation genetic testing for aneuploidy

PP: Per protocol

PPV: positive predictive value

pRPL: primary recurrent pregnancy loss

PS: percentile subgroup

ROC: receiver operating characteristic

RPL: recurrent pregnancy loss

RR: relative risk

SAE: serious adverse event

SAP: statistical analysis plan

SAR: serious adverse reaction

SD: standard deviation

SGA: small for gestational age

sRPL: secondary recurrent pregnancy loss

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SUSAR: suspected unexpected serious adverse reaction

Tc: cytotoxic T

Th: T-helper

TPO: thyroid peroxidase

Treg: regulatory T

uRPL. Unexplained recurrent pregnancy loss

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SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	2, 15
	2b	All items from the World Health Organization Trial Registration Data Set	Appendix
Protocol version	3	Date and version identifier	2
Funding	4	Sources and types of financial, material, and other support	16
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	16
	5b	Name and contact information for the trial sponsor	16
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	17
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	N/A

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Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	3
	6b	Explanation for choice of comparators	7
Objectives	7	Specific objectives or hypotheses	4
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	4
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	4
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	4
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	7
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	7
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	6
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	4, 9
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	11
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	7

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Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	12
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	4
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	6
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	6
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	6
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	6
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	6
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	9
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	10-11

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Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	9
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	13-14, and SAP in appendix
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	13-14, and SAP in appendix
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	13-14
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	14
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	13
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	11-12
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	14
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	15
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	15

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Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	6
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	15
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	10
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	16
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	16
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	8, 15
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	15-16
	31b	Authorship eligibility guidelines and any intended use of professional writers	16
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	16
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Appendix
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	Appendix

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

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