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Estradiol and progesterone levels in early pregnancy after natural, estradiol + progesterone or gonadotrophin stimulated frozen embryo transfer cycle: A randomized controlled trial.
Estradiol and progesterone levels in early pregnancy after natural, estradiol + progesterone or gonadotrophin stimulated frozen embryo transfer cycle: A randomized controlled trial.

The ESTRO-FET study

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Abstract

Introduction: Frozen embryo transfer is being increasingly used for assisted reproductive therapy and offers similar pregnancy rates as treatment with fresh embryo transfer. In women with regular menstrual cycles transfer of a frozen thawed blastocyst can be performed in either a natural cycle or substituted cycle. Anovulatory women can only be offered a substituted cycle. Knowledge on fetal exposure to estradiol in early pregnancy is very limited but studies on mice and rats have shown hormonal and metabolic disturbances in cubs born from estradiol exposed mothers. We aim to investigate serum estradiol and progesterone levels in women conceived after natural, estradiol and progesterone or gonadotrophin stimulated frozen embryo transfer.

Methods and analysis: The study is an open label randomized controlled trial with normo-ovulatory women being randomized to natural cycle or estradiol and progesterone stimulation and anovulatory women being randomized to estradiol and progesterone or gonadotrophin stimulation. Serum estradiol and progesterone will be measured every two weeks from cycle day 2-3 until gestational age 9+6. Serum levels will be compared according to treatment regimens and cycle length. Furthermore, obstetric outcomes (live birth rates, birth weight, gestational age at birth, complications and malformations) and a possible association to serum estradiol and progesterone levels will be evaluated.

Ethics and dissemination: The three treatment regimens are all standard treatments and are comparable with regards to pregnancy rates (1). Patients will be following routine treatments, thus discomforts are limited to routine transvaginal ultrasound scans and additional blood testing. The study will be carried out in accordance with the Declaration of Helsinki and monitored by a GCP-unit. Positive, negative and inconclusive findings will be published in international peer-reviewed journals.

Registration: The trial is registered with clinicaltrials.gov under unique protocol ID 2020-001218-39.

Abbreviations

AE = adverse events
ESTRO-FET: Estradiol and progesterone levels in early pregnancy after natural, estradiol + progesterone or gonadotrophin stimulated frozen embryo transfer cycle

Strengths and limitations of this study

- An open label randomized controlled study design
- Separate randomization for normo-ovulatory and anovulatory women
- Regular blood sampling of se-estradiol and -progesterone from cycle day 2-3 until GA 9+6
- Concurrent creation of a biobank containing plasma, serum and full blood samples
Introduction

During the last three decades, frozen embryo transfer has been increasingly used in assisted reproductive technology (ART). Cryopreserved embryos are usually thawed and replaced in the uterus in either a natural cycle spontaneous ovulation or in a hormone replacement therapy cycle. Due to refinement of the cryopreservation technique pregnancy rates after frozen embryo transfer are in line with or according to some authors even better, than after a fresh cycle (2;2-4).

Normo-ovulatory women can be offered a natural or a hormonal stimulated cycle for endometrial preparation prior to frozen embryo transfer. Studies have shown that the two treatment modalities are comparable in regard to pregnancy rates (5). Hormonal stimulation is mandatory in anovulatory women, and most women are treated with estradiol for endometrial preparation and progesterone for luteal phase support. Women with unsatisfactory endometrial growth to estradiol stimulation can be treated with low dose gonadotrophin.

Estradiol is administered in a dose of 6-8 mg daily from cycle day 2-3 until gestational age 9 + 6. Progesterone is added four days before transfer and continued until gestational age 9+6. The optimal level of estradiol before transfer has been debated. Tonguc et al. found that administration of only 2 mg of estradiol was associated with increased risk of miscarriage compared to administration of or 6 mg (6).

Fetal exposure to estradiol in early pregnancy has been sparsely investigated. Animal studies have found that rats exposed to high levels of estradiol developed hyperinsulinemia measured in umbilical cord blood (7;8).

It may be a matter of clinical concern that high estradiol levels may affect the fetus and cause long term hormonal and metabolic disturbances. The aim of the present study is therefore to investigate serum estradiol levels in women conceived after natural, estradiol + progesterone or gonadotrophin stimulated FET.

Methods and analysis

Hypothesis

Women treated with estradiol in FET cycles have higher estradiol levels in the first trimester of pregnancy compared to women in a natural FET cycle or an FSH stimulated cycle.

Objectives
**Primary objectives:** To compare serum estradiol levels in early pregnancy in women conceived following FET in either unstimulated, FSH stimulated or estradiol + progesterone stimulated cycle.

**Secondary objectives:** To compare pregnancy outcome in the different treatment regimens.

- Live birth rates
- Gestational age at delivery
- Birth weight
- Obstetric complications (preeclampsia, gestational diabetes, intrauterine growth retardation)
- Malformations

**Design**

An open label randomized controlled trial including 300 women aged 18-40 years referred for IVF treatment. Patients will be recruited from the fertility clinic in Herlev and Hvidovre University Hospital. The patients will be randomized to either natural, estradiol + progesterone or FSH stimulated FET cycle.

**Patient population**

**Recruitment:** The women will be recruited from the existing patient populations in the fertility clinic of Herlev and Hvidovre University Hospital. The women have frozen blastocysts from prior oocyte retrieval. The patients will be recruited when they sign up for a FET cycle on the 1st or 2nd day of menstruation. Routine baseline data (visit 0) have been collected prior to recruitment to the present study, since the included women already have been referred to IVF/ICSI treatment. Thus, data from visit 0 are collected retrospectively after informed consent.

**Inclusion criteria**

- Age ≥ 18 years ≤ 40 years
- BMI ≤ 35 kg/m²
- Normal wet smear within the past three years
- Thawed day 5 or day 6 blastocysts after either IVF or ICSI treatment
Exclusion criteria

- Age < 18 years or > 40 years
- BMI > 35 kg/m²
- Oocyte donation
- HIV/ hepatitis
- Undiagnosed vaginal bleeding
- Uterine malformations
- Persisting ovarian cysts
- Tumors in hypothalamus, pituitary, thyroid or adrenal
- Previous breast cancer
- BRCA1/2
- Unregulated thyroid disease
- Cardiovascular disease
- Breast feeding
- Present or previous chemotherapy/radiation therapy
- Present or previous malignant disease
- Smoking
- Alcohol/drug abuse
- Hypersensitivity to estradiol, Bemfola, Ovitrelle or Cyclogest
- Porphyria
- Known missed abortion or ectopic pregnancy
- Serious hepatic dysfunction/disease
- Enlarged ovaries or ovarian cysts not caused by PCOS

Criteria for discontinuation of study drugs

- Safety considerations as assessed by the PI
- Withdrawal of informed consent

Routine measures

- Laboratory analyses at the clinical biochemical department: p-thyroid stimulation hormone
(p-TSH), p-estradiol, p-progesterone, p-follicle-stimulating hormone (p-FSH), p-luteinizing hormone (p-LH), p-prolactin, p-anti-Mullerian hormone (p-AMH), HIV, rubella and hepatitis at visit 0.

- Gynecological examination and transvaginal ultrasound including evaluation of AFC performed at the fertility clinic (Herlev or Hvidovre at visit 0).
- Endocervical culture (chlamydia, gonorrhea) performed by the general practitioner before referral.
- General physical examination: Height, weight, blood pressure, heart rate performed at the fertility clinic (Herlev or Hvidovre at visit 0 and visit 1)

**Randomization**

There is no theoretical difference in estradiol levels during early pregnancy in normo- and anovulatory women.

Normo-ovulatory women are randomized to either:

1. Natural FET cycle
2. Estradiol and progesterone stimulated FET cycle

Anovulatory women will be randomized to either:

1. Estradiol and progesterone stimulated FET
2. FSH stimulated FET

**Randomization procedure:** The “Randomization Module” in Research electronic data capture (Redcap) is used. The computer-generated allocation table was created by an independent statistician and randomization is stratified by site. Patients can be reincluded and re-randomized in the study if they fail to obtain pregnancy in the first or second treatment cycle.

**Blinding**

Due to the nature of the intervention it was deemed unrealistic to blind study participants and clinical staff, why the study is non-blinded.

**Study visits**
Visit 0 and 1, see overview

Visit 2

1. **Natural cycle**: Transvaginal ultrasound examination with measurement of the leading follicle is performed on cycle day 10. If the leading follicle is 17-18 mm the women are instructed in administration of subcutaneous HCG injection (250 microgram) at 10 p.m., and embryo transfer is planned 7 days later. If the leading follicle is less than 17-18 mm the women will be scheduled for a new scan a few days later.

2. **Estradiol + progesterone stimulated cycle**: Transvaginal ultrasound examination with measurement of endometrial thickness. If the endometrium is 7 mm or more embryo transfer is planned, and the women are instructed in administration of progesterone 400 mg vaginally morning and noon and one rectal administration at night from four days before embryo transfer. Se-hCG is measured 11 days after embryo transfer. In case of positive se-hCG, both estradiol and progesterone are continued until gestational week 9+6. If the endometrium is less than 7 mm the women will be scheduled for a new scan a few days later.

3. **FSH stimulated cycle**: Transvaginal ultrasound examination with measurement of the leading follicle is performed on cycle day 10. If the leading follicle is 17-18 mm the women are instructed in administration of subcutaneous HCG injection (250 microgram) at 10 p.m., and embryo transfer is planned 7 days later. If the leading follicle is less than 17-18 mm the women will be scheduled for a new scan a few days later.

All treatment regimens are standard treatments. In the daily clinical setting ovulatory women can choose between treatment 1 or 2. Most women choose 2 because it is more flexible with lower risk of cancellation. Anovulatory women are treated with treatment 2. Treatment three is primarily used for women, in whom the endometrium does not respond to estradiol tablets. When embryo transfer is planned the women will also have se-estradiol and -progesterone measured.

Visit 3 to 7, see visit overview

Visit overview
ESTRO-FET: Estradiol and progesterone levels in early pregnancy after natural, estradiol + progesterone or gonadotrophin stimulated frozen embryo transfer cycle

Version: 5.1 Date 30.08.2021

| General | Visit 0 | Visit 1 | Visit 2 | Visit 3 | Visit 4 | Visit 5 | Visit 6 | Visit 7 |
|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| Assessment of inclusion and exclusion criteria | x | | | | | | | |
| Signed consent for participation in the study | | x | | | | | | |
| Signed consent for thawing of frozen blastocyst | | | x | | | | | |
| Demography | | | | x | | | | |
| Medical history | | | | x | | | | |
| Concomitant medication | | | | | x | | | |
| Menstrual cycle registration | | | | | | x | | |

| Clinical examination | Visit 0 | Visit 1 | Visit 2 | Visit 3 | Visit 4 | Visit 5 | Visit 6 | Visit 7 |
|----------------------|---------|---------|---------|---------|---------|---------|---------|---------|
| Blood pressure, heart rate | x | x | | | | | | |
| Height/ weight | x | x | | | | | | |
| Transvaginal ultrasound | x | x | x | | x | x | | |

| Bio samples | Visit 0 | Visit 1 | Visit 2 | Visit 3 | Visit 4 | Visit 5 | Visit 6 | Visit 7 |
|-------------|---------|---------|---------|---------|---------|---------|---------|---------|
| Se-estradiol | x | x | x | x | x | x | x | x |
| Se-progesterone | x | x | x | x | x | x | x | x |
| Se-HCG | | | | | | | | x |

| Procedures | Visit 0 | Visit 1 | Visit 2 | Visit 3 | Visit 4 | Visit 5 | Visit 6 | Visit 7 |
|-------------|---------|---------|---------|---------|---------|---------|---------|---------|
| Randomization | x | | | | | | | |
| Transfer of a frozen, thawed blastocyst | | | | | | | | x |

**Treatment regimens and study medication**

1. **Natural cycle:** HCG (250 microgram) subcutaneous injection when the leading follicle is 17-18 mm.

2. **Estradiol + progesterone stimulated cycle:** Estradiol 6-8 mg (oral administration) daily from cycle day 2-3. Progesterone 400 mg x 3 daily (vaginal administration x2, rectal administration x1) from 4 days before embryo transfer. Both are continued until gestational week 9+6.
3. FSH-stimulated cycle: Subcutaneous injection of 50-75 IE recombinant FSH from cycle day 2-3 and until the leading follicle is 17-18 mm, where 250 microgram subcutaneous HCG is administered. Embryo transfer is planned 7 days after HCG administration.

The study medication will be collected from a pharmacy by the patient and the medication packaging will be brought to the next study visit for inspection.

**Compliance to study medication**

Compliance to study medication will be evaluated orally with the individual patient for every visit at the fertility clinic.

**Data management**

*Data collection and processing:* Source data will be recorded in the patient record or on specific worksheets. Data will be stored in Redcap. A Case Report Form (CRF) will be constructed in RedCap for data capture. Data will be stored in coded form in 15 years according to recommendations from "Videnscenter for dataanmeldelser", Rigshospitalet. Before recruitment and signed consent information on prior hospitalizations, chronic disease and medication will be obtained from the patient’s electronic medical journal. After signed consent and termination of the study/delivery obstetric information will be obtained from the patient’s electronic medical journal. A separate log for re-included women is kept, in order to prevent data from the same women to appear twice in the analyses. If a reincluded women obtains pregnancy only data from that cycle will be used.

*Biobank:* A bio bank will be established at the Fertility Clinic, Herlev University Hospital. This bio bank will store blood samples in coded form for later analysis of biomarkers. Samples will be stored at -80°C (in total 24 ml blood). Blood samples from the biobank will be stored for 15 years provided patient consent. Hereafter the material will be destroyed. Additional analyses will only be performed after approval from the Ethics Committee and the material will not be carried out of the country. The patients can participate in the study without having biologic material stored in the biobank.
**Data analysis:** The per protocol population will consist of all patients who completed the study with a documented valid baseline and pregnancy rates, without any major protocol violations. Analysis of the primary outcome parameter is difference in se-estradiol levels in early pregnancy after FET in either natural, estradiol + progesterone or gonadotrophin stimulated cycle. Se-estradiol levels will be compared after the two different treatments within the groups and between the groups. Obstetric outcomes (secondary objectives) will only be compared within the two groups as anovulatory women have an a priori increased risk of obstetric complications. The absolute values of serum estradiol for the different groups, and the 95 % confidence interval will be presented. Normally distributed variables will be presented as mean ± SD, non-parametric statistics and appropriate log-transformation will be performed if assumption of normality is not met. After log transformation the parameter will be further tested for normality distribution as indicated. A two-tailed p value of 0.05 or less is considered statistically significant. Comparisons between treatment groups will be performed by an unpaired two-sample t-test, Mann-Whitney test or Chi-squared test as appropriate.

**Power analysis:** A statistical power analysis was performed for sample size estimation, based on data from previous studies regarding serum estradiol levels in early, naturally conceived, pregnancies (9). The median serum estradiol level in gestational week 7 is 3.3 nmol/L. Using the clinically relevant effect size of 20% absolute increase in serum estradiol levels in estradiol treated FET with a two-sided significance level of 0.05 and with 80% power, the projected sample size needed is N = 83 women. To allow for an estimated drop-out or major protocol deviations rate of 5 % we will need to include 100 patients in each arm of this study. Dropouts will be replaced by new patients.

**Quality control and assurance**

The study will be carried out in accordance with the Helsinki Declaration, EU Directive on GCP and ICH-GCP guidelines after approval by the Regional Scientific Ethics Committee, Danish Medicines Agency and the “Videnscenter for dataanmeldelse”, Rigshospitalet. The study will be registered on www.clinicaltrials.gov and monitored by the GCP unit at Frederiksberg Hospital. Audits will be planned and executed in collaboration with sponsor and PI.
Financial remuneration

The study subjects will receive no financial remuneration for participation in the study, as all the treatments investigated are standard treatments.

Study time line

Approval from the Ethics Committee Dec. 2020
Approval from the Danish Medicines Agency Nov. 2020
Approval from GCP Jan. 2021
Study start (First patient first visit) April 2021
Completion of the study (last patient last visit) April. 2023
Data analysis May 2023
Publications July-October 2023

Ethics and dissemination

Patient discomforts and risks

Transvaginal ultrasound is a non-invasive procedure without any known side effect from the sound waves used. The procedure can be associated with minor discomfort in some women and there is a minimal risk of allergic reaction to the examination gel. Since this study is an evaluation of standard procedures additional discomfort is limited to weekly blood sampling during the first 10 weeks of pregnancy. An estimated 150 ml of blood will be collected in total.

Informed consent

Subjects recruited will attend an outpatient clinic at the Fertility Clinic. The PI or subinvestigator will ensure that the subject is adequately informed about the study background and design, in orally and in writing. The written patient information will be sent out to all potentially eligible subjects along with the brochure: "Your rights as a participant in biomedical research".

Before signing the consent form, the subject will be given 24 hours to reflect. The subjects are informed that they may, at any time, withdraw their informed consent to participate in the study without it having consequences for their future treatment at the study site. The subjects will sign
three different consent forms: a form concerning the woman’s participation in the study, a form concerning access to the medical journal of the child and a form concerning the creation of a biobank. Consent to store blood samples in a biobank is optional and subjects can participate regardless. No study-related examinations will be conducted until after the informed consent has been obtained.

Insurance

Patients are covered under existing law of product liability insurance for the study drug, and “Patienterstatningen” (Patient insurance).

Publication plan

Positive, negative and inconclusive study results will be published in international peer reviewed scientific journals and made publicly available at www.clinicaltrials.gov. The PhD student, Nina Freiesleben Mørch, will be first author and Pernille F. Svendsen last author.

Patient and public involvement

Patients and public were not involved in the planning of this study.

Discussion

The use of FET is increasing in fertility treatment due to refinement in techniques and equipment. The women receiving stimulation with estradiol and progesterone are exposed to a high dosage of both hormones during the first 10 weeks of pregnancy and the literature on concurrent fetal exposure is very limited. Likewise, the long-term effect of exposure to high levels of estradiol in the early human fetal development has been scarcely investigated. The findings of hormonal and metabolic disturbances in animal studies constitutes a concern for children born after estradiol and progesterone stimulated FET. If the present study finds a significant difference in serum levels between treatment groups, it will be relevant to examine possible hormone and metabolic disturbances in off springs born after exposure to estradiol in the first trimester.
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Authors’ contributions

The original idea and conceptualization was provided by Pernille Fog Svendsen, who also drafted the original protocol and obtained approvals from the Ethics Committee and Danish Medical Agencies. Nina Freiesleben Mørch contributed with revisions to the protocol, practical design and
assistance in obtaining GCP approval. Mette Petri Lauritsen contributed in drafting of the original protocol.

**Funding statement**

This work is supported by Gedeon Richter with a grant of 400,000 DKK. The study has also received a grant of 400,000 DKK by Gangsted-Rasmussen Group. The local reasearch board of Herlev and Gentofte University Hospital has provided a grant of 60,000 DKK. Funders had no role in the design of the study.

**Competing Interests Statement**

Gedeon Richter is the market holder of two types of the study medication; Bemfola and Cyclogest. The company is also a financial supporter of the trial and thus represents a conflict of interest. The company is not invovled in execution of the study and will not be involved in the following data mangement and publication process of the study.
# Reporting checklist for protocol of a clinical trial.

Based on the SPIRIT guidelines.

## Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

Your article may not currently address all the items on the checklist. Please modify your text to include the missing information. If you are certain that an item does not apply, please write "n/a" and provide a short explanation.

Upload your completed checklist as an extra file when you submit to a journal.

In your methods section, say that you used the SPIRIT reporting guidelines, and cite them as:

Chan A-W, Tetzlaff JM, Gøtzsche PC, Altman DG, Mann H, Berlin J, Dickersin K, Hróbjartsson A, Schulz KF, Parulekar WR, Krleža-Jerić K, Laupacis A, Moher D. SPIRIT 2013 Explanation and Elaboration: Guidance for protocols of clinical trials. BMJ. 2013;346:e7586

| Reporting Item | Page Number |
|----------------|-------------|
| **Administrative information** | |
| Title | #1 | Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym | 1 |
Trial registration

Trial registration: All items from the World Health Organization Trial Registration Data Set

Protocol version

Funding

Roles and responsibilities:

Roles and responsibilities:

Roles and responsibilities:

Roles and responsibilities:

Roles and responsibilities:
other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)

Introduction

Background and rationale

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

Background and rationale: choice of comparators

Explanation for choice of comparators

Objectives

Specific objectives or hypotheses

Trial design

Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, non-inferiority, exploratory)

Methods:

Participants, interventions, and outcomes

Study setting

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
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)

Interventions:  #11a Interventions for each group with sufficient detail to allow replication, including how and when they will be administered

Interventions:  #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)

Interventions:  #11c Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)

Interventions:  #11d Relevant concomitant care and interventions that are permitted or prohibited during the trial

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
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)

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

Recruitment #15  Strategies for achieving adequate participant enrolment to reach target sample size

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

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

Allocation: #16c Who will generate the allocation sequence, who will enrol
implementation participants, and who will assign participants to
interventions

Blinding (masking) #17a Who will be blinded after assignment to interventions (eg,
emergency trial participants, care providers, outcome assessors, data
unblinding analysts), and how

Blinding (masking): #17b If blinded, circumstances under which unblinding is
emergency permissible, and procedure for revealing a participant’s
unblinding allocated intervention during the trial

Methods: Data
collection,
management, and
analysis

Data collection plan #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
Data collection plan: 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

Data management 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

Statistics: outcomes 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

Statistics: additional analyses Methods for any additional analyses (eg, subgroup and adjusted analyses)

Statistics: analysis Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)

Methods: Monitoring

Data monitoring: 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.

Data monitoring: **#21b** Description of any interim analyses and stopping interim analysis guidelines, including who will have access to these interim results and make the final decision to terminate the trial.

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.

Auditing **#23** Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor.

Ethics and dissemination

Research ethics **#24** Plans for seeking research ethics committee / institutional review board (REC / IRB) approval.

Protocol amendments **#25** Plans for communicating important protocol modifications (e.g., changes to eligibility criteria, outcomes, analyses) to relevant parties (e.g., investigators, REC / IRBs, trial participants, trial registries, journals, regulators).
Consent or assent: **#26a** Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)

Consent or assent: **#26b** Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable

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

Declaration of interests: **#28** Financial and other competing interests for principal investigators for the overall trial and each study site

Data access: **#29** Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators

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

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
Dissemination policy: \#31b Authorship eligibility guidelines and any intended use of authorship professional writers

Dissemination policy: \#31c Plans, if any, for granting public access to the full reproducible research protocol, participant-level dataset, and statistical code

Appendices

Informed consent \#32 Model consent form and other related documentation materials given to participants and authorised surrogates

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

None The SPIRIT Explanation and Elaboration paper is distributed under the terms of the Creative Commons Attribution License CC-BY-NC. This checklist can be completed online using https://www.goodreports.org/, a tool made by the EQUATOR Network in collaboration with Penelope.ai
Estradiol and progesterone levels in early pregnancy after natural, estradiol + progesterone or gonadotrophin stimulated frozen embryo transfer cycle: A randomized controlled trial protocol.

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Estradiol and progesterone levels in early pregnancy after natural, estradiol + progesterone or gonadotrophin stimulated frozen embryo transfer cycle: A randomized controlled trial protocol.

The ESTRO-FET study

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Abstract

Introduction: Frozen embryo transfer is being increasingly used for assisted reproductive therapy and offers similar pregnancy rates as treatment with fresh embryo transfer. In women with regular menstrual cycles transfer of a frozen thawed blastocyst can be performed in either a natural cycle or substituted cycle. Anovulatory women can only be offered a substituted or stimulated cycle. Knowledge on fetal exposure to estradiol in early pregnancy is very limited but studies on mice and rats have shown hormonal and metabolic disturbances in cubs born from estradiol exposed mothers. We aim to investigate serum estradiol and progesterone levels in women conceived after natural, estradiol and progesterone or gonadotrophin stimulated frozen embryo transfer.

Methods and analysis: The study is an open label randomized controlled trial with normo-ovulatory women being randomized to natural cycle or estradiol and progesterone substitution and anovulatory women being randomized to estradiol and progesterone substitution or gonadotrophin stimulation. Serum estradiol and progesterone will be measured every two weeks from cycle day 2-3 until gestational age 9+6. Serum levels will be compared according to treatment regimens and cycle length. Furthermore, obstetric outcomes (live birth rates, birth weight, gestational age at birth, complications and malformations) and a possible association to serum estradiol and progesterone levels will be evaluated.

Ethics and dissemination: The three treatment regimens are all standard treatments and are comparable with regards to pregnancy rates. Patients will be following routine treatments, thus discomforts are limited to routine transvaginal ultrasound scans and additional blood testing. The study is approved by the Scientific Ethical Committee of the Capital Region of Denmark and the Danish Medicines Agency. The study will be carried out in accordance with the Declaration of Helsinki and monitored by a GCP-unit. Positive, negative and inconclusive findings will be published in international peer-reviewed journals.

Registration details: The trial is registered with ClinicalTrials.gov under unique protocol-ID NCT04997525 and with EU Clinical Trials Register under trial-ID 2020-001218-39

Abbreviations
**Strengths and limitations of this study**

- An randomized controlled study design
- Separate randomizations for normo-ovulatory and anovulatory women
- The interventions are not blinded to subjects or clinicians
- The trial will be multicenter based for a more representative study population
Introduction

During the last three decades, frozen embryo transfer has been increasingly used in assisted reproductive technology (ART). Cryopreserved embryos are usually thawed and replaced in the uterus in either a natural cycle spontaneous ovulation or in a hormone replacement therapy cycle. Due to refinement of the cryopreservation technique pregnancy rates after frozen embryo transfer are in line with or according to some authors even better, than after a fresh cycle (1;1-3).

Normo-ovulatory women can be offered a natural or a hormone substituted cycle for endometrial preparation prior to frozen embryo transfer. Hormonal stimulation or substitution is mandatory in anovulatory women, and most women are treated with estradiol for endometrial preparation and progesterone for luteal phase support. Women with unsatisfactory endometrial growth to estradiol substitution can be treated with low dose gonadotrophin. The three treatment regimens are all standard treatments and are comparable with regards to pregnancy rates (4).

Estradiol is administered in a dose of 6-8 mg daily from cycle day 2-3 until gestational age 9 + 6. Progesterone is added four days before the day of transfer and continued until gestational age 9+6. The optimal level of estradiol before transfer has been debated. Tonguc et al. found that administration of only 2 mg of estradiol was associated with increased risk of miscarriage compared to administration of 6 mg (5).

Fetal exposure to estradiol in early pregnancy has been sparsely investigated. Animal studies have found that rats exposed to high levels of estradiol developed hyperinsulinemia measured in umbilical cord blood (6;7).

It may be a matter of clinical concern that high estradiol levels may affect the fetus and cause long term hormonal and metabolic disturbances. The aim of the present study is therefore to investigate serum estradiol levels in women conceived after natural, estradiol + progesterone or gonadotrophin stimulated FET.

Methods and analysis

Hypothesis

Women treated with estradiol in FET cycles have higher estradiol levels in the first trimester of pregnancy compared to women in a natural FET cycle or an FSH stimulated cycle.

Objectives
**Primary objectives:** To compare serum estradiol levels in early pregnancy in women conceived following FET in either unstimulated, FSH stimulated or estradiol + progesterone substituted cycle.

**Secondary objectives:** To compare pregnancy outcome in the different treatment regimens.
- Live birth rates defined as one or more neonates born after 24 weeks of gestation.
- Gestational age at delivery
  - Full term defined as > 37 weeks of gestation, preterm defined as 32 to 37 weeks of gestation, very preterm defined as 28 to 32 weeks of gestation and extremely preterm defined as < 28 weeks of gestation.
- Birth weight
  - Low birth weight defined as < 2500 g and very low birth weight defined as < 1500 g
- Obstetric complications (preeclampsia, gestational diabetes, intrauterine growth retardation)
- Malformations detected at routine fetal ultrasound scans or at birth

**Design**
An open label randomized controlled trial including 300 women aged 18-40 years referred for IVF treatment. Patients will be recruited from the fertility clinic in Herlev and Hvidovre University Hospital. The patients will be randomized to either natural, estradiol + progesterone or FSH stimulated FET cycle.

**Patient population**
*Recruitment:* The women will be recruited from the existing patient populations in the fertility clinic of Herlev and Hvidovre University Hospital. The women have frozen blastocysts from prior oocyte retrieval. The patients will be recruited when they sign up for a FET cycle on the 1st or 2nd day of menstruation. Routine baseline data (visit 0) have been collected prior to recruitment to the present study, since the included women already have been referred to IVF/ICSI treatment. Thus, data from visit 0 are collected retrospectively after informed consent.

**Inclusion criteria**
- Age ≥ 18 years ≤ 40 years
BMI \leq 35 \text{ kg/m}^2

- Normal wet smear within the past three years
- Thawed day 5 or day 6 blastocysts after either IVF or ICSI treatment

**Exclusion criteria**

- Age < 18 years or > 40 years
- BMI > 35 \text{ kg/m}^2
- Oocyte donation
- HIV/ hepatitis
- Undiagnosed vaginal bleeding
- Uterine malformations
- Persisting ovarian cysts
- Tumors in hypothalamus, pituitary, thyroid or adrenal glands
- Previous breast cancer
- BRCA1/2
- Unregulated thyroid disease
- Cardiovascular disease
- Breast feeding
- Present or previous chemotherapy/radiation therapy
- Present or previous malignant disease
- Smoking
- Alcohol/drug abuse
- Hypersensitivity to estradiol, Bemfola, Ovitrelle or Cyclogest
- Porphyria
- Known missed abortion or ectopic pregnancy
- Serious hepatic dysfunction/disease
- Enlarged ovaries or ovarian cysts not caused by PCOS

**Criteria for discontinuation of study drugs**

- Safety considerations as assessed by the PI
- Withdrawal of informed consent
Routine measures

- Laboratory analyses at the clinical biochemical department: p-thyroid stimulation hormone (p-TSH), p-estradiol, p-progesterone, p-follicle-stimulating hormone (p-FSH), p-luteinizing hormone (p-LH), p-prolactin, p-anti-Mullerian hormone (p-AMH), HIV, rubella and hepatitis at visit 0.
- Gynecological examination and transvaginal ultrasound including evaluation of AFC performed at the fertility clinic (Herlev or Hvidovre at visit 0).
- Endocervical culture (chlamydia, gonorrhea) performed by the general practitioner before referral.
- General physical examination: Height, weight, blood pressure, heart rate performed at the fertility clinic (Herlev or Hvidovre at visit 0 and visit 1)

Randomization

There is no theoretical difference in estradiol levels during early pregnancy in normo- and anovulatory women.

Normo-ovulatory women are randomized to either:
1. Natural FET cycle
2. Estradiol and progesterone substituted FET cycle

Anovulatory women will be randomized to either:
1. Estradiol and progesterone substituted FET cycle
2. FSH stimulated FET cycle

Randomization procedure: The “Randomization Module” in Research electronic data capture (Redcap) is used. The computer-generated allocation table was created by an independent statistician and randomization is stratified by site. Patients can be reincluded and re-randomized in the study if they fail to obtain pregnancy in the first or second treatment cycle.

Blinding

Due to the nature of the intervention it was deemed unrealistic to blind study participants and
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clinical staff, why the study is non-blinded.

Study visits

Visit 0 and 1, see overview

Visit 2

1. **Natural cycle:** Transvaginal ultrasound examination with measurement of the leading follicle is performed on cycle day 10. If the leading follicle is 17-18 mm the women are instructed in administration of subcutaneous HCG injection (250 microgram) at 10 p.m., and embryo transfer is planned 7 days later. If the leading follicle is less than 17-18 mm the women will be scheduled for a new scan a few days later.

2. **Estradiol + progesterone substituted cycle:** Transvaginal ultrasound examination with measurement of endometrial thickness. If the endometrium is 7 mm or more embryo transfer is planned, and the women are instructed in administration of progesterone 400 mg vaginally morning and noon and one rectal administration at night from four days before embryo transfer. Se-hCG is measured 11 days after embryo transfer. In case of positive se-hCG, both estradiol and progesterone are continued until gestational week 9+6. If the endometrium is less than 7 mm the women will be scheduled for a new scan a few days later.

3. **FSH stimulated cycle:** Transvaginal ultrasound examination with measurement of the leading follicle is performed on cycle day 10. If the leading follicle is 17-18 mm the women are instructed in administration of subcutaneous HCG injection (250 microgram) at 10 p.m., and embryo transfer is planned 7 days later. If the leading follicle is less than 17-18 mm the women will be scheduled for a new scan a few days later.

All treatment regimens are standard treatments. In the daily clinical setting ovulatory women can choose between treatment 1 or 2. Most women choose treatment 2 because it is more flexible with lower risk of cancellation. Anovulatory women are treated with treatment 2. Treatment 3 is primarily used for women, in whom the endometrium does not respond to estradiol tablets. When embryo transfer is planned the women will also have se-estradiol and -progesterone measured.
Visit 3 to 7, see visit overview

Table 1: Visit overview

|                        | Visit 0 Before IVF/ICSI | Visit 1 | Visit 2 | Visit 3 | Visit 4 | Visit 5 | Visit 6 | Visit 7 |
|------------------------|-------------------------|---------|---------|---------|---------|---------|---------|---------|
| **General**            |                         |         |         |         |         |         |         |         |
| Assessment of inclusion and exclusion criteria | x                      |         |         |         |         |         |         |         |
| Signed consent for participation in the study | x                      |         |         |         |         |         |         |         |
| Signed consent for thawing of frozen blastocyst | x                      |         |         |         |         |         |         |         |
| Demography             |                         | x       |         |         |         |         |         |         |
| Medical history        |                         |         | x       |         |         |         |         |         |
| Concomitant medication |                         |         |         | x       |         |         |         |         |
| Menstrual cycle registration | x                      |         |         |         |         |         |         |         |
| **Clinical examination** |                         |         |         |         |         |         |         |         |
| Blood pressure, heart rate |                         | x       |         | x       |         |         |         |         |
| Height/ weight         |                         | x       |         | x       |         |         |         |         |
| Transvaginal ultrasound |                         | x       | x       | x       | x       | x       | x       |         |
| **Bio samples**        |                         | x       | x       | x       | x       | x       | x       | x       |
| Se-estradiol           |                         |         |         |         |         |         |         |         |
| Se-progesterone        |                         |         |         |         |         |         |         |         |
| Se-HCG                 |                         |         |         |         |         |         |         |         |
| **Procedures**         |                         |         |         |         |         |         |         |         |
| Randomization          |                         |         |         |         |         |         |         |         |
| Transfer of a frozen, thawed blastocyst | x                      |         |         |         |         |         |         |         |

The Visit Overview Table 1 shows the general information, clinical examinations, bio samples and procedures that are performed or collected at each of the study visits.
### Treatment regimens and study medication

1. **Natural cycle:** HCG (250 microgram) subcutaneous injection when the leading follicle is 17-18 mm.
2. **Estradiol + progesterone substituted cycle:** Estradiol 6-8 mg (oral administration) daily from cycle day 2-3. Progesterone 400 mg x 3 daily (vaginal administration x2, rectal administration x1) from 4 days before embryo transfer. Both are continued until gestational week 9+6.
3. **FSH stimulated cycle:** Subcutaneous injection of 50-75 IE recombinant FSH from cycle day 2-3 and until the leading follicle is 17-18 mm, where 250 microgram subcutaneous HCG is administered. Embryo transfer is planned 7 days after HCG administration.

The study medication will be collected from a pharmacy by the patient and the medication packaging will be brought to the next study visit for inspection.

### Compliance to study medication

Compliance to study medication will be evaluated orally with the individual patient for every visit at the fertility clinic.

### Data management

*Data collection and processing:* Source data will be recorded in the patient record or on specific worksheets. Data will be stored in Redcap. A Case Report Form (CRF) will be constructed in RedCap for data capture. Data will be stored in coded form in 15 years according to recommendations from Danish Knowledge Center for Data Reporting. Before recruitment and signed consent information on prior hospitalizations, chronic disease and medication will be obtained from the patient’s electronic medical journal. After signed consent and termination of the study/delivery obstetric information will be obtained from the patient’s electronic medical journal.

A separate log for re-included women is kept, in order to prevent data from the same women to appear twice in the analyses. If a reincluded women obtains pregnancy only data from that cycle will be used.
Biobank: A bio bank will be established at the Fertility Clinic, Herlev University Hospital. This bio bank will store blood samples in coded form for later analysis of biomarkers. Samples will be stored at -80°C (in total 24 ml blood). Blood samples from the biobank will be stored for 15 years provided patient consent. Hereafter the material will be destroyed. Additional analyses will only be performed after approval from the Ethics Committee and the material will not be carried out of the country. The patients can participate in the study without having biologic material stored in the biobank.

Data analysis: The per protocol population will consist of all patients who completed the study with a documented valid baseline and pregnancy rates, without any major protocol violations. Analysis of the primary outcome parameter is difference in se-estradiol levels in early pregnancy after FET in either natural, estradiol + progesterone or gonadotrophin stimulated cycle. Se-estradiol levels will be compared after the two different treatments within the groups and between the groups. Obstetric outcomes (secondary objectives) will only be compared within the two groups as anovulatory women have an a priori increased risk of obstetric complications. The absolute values of serum estradiol for the different groups, and the 95 % confidence interval will be presented. Normally distributed variables will be presented as mean ± SD, non-parametric statistics and appropriate log-transformation will be performed if assumption of normality is not met. After log transformation the parameter will be further tested for normality distribution as indicated. A two-tailed p value of 0.05 or less is considered statistically significant. Comparisons between treatment groups will be performed by an unpaired two-sample t-test, Mann-Whitney test or Chi-squared test as appropriate. Additional analysis due to loss of follow-up: Data from the Intention to Treat Population will be analyzed to determine the validity of the conclusions of the per protocol population. Analysis will include duration in study and reason for discontinuation as co-variables. Discontinuation of the study solely due to the woman not becoming pregnant, will not be considered a dropout since this will be the predictable outcome for approximately 50% of the included women.

Power analysis: A statistical power analysis was performed for sample size estimation, based on data from previous studies regarding serum estradiol levels in early, naturally conceived, pregnancies (8). The median serum estradiol level in gestational week 7 is 3.3 nmol/L. Using the assumed clinically relevant effect size of 20% absolute increase in serum estradiol levels in estradiol treated FET with a two-sided significance level of 0.05 and with 80% power, the projected
sample size needed is \( N = 83 \) women. Approximately 50% of FET treatments result in pregnancy leading to a total of 42 pregnant women in each arm. To allow for an estimated drop-out or major protocol deviations rate of 5% we will need to include 100 patients in each arm of this study. Dropouts will be replaced by new patients.

**Patient and public involvement**

Patients and public were not involved in the planning and development of this study and will not be involved in the conduct of the trial. Dissemination of the results to study participants will be conveyed through links to online articles on the websites of the involved fertility clinics.

**Financial remuneration**

The study subjects will receive no financial remuneration for participation in the study, as all the treatments investigated are standard treatments.

**Study time line**

- Approval from the Ethics Committee: Dec. 2020
- Approval from the Danish Medicines Agency: Nov. 2020
- Approval from GCP: Jan. 2021
- Study start (First patient first visit): April 2021
- Completion of the study (last patient last visit): April 2023
- Data analysis: May 2023
- Publications: July-October 2023

**Ethics and dissemination**

**Patient discomforts and risks**

Transvaginal ultrasound is a non-invasive procedure without any known side effect from the sound waves used. The procedure can be associated with minor discomfort in some women and there is a minimal risk of allergic reaction to the examination gel. Since this study is an evaluation of standard procedures additional discomfort is limited to weekly blood sampling during the first 10 weeks of pregnancy. An estimated 150 ml of blood will be collected in total.
Informed consent

Subjects recruited will attend an outpatient clinic at the Fertility Clinic. The PI or subinvestigator will ensure that the subject is adequately informed about the study background and design, orally and in writing. The written patient information will be sent out to all potentially eligible subjects along with the brochure: "Your rights as a participant in biomedical research".

Before signing the consent form, the subject will be given 24 hours to reflect. The subjects are informed that they may, at any time, withdraw their informed consent to participate in the study without it having consequences for their future treatment at the study site. The subjects will sign three different consent forms: a form concerning the woman’s participation in the study, a form concerning access to the medical journal of the child and a form concerning the creation of a biobank. Consent to store blood samples in a biobank is optional and subjects can participate regardless. No study-related examinations will be conducted until after the informed consent has been obtained.

Adverse events

Data on other AEs, SAEs and SUSAR, including abnormal laboratory values, as assessed by the investigator as clinically significant will be collected and recorded on standardized forms at each contact. These data are reported to relevant authorities in accordance with applicable laws and ICH-GCP guidelines.

Quality control and assurance

The study will be carried out in accordance with the Helsinki Declaration, EU Directive on GCP and ICH-GCP guidelines. The trial is approval by the Scientific Ethical Committee of the Capitol Region of Denmark, the Danish Medicines Agency and the Danish Knowledge Center for Data Reporting.

The study has been registered on www.clinicaltrials.gov (NCT04997525) and with EU Clinical Trials Register under trial-ID 2020-001218-39. Monitoring will be carried out by the GCP unit at Frederiksberg Hospital. Audits will be planned and executed in collaboration with sponsor and PI.

Insurance
Patients are covered under existing law of product liability insurance for the study drug, and “Patienterstatningen” (Patient insurance).

Data sharing and publication plan

Data obtained from this trial will only be shared according to the ICMJE guidelines. Positive, negative and inconclusive study results will be published in international peer reviewed scientific journals and made publicly available at www.clinicaltrials.gov. The PhD student, Nina Freiesleben Mørch, will be first author and Pernille F. Svendsen last author.

Discussion

The use of FET is increasing in fertility treatment due to refinement in techniques and equipment. The women receiving substitution with estradiol and progesterone are exposed to a high dosage of both hormones during the first 10 weeks of pregnancy and the literature on concurrent fetal exposure is very limited. Likewise, the long-term effect of exposure to high levels of estradiol in the early human fetal development has been scarcely investigated. The findings of hormonal and metabolic disturbances in animal studies constitutes a concern for children born after estradiol and progesterone substituted FET. If the present study finds a significant difference in serum levels between treatment groups, it will be relevant to examine possible hormonal and metabolic disturbances in off spring born after exposure to estradiol in the first trimester.

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Authors’ contributions

The original idea and conceptualization was provided by Pernille Fog Svendsen, who also draftet the original protocol and obtained approvals from the Ethics Committee and Danish Medical Agencies. Nina Freiesleben Morch contributed with revisions to the protocol, practical design and assistance in obtaining GCP approval. Mette Petri Lauritsen contributed in drafting of the original protocol.

Funding statement

This work is supported by Gedeon Richter with a grant of 625,000 DKK. The study has also received a grant of 400,000 DKK by Gangsted-Rasmussen Group. The local research board of Herlev and Gentofte University Hospital has provided a grant of 60,000 DKK. Funders had no role in the design of the study.
Competing Interests Statement

Gedeon Richter is the market holder of two of the study drugs used in this trial; Bemfola and Cyclogest. The company is also a financial supporter of the study and thus represents a conflict of interest. The company is not involved in the execution of the trial and will not be involved in the following data management and publication process.
Reporting checklist for protocol of a clinical trial.

Based on the SPIRIT guidelines.

**Instructions to authors**

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

Your article may not currently address all the items on the checklist. Please modify your text to include the missing information. If you are certain that an item does not apply, please write "n/a" and provide a short explanation.

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In your methods section, say that you used the SPIRIT reporting guidelines, and cite them as:

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| Reporting Item | 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 |
| Trial registration: dataset | #2b | All items from the World Health Organization Trial Registration Data Set | N/A |
| Protocol version | #3 | Date and version identifier | 1 |
| Funding | #4 | Sources and types of financial, material, and other support | 15 |
| Roles and responsibilities: contributorship | #5a | Names, affiliations, and roles of protocol contributors | 15 |
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

Background and rationale: choice of comparators #6b Explanation for choice of comparators

Objectives #7 Specific objectives or hypotheses 4-5

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, non-inferiority, exploratory)

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.

| Eligibility criteria | #10 | Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (e.g., surgeons, psychotherapists) |
|----------------------|-----|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Interventions:       | #11a| Interventions for each group with sufficient detail to allow replication, including how and when they will be administered.                                                                                                                                  |
| description          |     |                                                                                                                                                                                                                                                            |
| Interventions:       | #11b| Criteria for discontinuing or modifying allocated interventions for a given trial participant (e.g., drug dose change in response to harms, participant request, or improving/worsening disease)                                                                                     |
| modifications        |     |                                                                                                                                                                                                                                                            |
| Interventions:       | #11c| Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (e.g., drug tablet return; laboratory tests)                                                                                                               |
| adherence            |     |                                                                                                                                                                                                                                                            |
| Interventions:       | #11d| Relevant concomitant care and interventions that are permitted or prohibited during the trial.                                                                                                                                                                 |
| concomitant care     |     |                                                                                                                                                                                                                                                            |
| Outcomes             | #12 | Primary, secondary, and other outcomes, including the specific measurement variable (e.g., systolic blood pressure), analysis metric (e.g., change from baseline, final value, time to event), method of aggregation (e.g., median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended |
| 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)                                                                                                                     |
| 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                                                                                                      |
| Recruitment          | #15 | Strategies for achieving adequate participant enrolment to reach target sample size.                                                                                                                                                                         |
|                      |     |                                                                                                                                                                                                                                                            |
**Methods:**

**Assignment of interventions (for controlled trials)**

Allocation: sequence generation  
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.

Allocation concealment mechanism  
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.

Allocation: implementation  
Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions.

Blinding (masking)  
Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how.

Blinding (masking): emergency unblinding  
If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant’s allocated intervention during the trial.

**Methods: Data collection, management, and analysis**

Data collection plan  
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

Data collection plan: retention #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

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

Statistics: outcomes #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

Statistics: additional analyses #20b Methods for any additional analyses (eg, subgroup and adjusted analyses)

Statistics: analysis population and missing data #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)

Methods: Monitoring

Data monitoring: formal committee #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

Data monitoring: interim analysis #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

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

| Auditing          | #23 | Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor |
|-------------------|-----|--------------------------------------------------------------------------------------------------|
| Ethics and        |     |                                                                                                  |
| dissemination     |     |                                                                                                  |
| Research ethics   | #24 | Plans for seeking research ethics committee / institutional review board (REC / IRB) approval |
| approval          |     |                                                                                                  |
| 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) |
| Consent or assent | #26a| Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32) |
| Consent or assent: ancillary studies | #26b| Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable |
| 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 |
| Declaration of interests | #28 | Financial and other competing interests for principal investigators for the overall trial and each study site |
| Data access       | #29 | Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators |
| 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 |
| Dissemination policy: trial results | #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

Dissemination policy: #31b Authorship eligibility guidelines and any intended use of professional writers

Dissemination policy: #31c Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code

Appendices

Informed consent materials #32 Model consent form and other related documentation given to participants and authorised surrogates Appx 1

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 10-11

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