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TITLE PAGE

PROTOCOL ARTICLE

A "Freeze-all" versus a "Fresh embryo transfer"-strategy in assisted reproductive technology (ART): Study protocol for a multicentre randomised controlled trial

Authors

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ABSTRACT

Introduction Pregnancy rates after frozen-thawed embryo transfer (FET) have improved in recent years and are now approaching or even exceeding those obtained after fresh embryo transfer. This is partly due to improved laboratory techniques, but may also be caused by a more physiological hormonal and endometrial environment in FET cycles. Furthermore, the risk of ovarian hyperstimulation syndrome (OHSS) is practically eliminated in segmentation cycles followed by FET and the use of natural cycles in frozen-thawed embryo transfers may be beneficial for the post-implantational conditions of foetal development. However, a freeze-all strategy is not yet implemented as standard care due to limitations of large randomised trials showing a benefit of such a strategy. Thus, there is a need to test the concept against standard care in a randomised controlled design. This study aims to compare ongoing pregnancy and live birth rates between a freeze-all strategy with GnRH agonist triggering versus hCG trigger and fresh embryo transfer in a multicentre randomised controlled trial.

Methods and analysis Multicentre randomised, controlled, double-blinded trial of women undergoing ART treatment including 424 normo-ovulatory women aged 18 to 40 from Denmark and Sweden. Participants will be randomised (1:1) to one of two treatment groups of either A. hCG trigger and single blastocyst transfer in the fresh (stimulated) cycle or B. GnRH agonist trigger and single vitrified-warmed blastocyst transfer in a subsequent hCG triggered natural menstrual cycle. The primary endpoint is to compare ongoing pregnancy rates in the two treatment groups after the first single blastocyst transfer.

Ethics and dissemination

The study will be performed in accordance with the ethical principles in the Helsinki Declaration. The study is approved by the Scientific Ethical Committees in Denmark and Sweden. The results of the study will be publically disseminated.

Trial registration numbers: ClinicalTrials.gov identifier: NCT02746562; Ethical Approval, Denmark: H-1600-1116, Ethical Approval, Sweden: Dnr. 2016/654

Strengths and limitations of this study

- The design: A multicentre, randomised controlled double-blinded trial
- Superiority study powered to identify an increase in ongoing pregnancy rate in the freeze-all group compared to the conventional fresh blastocyst transfer group
- The dose and type of gonadotropin are decided and entered in the randomisation program by the doctor before randomisation is performed
- Doctors and patients are blinded to the randomisation result until the end of the controlled ovarian stimulation, which avoids bias from adjustments in gonadotropin stimulation dose
- The study includes normo-ovulatory women aged 18-39 years with a BMI < 35 thus results can be extrapolated to the majority of the normo-ovulatory infertile population
- GnRH-agonist trigger in the freeze-all group adds a concept of an OHSS-free strategy
- As both GnRH-agonist trigger and elective freeze-all are new treatment approaches, we will not be able to distinguish the two effects from each other, but compare an OHSS-free strategy to a conventional fresh transfer strategy

INTRODUCTION

The use of assisted reproductive technology (ART) is increasing and presently up to 5 % of birth cohorts in certain countries are conceived by ART.[1] In recent years, pregnancy rates following frozen embryo transfer (FET) have rapidly increased and may now be a viable and appropriate alternative to the conventional fresh embryo transfer in ART. The main reason is the introduction of vitrification, increasing post-thawing survival rates after blastocyst culture significantly as compared to previous years.[2-3] Implantation as well as clinical and on-going pregnancy rates are correspondingly improving in frozen cycles and approaching or even exceeding those associated with fresh embryo transfer.[4-6]

A freeze-all strategy has been suggested as a way to further improve success rates in ART, arguing that the use of the best embryo in frozen cycles instead of in fresh cycles, as is conventionally done, may potentially increase pregnancy rates and live birth rates.[6-7] The rationale is that transfer of a frozen-thawed embryo in a subsequent natural menstrual cycle has the advantage of an endometrium that has not been exposed to the supraphysiological levels of estradiol and progesterone following controlled ovarian stimulation (COS) in fresh cycles, which may negatively affect endometrial receptivity.[5 8] Elective FET (eFET) moreover has the benefit of essentially eliminating the risk of developing late ovarian hyperstimulation syndrome (OHSS) associated with the pregnancy-related rise in human chorionic gonadotropin (hCG) levels.[9] If ovulation is induced with a GnRH agonist instead of hCG and all embryos are subsequently frozen, even early OHSS is minimized making the overall OHSS risk extremely low.[10] Freezing and thawing of embryos additionally enables an elective single embryo transfer policy with cumulative pregnancy rates similar to those seen after double embryo transfer, encouraging an elective single embryo transfer policy.[11-12]

Despite evidence suggesting that ART outcomes may be further improved with the adaptation of a freeze-all strategy, the implementation remains a topic of ongoing debate and only one in five transfers in Europe on average was performed with frozen-thawed embryos in 2012.[1] In a large recent study, including 1508 patients with polycystic ovary syndrome comparing the freeze-all strategy with conventional fresh embryo transfer, the authors found a significantly higher frequency of live birth after the first frozen embryo transfer compared with fresh-embryo transfer (49.3% vs. 42.0%).[7] Correspondingly, in a meta-analysis including three trials accounting for 633 cycles in women aged 27-33 years, eFET resulted in significantly higher clinical and ongoing pregnancy rates compared with fresh embryo transfer.[6] However, the included studies showed heterogeneity and one of the included publications was later retracted due to serious methodological flaws. In addition, the vast majority of the participants were high responders (496 out of 633) accounting for

a highly selected group of patients, mostly consisting of PCOS patients or patients with and ovarian PCO like morphology.[6] Moreover, previous studies were performed in China, US and Japan making them less generalizable to a European ART setting. According to Clinicaltrials.gov there are a few ongoing European RCT´s on the freeze-all strategy, however none of these studies include a complete OHSS-free strategy with GnRH-agonist trigger in the freeze-all group. This underlines the need for a large multicentre randomised controlled trial exploring a freeze-all strategy in a broader population of ART patients. The present study will explore this approach in a bi-national multicentre randomised controlled trial setting providing information on the prospect of a freeze-all strategy.

Objectives

Primary objective

The primary objective of the study is to investigate if the ongoing pregnancy rate after single blastocyst transfer is superior in a freeze-all and transfer later- strategy compared to the conventional hCG trigger and fresh transfer strategy. Ongoing pregnancy and live birth rates will be calculated per randomized patient and per transfer according to the intention-to-treat principle. Additional calculations will be performed also according to the per-protocol principle. Ongoing pregnancy rate is defined as an intrauterine pregnancy with a foetal heart beat at transvaginal ultrasound in gestational week 7-8.

Secondary objectives

Secondary objectives include:

1. To assess cumulative live birth rates after *one complete cycle* including consecutive single blastocyst transfers of all embryos deriving from that oocyte retrieval (fresh and frozen) in the two study groups
2. To assess the transfer cancellation rate in the two study groups
3. To compare neonatal outcomes (preterm birth, low birth weight, SGA (small-for-gestational age), LGA (large-for-gestational age) and perinatal mortality) and the incidence of preeclampsia in the two study groups
4. To measure time-to-pregnancy from the date of start of COS to the date of the first ongoing pregnancy in the two study groups
5. To assess quality of life for both female and male partners during the two treatment protocols
6. To assess physical well-being by way of questionnaires and VAS scores regarding pain and discomfort at four and 16 days after oocyte retrieval in the two study groups

METHODS AND ANALYSIS

Study design

The study is designed as a multicentre randomised, controlled double-blinded trial with seven fertility clinics in Denmark and Sweden participating. All seven clinics are part of a University Hospital setting and perform standardized treatments according to the public health care system in Denmark and Sweden. Patient enrolment started in May 2016 and completion is expected in May 2018.

Study population/Participants and recruitment

The study participants will consist of women and their partners initiating ART treatment at one of the seven participating public clinics in Denmark and Sweden. Before initiating treatment patients will attend an information meeting, where they will be informed about the standard ART procedures, treatment regimens as well as ongoing clinical studies at the treatment sites. Those patients not able to participate in the information meeting will instead be informed by a doctor at an outpatient clinic consultation. Recruitment will be carried out by the doctors and study nurses at the fertility clinics. Prior to the initiation of treatment, patient files will be browsed by investigators at the clinics to asses if the patient fulfills the immediate inclusion criteria. Screening, including ultrasound examination of the uterus and ovaries is done on menstrual cycle day two or three securing that all inclusion criteria are met. Patients fulfilling the study criteria will start COS using a GnRH antagonist co-treatment in accordance with the standard routines of the trial site.

Eligibility criteria

To participate in the study, women will be required to meet the following inclusion criteria: Female age 18 to 39 years; eligibility to initiate the first, second or third ART cycle with oocyte aspiration (IVF or ICSI); AMH level > 6.28 pmol/L (Roche Elecsys assay) corresponding to the AMH threshold level used in the Bologna criteria to characterize poor responders; regular menstrual cycle ≥ 24 days and ≤ 35 days: body mass index 18–35 kg/m^2; preservation of both ovaries and capability of signing informed consent. For specific exclusion criteria see Table 1.

Randomisation and blinding

Patients who meet the inclusion criteria are randomised 1:1 to one of the two treatment groups: A. Freeze-all including GnRH agonist trigger, blastocyst vitrification and subsequent FET in a hCG triggered natural cycle or B. Traditional hCG trigger and fresh blastocyst transfer.
The randomisation is carried out by a study nurse or a non-treating doctor using a computerised randomisation program that runs a minimization algorithm, initially seeded using a random block

sequence for the first subjects. The minimization algorithm is balancing the following variables: Female age (mean, and frequency of age ≥37 years), previously performed cycles (frequency of 0/1/2/3 cycles), nulliparous (frequency of yes/no), fertilisation method (frequency of IVF/ICSI), smoking (frequency of yes/no), AMH (≤ 12 pmol/L, 13-28 pmol/L, >28 pmol/L) and mean BMI. It selects with high (but less than 1.0) probability the treatment arm that provides the optimal balance between the arms. It also enforces predefined maximum allowed differences in number of subjects in each treatment arm at each study site (fertility clinic) and within the whole study.
Furthermore, the starting dose of FSH is entered into the randomisation program before randomisation is performed to make sure that the FSH dose is decided upon before randomisation. Both the treating consultants and patients are blinded to the randomisation results during the controlled ovarian stimulation until the day when ovulation trigger is planned.

Treatment arms and interventions

The short GnRH antagonist protocol and blastocyst culture is applied to both treatment arms. The starting dose and type of gonadotropin is decided by the doctor on stimulation day one (cycle day two or three) and entered into the randomisation program prior to randomisation. Recombinant follicular stimulating hormone (rFSH) or human menopausal gonadotropin (hMG) can be used according to the preference of the site, but the daily dose cannot exceed 300 IU. The gonadotropin stimulation will be performed according to the routine in the clinics and can be changed during the treatment according to the ovarian response to stimulation evaluated through ultrasound examination. GnRH antagonist co-treatment is initiated at a daily dose of 0.25 mg on stimulation day five or six according to the general standards in each clinic and is continued throughout the rest of the gonadotropin stimulation period.
Ultrasound examination is performed on cycle day two or three (baseline), stimulation day six or seven and subsequently every second to third day until ovulation trigger is decided according to the hCG/GnRH agonist trigger criterion: as soon as three follicles are ≥ 17 mm or one day later. At baseline a comprehensive ultrasound examination will estimate endometrial thickness, ovarian volume as well as number and size of antral follicles divided into the following three subclasses: 2-4 mm, 5-7 mm and 8-10 mm. On the day of ovulation trigger endometrial thickness and morphology as well as follicular development with number and size of follicles > 10 mm are registered.
When ovulation trigger is decided, the result of the randomisation is disclosed to both doctors and patients and ovulation and oocyte maturation is triggered with a single injection of 250 μg of hCG in the fresh embryo transfer group or a GnRH agonist trigger injection (0.5 mg Buserelin) in the freeze-all group. If > 18 follicles with a diameter > 11 mm are observed in the fresh embryo transfer group GnRH agonist triggering with Buserelin and vitrification of all embryos will be

performed to avoid severe OHSS. All fertilised oocytes are cultured to the blastocyst stage and the embryos are scored and ranked according to standardised criteria ascribed to this study. The ranking will assure that the blastocyst with the highest implantation potential is transferred first in both groups. In the fresh transfer group, single blastocyst transfer is performed on day five after oocyte retrieval if a good quality blastocyst has developed. Surplus good quality blastocysts will be vitrified on day five or six. Luteal phase support is administered as vaginal progesterone according to the clinics standard procedures from day two after oocyte retrieval until the day of hCG test; thus luteal support is not extended into early pregnancy. In the freeze-all group all blastocysts of good quality are vitrified on day five or six depending on when the blastocyst stage is reached. The blastocyst with the highest rank is marked and will be the first one used in a subsequent FET hCG triggered modified natural cycle. There should be *at least one completed menstrual cycle in between the stimulation and the embryo transfer*. In FET cycles a single injection of 250 µg hCG is administered, when the leading follicle is ≥ 17 mm. Blastocyst transfer is performed six or seven days after the hCG injection. No luteal phase support is given.

A serum beta-hCG test is performed 11 days after blastocyst transfer. Clinical pregnancy is confirmed by transvaginal ultrasound 3 to 4 weeks after a positive serum-hCG test at gestational age 7-8. For overview of study design see figure 1.

Data collection and management

Treatment related data is collected at 1) Baseline (cycle day two or three), 2) Day of ovulation trigger and 3) five days after oocyte retrieval. Data on blastocysts are collected at culture day five/six. Follow-up data on all pregnancies resulting from blastocysts transferred according to the study protocol will be followed from study inclusion and one year onwards. Data is transferred to an online eCRF system called MediCase with an underlying Microsoft SQL server database located in a guarded underground facility in Sweden. Data is backed up daily (one back-up to another computer in the same physical location as the server, and a second back-up to a physically separate location, also in Sweden). MediCase has a complete audit trail and is designed to only contain de-identified data and is entirely based on anonymous subject ID numbers used in the trial.

Sample collection

Blood samples will be collected three times during the treatment process: 1) Baseline (cycle day two or three), 2) Day of ovulation induction and 3) 16 days after oocyte retrieval (day of pregnancy test in the fresh embryo transfer group). For overview of samples taken see Table 2. Furthermore one serum, one plasma and one fullblood sample are taken at baseline and on the day of triggering and stored according to a trial specific laboratory manual in a project-specific biobank as

back-up for analysis of endocrine and immunological factors of relevance for pregnancy. The frozen samples will be kept anonymised in the biobank with only the patient specific project ID number and collection date marked on the sample. The samples will be store in the participating fertility clinics and destroyed 5 years after the end of the study period if not analysed.

Further blood samples will be collected during the luteal phase for a smaller subgroup of 30 patients in each treatment group as part of a luteal phase subgroup analysis of differences in hormone levels in the two groups. The following blood samples will be collected at 1) Day of ovulation induction and 2) Day of ovulation induction, day of ovulation induction +7, +11, +14, +16 and +19: Estradiol, Inhibin-A, OH-Progesterone, Progesterone, LH and hCG.

Questionnaires

Women as well as male partners will be asked to fill in quality of life validated questionnaires twice during the treatment process: 1) Four days after oocyte retrieval and 2) 16 days after oocyte retrieval. The questionnaires consist of standardized questions specially developed to explore emotional aspects as well as quality-of life related aspects of the treatment process. The women will at the same time be asked to fill in questionnaires regarding physical discomfort including a VAS score of physical pain in relation to the treatment.

Statistics

Sample size calculation

The trial is designed as a superiority study. Sample size calculation indicates that 424 participants (n = 212 in each arm) are required to have a 80 % chance of detecting, at a significance level at 0.05, an increase in the primary outcome measure (ongoing pregnancy rate per transfer) from 30% in the control group (fresh embryo transfer) to 43 % in the experimental group (freeze-all).

Outcome measurements (primary and secondary)

The primary endpoint is the ongoing pregnancy rate per transfer of the first blastocyst.
Ongoing pregnancy is defined as a pregnancy with a positive foetal heart beat at gestational week 7-8. Secondary endpoints are shown in Table 3.

Statistical analyses

Analyses of cumulative pregnancy rates and live birth rates after one oocyte retrieval including fresh and all frozen embryo transfer cycles will be compared by Cox-regression analyses. Comparisons between treatment groups will be performed primarily according to the intention-to-treat (ITT) principle but per-protocol analyses will also be done. Continuous data will be compared

by students *t*-test or Mann-Whitney U test and Kruskal-Wallis test as appropriate. Proportions will be compared with chi-square test. Predictive factors for ongoing pregnancy in the two treatment groups will be tested with multivariate logistic regression analyses. A p-value of < 0.5 will be considered as statistically significant.

ETHICS, SAFETY AND DISSEMINATION

The study has been approved by the Scientific Ethical committees in both Denmark and Sweden. Following oral and written information outlining the rationale, trial design, aims and treatment procedures written informed consent will be obtained from women and male partners prior to the enrolment in the study.

The participants are stimulated using individualised doses of gonadotropin stimulation in accordance with the clinical practice at each site. In all clinics serum AMH is considered when the FSH dose is determined. All medicine used in the study is part of standard ART care.

The overall safety of the patients is high in both treatment groups. The risk of OHSS is expected to be similar to the standard clinical protocol in the fresh embryo transfer group and lower in the freeze-all group in which GnRH agonist is used for ovulation trigger. In women in the fresh embryo transfer group with a risk of OHSS development (more than 18 follicles with a diameter over 11 mm), GnRH agonist will be used for trigger instead of hCG and all blastocysts will be vitrified and the transfer postponed.

No financial incentive exists for the participants as all couples are reimbursed for their first three ART treatments in the public health care system in the Nordic countries.

The results of the study will be publically disseminated in peer-reviewed scientific journals and presented at relevant international scientific meetings such as ESHRE (European Society of Human Reproduction and Embryology) and ASRM (American Society for Reproductive Medicine). In addition results will be published in popular science journals and other media.

DISCUSSION

The increasing interest in possible benefits of a freeze-all strategy and the limitations of existing randomised controlled trials comparing this strategy with conventional fresh embryo transfer underline the need for additional studies. Previous studies investigating FET cycles concluded that

a freeze-all strategy resulted in significantly increased pregnancy rates, however these studies were performed in highly selected patient populations with poor generalizability.[6-7] Further, the complete OHSS-free strategy combining GnRH agonist trigger and freeze-all has not yet been investigated in a RCT setting. As GnRH agonist trigger does not hamper the yield of mature oocytes[12] and reduces the risk of OHSS to an absolutely minimum, it seems rational to include GnRH agonist trigger in the freeze-all concept.

The strengths of this study include the design as a multicenter randomised controlled double-blinded trial as well as preregistration and publication of the study protocol for more transparency. The investigation of several outcome measures related to different aspects of success parameters, including quality of life may furthermore add important information as regards the future potential of the freeze-all strategy in assisted reproduction.

Appendices

Table 1. Specific exclusion criteria
Endometriosis stage III to IV
Ovarian cysts with a diameter > 30 mm at day of start of stimulation
Submucosal fibroids
Women with severe co-morbidity (IDDM (insulin dependent diabetes mellitus), NIDDM (non-insulin dependent diabetes mellitus), gastrointestinal, cardiovascular, pulmonary, liver or kidney disease)
Dysregulated thyroid disease
Non-Danish or English speaking
Contraindications or allergies to use of gonadotropins or GnRH antagonists
TESA (testicular sperm aspiration)
OD (oocyte donation)
Previous inclusion in the study

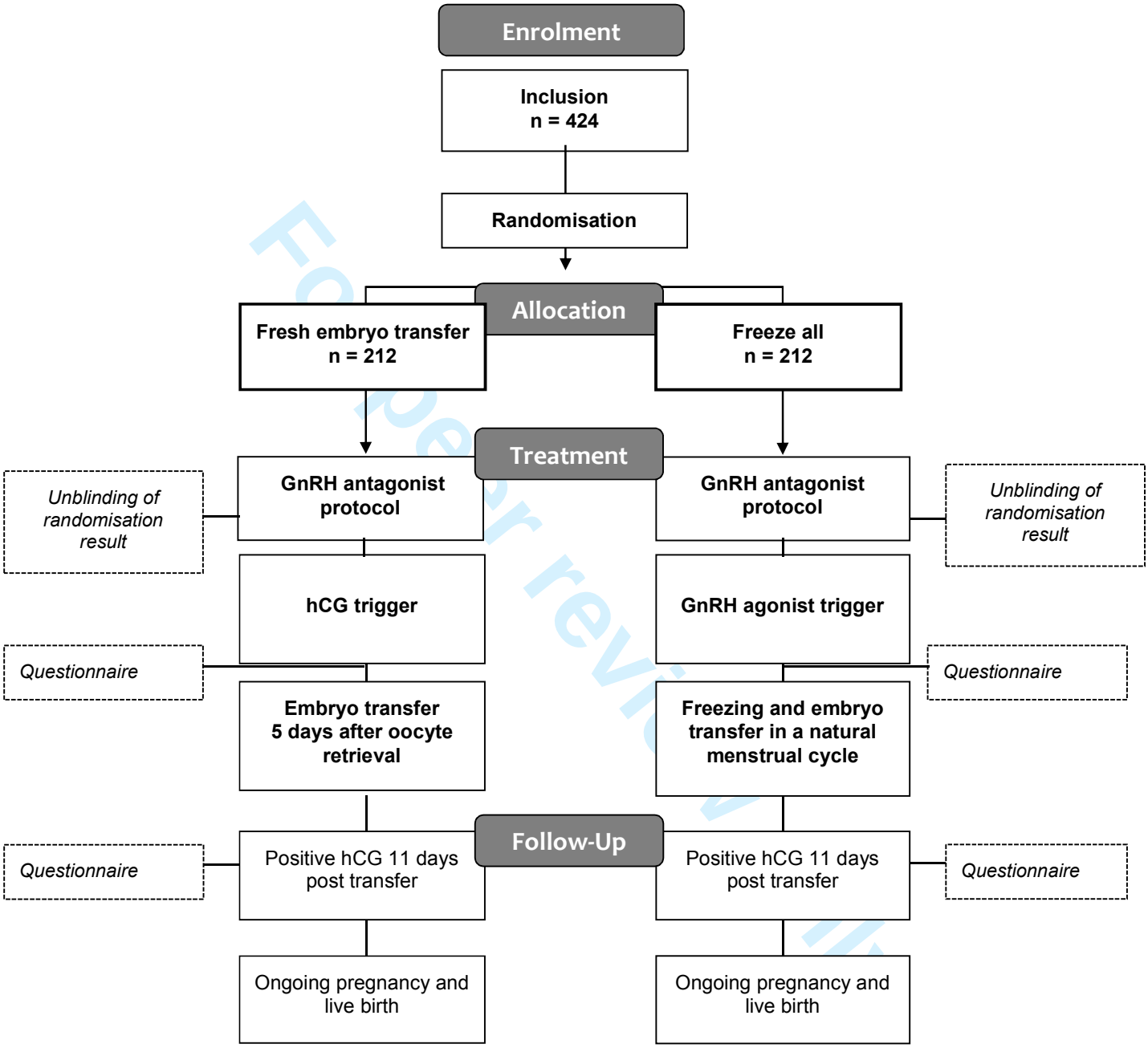
Table 2. Blood sample collection	
Baseline (cycle day 2 or 3)	AMH FSH LH Estradiol Progesterone TSH TPO-antibodies Vitamin D CRP suPAR*
Day of ovulation induction	FSH LH Estradiol Progesterone CRP suPAR*
16 days after oocyte retrieval	CRP suPAR* hCG**

* Soluble urokinase-type plasminogen activator receptor, only measured at Hvidovre Hospital

** only fresh embryo transfer group

Table 3. Secondary endpoints
♦ Ongoing pregnancy rate per start of *per randomized patient, per started ovarian stimulation and per oocyte retrieval*
♦ Live birth rate after the first blastocyst transfer calculated *per randomized patient, per started ovarian stimulation*, *per oocyte retrieval* and *per transfer*
♦ Cumulative live birth rate after one stimulated cycle with oocyte retrieval
♦ Cumulative live birth rate after use of all frozen blastocyst or after at least 1 year of follow-up
♦ Number of cycles with no embryo transfer
♦ Time-to-pregnancy (from start of ovarian stimulation to positive hCG)
♦ Time-to-delivery
♦ Cancelled embryo transfers
♦ Ovarian hyperstimulation syndrome (OHSS)
♦ Preterm birth
♦ Low birth weight
♦ Small-for-gestational age (SGA)
♦ Large-for-gestational age (LGA)
♦ Perinatal mortality
♦ Preeclampsia
♦ Placental rupture
♦ Positive hCG 11 days post embryo transfer
♦ Miscarriage, biochemical pregnancies, ectopic pregnancies
♦ Quality of life for female and male partner
♦ Cost-effectiveness
Other outcome measurements
♦ Number of good blastocysts
♦ Number of fertilized oocytes
♦ Number of high quality embryos day 2
♦ Number of grade 1 blastocysts
♦ Number of frozen blastocyst
♦ Paraclinical data: Endocrine, genetic and immunological parameters

Figure 1. Flowchart of the Freeze-all study design



Contributor statement ANA, AP and SS participated in the conception, design, and writing of the study protocol. KL and HSN contributed to the revision and editing of the study protocol. AP, SS, KL, JB, LP, ANA, HSN, CB, PH, MB, ALM and SOS will be involved in the recruitment of patients and the acquisition of data. SS wrote the first draft of this manuscript. AZ was involved in developing the laboratory criteria for the study. SS, AP, ANA, PH, CB, KL, JB, LP, HSN, MB, ALM and SOS will AP, ANA, PH, CB, KL, AZ, JB, LP, HSN, MB, ALM and SOS were all involved in critical revision of the manuscript. All authors approved the final version of the manuscript to be submitted.

Competing interests None declared

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Data sharing statement This manuscript is a study protocol. Data from the final study will be shared according to the coming ICJME guidelines.

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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			Page 1 of protocol
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	_____
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	Page 17 of protocol _____
	2b	All items from the World Health Organization Trial Registration Data Set	All accounted for see items below _____
Protocol version	3	Date and version identifier	Page 1 of protocol (header) _____
Funding	4	Sources and types of financial, material, and other support	Page 18 of protocol _____
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	Page 1, 2, 3 of protocol _____

	5b	Name and contact information for the trial sponsor	Page 1 of protocol
	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	Not applicable
	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)	Not applicable
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	Page 3, 4 5 of protocol
	6b	Explanation for choice of comparators	Page 3, 4 5 of protocol
Objectives	7	Specific objectives or hypotheses	Page 5 of protocol
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)	Page 8-10 of protocol
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	Page 8 List of study sites; page 1-3
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)	Page 7-8
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	Page 11-13
	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)	Page 14-15
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	Page 11 (visits)
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	Not applicable
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	Page 5-7
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)	Page 5-13
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	Page 15
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	Page 8

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	Page 9
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	Page 9
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	Page 9, 11
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	Page 9
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	No circumstances
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	Page 9, 10, 11 and 14
	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	Page 13-14

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	Page 13,14, Page 16 Details data management can be obtained through contact to primary investigator ("projektansvarlig læge" of trial
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	Page 15 Details of planned statistical analysed can be obtained through contact to primary investigator ("projektansvarlig læge" of trial
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	Same as item 20a
	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)	Same as item 20a
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	No data monitoring committee used

	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	No interim analyses will be performed
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	Not applicable
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	Not available in protocol
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	Page 17
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)	Not available in protocol
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	Page 8 of protocol
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	Not 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	Page 14, 16
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	Page 18 No competing interests exists

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	Not available in protocol
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	Not applicable
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	Not available in protocol
	31b	Authorship eligibility guidelines and any intended use of professional writers	Not available in protocol
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	No current plans
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Not available in protocol – Standard consent forms from the Danish etichal committee is used as well s participant information approved by the Ethical Committee
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	Page 10

*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.

November 17th, 2016
Protokol_Freeze all and transfer later_V2_17112016

A multicentre randomized controlled trial of a "Freeze all and transfer later" versus a conventional "Fresh Embryo Transfer" strategy for assisted reproductive technology (ART) in women with a regular menstrual cycle

- A Multicentre Randomized Controlled Trial of patients undergoing IVF/ICSI

Multicenter studium med syv deltagende fertilitetsklinikker i Danmark og Sverige

Projektansvarlig læge

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1. BACKGROUND

In recent years improved cryopreservation techniques have made frozen embryo transfer (FET) a viable and promising alternative to fresh embryo transfer in assisted reproduction (ART). The optimization of cryopreservation techniques from slow-freezing to vitrification and prolonged embryo culture from cleavage to blastocyst state encourages the use of FET as the embryo survival rate following freezing and thawing is now significantly higher reaching 95-97% (Loutradi et al., 2008). Success rates including implantation as well as clinical and on-going pregnancy rates in FET are also significantly improving and approaching or even exceeding those associated with fresh embryo transfer (Kupka et al., 2014; Roque et al., 2013; Shapiro et al., 2011; Zhu et al., 2011). This is partly due the improved laboratory techniques, but may also be due to the endometrial environment in the FET cycles, which mirrors the natural cycle. In the stimulated cycle supraphysiological levels of estradiol and progesterone are present and may cause impaired endometrial receptivity (Shapiro et al., 2011). Furthermore, obstetric and perinatal outcomes after cryopreservation of embryos have been investigated and follow-up data from children born after FET have shown lower perinatal morbidity compared with fresh embryo transfer (Kansal et al., 2011), but FET may also give rise to more large-for-gestational age babies (Pinborg et al., 2014). In addition, a recent systematic review and meta-analysis on data from 11 observational studies has shown better perinatal outcomes including lower perinatal mortality in singleton pregnancies following frozen-thawed embryo transfer

compared with pregnancies after fresh embryo transfer (Maheshwari et al., 2012). Moreover, FET has the benefits of minimizing the risk of ovarian hyperstimulation syndrome (OHSS), which is the most severe side effect of ART and potentially life threatening. Finally, improved cryopreservation techniques favour an elective single embryo transfer (eSET) policy minimizing multiple pregnancies after ART (Pinborg, 2012).

Despite the noticeable advantages of embryo cryopreservation, fresh embryo transfer has persistently been the conventional in vitro fertilisation (IVF) procedure as only one in five transfers were made using frozen-thawed embryos in Denmark in 2013 (www.fertilitetsselskab.dk). This favour of a fresh embryo transfer strategy is however reflected in other European countries including Finland, Sweden and Iceland where approximately every third ART child is born after FET (Kupka et al., 2014). Some evidence suggests that IVF outcomes can be further improved with the adaptation of a `freeze-all´ or elective frozen embryo transfer (eFET) strategy with replacement of thawed embryos in natural cycles (Evans et al., 2014; Devroey et al., 2011; Maheshwari et al., 2013; Roque et al.,2013).

In a recent meta-analysis including three trials accounting for 633 cycles in women aged 27–33 years (Roque et al., 2013), FET resulted in significantly higher ongoing pregnancy rates (RR 1.32, 95% CI 1.10–1.59) and clinical pregnancy rates (RR 1.31, 95% CI 1.10–1.56). The studies showed heterogeneity and only 137 of the participants were normal responders, while the rest was high responders. Moreover one study included only cleavage stage embryo transfer while the other two included blastocyst transfers only. The studies were performed in Japan and in the US, while no European RCT has been published yet. Further, one of the included papers (Aflatoonian et al., 2010) was later retracted based on findings of serious methodological flaws in the study. This accentuates the need for a large multicentre, randomized controlled trial to evaluate the prospect and clinical consequences of a “freeze all embryos and transfer later” policy compared with conventional fresh embryo transfer.

The aim of this multicentre randomized controlled trial is to compare a “freeze-all” embryo strategy with a conventional single fresh embryo transfer strategy in women 18 to 40 years of

age undergoing their first to third IVF/ICSI cycle women with regard to treatment outcomes, risks for mother and child, quality of life and cost-effectiveness aspects of the two treatment modalities in a short GnRH antagonist protocol with blastocyst transfer and vitrification as the freezing method.

2. STUDY AIMS

1. The primary aim is to compare ongoing pregnancy rates and live birth rates after the first single blastocyst transfer in the "freeze-all" versus "fresh embryo transfer" group.

2. To assess cumulative live birth rates after one stimulated cycle with oocyte retrieval in the two study arms.

3. To compare perinatal outcomes (preterm birth, low birth weight, small-for-gestational age, large-for-gestational-age, preeclampsia and perinatal mortality) in the two groups.

4. To measure time to pregnancy from start of ovarian stimulation and quality of life in both females and males in the two groups.

5. To explore VAS scores regarding pain and discomfort at the day of embryo transfer and 11 days post transfer in the two study arms

6. To assess female physical well-being during the two treatment modalities and to assess quality of life for both female and male partners during the two treatment protocols.

3. ENDPOINTS

Primary endpoints

- Ongoing pregnancy rate *per transfer* of the first blastocyst
  (pregnancy with positive fetal heart beat in gestational week 7-8)
- Ongoing pregnancy rate *per oocyte pick-up*

(pregnancy with positive fetal heart beat in gestational week 7-8)

- Ongoing pregnancy rate *per start of ovarian stimulation*
  (pregnancy with positive fetal heart beat in gestational week 7-8)
- Ongoing pregnancy rate *per randomized patient*
  (pregnancy with positive fetal heart beat in gestational week 7-8)

Secondary endpoints

- Live birth rates after the first blastocyst transfer calculated per randomized patient, per started ovarian stimulation, per oocyte pick-up and per transfer
- Cumulative live birth rate after one stimulated cycle with oocyte retrieval
- Cumulative live birth rate after use of all frozen blastocysts or after at least 1 year of follow-up.
- Number of cycles with no embryo transfer
- Time-to-pregnancy (from start of ovarian stimulation to positive hCG)
- Time-to-delivery
- Cancelled embryo transfers
- OHSS
- Preterm birth
- Low birth weight
- Small-for-gestational age (SGA)
- Large-for-gestational age (LGA)
- Perinatal mortality
- Preeclampsia
- Placental rupture
- Positive hCG 11 days post embryo transfer
- Miscarriage, biochemical pregnancies, ectopic pregnancies
- Quality of life for female and male partner
- Cost-effectiveness

Other outcomes

- Number of good quality blastocyst
- Number of fertilized oocytes
- Number of high quality embryos day 2
- Number of grade 1 blastocysts?
- Number of frozen blastocysts
- Para-clinical data: Endocrine, genetic and immunological parameters influencing pregnancy

4. STUDYPOPULATION

Inclusion criteria

- Women > 6.28 pmol/L with the Roche Elecsys assay* (AMH > 1.1 ng/ml ~ 7.85 pmol/L old assay). This is according to the Bologna criteria for POR; AMH < 0.5–1.1 ng/ml (3.57-7,85 pmol/l (old assay) ~ 2,86 – 6,28 pmol/l Elecsys)(Ferraretti et al., 2011)
- Female age 18 year to less than 40 years
- 1.-3. IVF/ICSI cycle with oocyte aspiration
- Regular menstrual cycle $\geq$ 24 days and $\leq$ 35 days
- BMI $\geq$ 18 or < 35 kg/m^2
- Two ovaries
- Can and will sign the informed content

- **Exclusion criteria**
- Women who do not fulfil the inclusion criteria
- Endometriosis stage III to IV
- Ovarian cysts with diameter > 30 mm at day of start of stimulation
- Submucosal fibroids
- Women with severe co-morbidity (IDDM, NIDDM, gastrointestinal, cardio-vascular, pulmonary, liver or kidney disease)
- Dysregulation of thyroid disease
- Not Danish or English speaking women

- Contraindications or allergies to use of gonadotrophins or GnRH antagonists
- TESA (testicular sperm aspiration)
- OD (oocyte donation)
- Previous inclusion in the study

5. METHODS

Inclusion of patients

- All couples or single/lesbian women starting IVF/ICSI treatment participate in a standard information meeting arranged by the clinic. During this 2 hour meeting patients and their partners are informed about the normal IVF/ICSI procedures, treatments and research in the clinic as well as this study. If patients are not attending the information meeting, the will be informed about the study at their first outpatient visit at the clinic.
- Few patients do not participate in the information meeting and they will have an appointment at the outpatient clinic in the Fertility clinic, where they will receive information about the IVF/ICSI treatment and be informed about this study.
- Patient files are browsed by one of the investigators, who decide if the patient is eligible. After the information meeting all patients receives a phone call from a doctor/study nurse, where they are informed about the treatment plan. If the inclusion criteria are fulfilled, the couples will receive oral information about the study and asked if they are interested in participating in the study, if so, the written patient information is sent to the couples by email. If the couples are interested, a visit is planned on menstrual cycle day 2-4. The couple is informed that they can bring an assessor to the oral information visit. The written information is send by email, which leaves possibility of reading and reflection.

Informed consent

At the fertility clinic the patient will be seen by one of the investigators. Patients will be informed about the aim of the project and risks in accordance with the guidelines from the Scientific Ethical

Committee. In case of questions, these will be answered. If the patients need more time for reflection, a new visit will be arranged. After signing the informed consent, the patient will be screened.

Screening – cycle day 2-4

- Medical and gynaecological history inclusive reproductive history including menstrual cycle length, smoking (yes/no), years of infertility
- Transvaginal ultrasound examination including ovarian volume, antral follicle count (AFC), and endometrial thickness and morphology and exclusion of pathology
- Height and weight
- Blood samples: AMH, FSH, LH, estradiol, progesterone, TSH, TPO antibodies, vitamin D, CRP and suPAR* (*only done at Hvidovre Hospital)
- One full blood, one plasma and one serum sample is cryopreserved as back-up and for analysis of endocrine and immunological factors of relevance for pregnancy

Screening should be performed no later than 3 months before randomization.

Randomization

When the patient has signed the informed consent, has been screened and it is confirmed that the inclusion criteria are meet, the patient is randomized to one of the two arms:

I. hCG arm with traditional hCG triggering and fresh blastocyst transfer
II. GnRH agonist triggering arm with blastocyst cryopreservation and subsequent transfer in a natural cycle.

Computerized randomization is performed according to the 1) Trial site and to 2) Female age <= 37 years or >37 years. The gonadotrophin stimulation dose is decided upon before randomization and entered into the database before randomization. The doctor and patient are blinded to the randomization until the day of hCG or GnRH agonist triggering.

Blood samples

Blood samples are collected at

- Baseline before the first gonadotrophin injection (cycle day 2-4): AMH, FSH, LH, estradiol, progesterone, TSH, TPO-antibodies, vitamin D CRP and suPAR* (*only done at Hvidovre Hospital)
- Day of trigger-injection: FSH, LH, estradiol, progesterone, CRP and suPAR* (*only done at Hvidovre Hospital)
- Day 16 after oocyte pick-up: hCG (only in the fresh embryo transfer group), CRP and suPAR* (*only done at Hvidovre Hospital)

At baseline and at day of trigger an extra full blood, plasma and serum sample is collected and stored according to a trail-specific laboratory manual. This will be stored in the freezer as back-up and for analysis of endocrine and immunological factors of relevance for this study.

Subgroup analyses in the luteal phase

During the lutealphase with embryo transfer of the stimulated fresh cycle and non-stimulated FER cycle blood samples are taken on day of hCG injection, hCG injection day+7, +11, +14, +16 and +19 for patients included at Hvidovre Hospital until 30 patient in each arm has been achieved. The following blood samples are collected; Estradiol, Inhibin-A, OH-progesterone, Progesterone, LH and hCG.

All blood samples are confidential. The frozen samples are anonymous, so no person identifiable date is left on the sample. Only the patient project ID number and the collection date identifies the sample. The study will be approved by the Danish Data Protection Agency and the Scientific Ethical Committee of the Capital Region, Region Zeeland and the Region Skäne in Sweden. The blood samples will be stored in the participating fertility clinics and if not used then five years after end of the study, at December 1st, 2023 blood samples will be definitively destroyed.

Gonadotropin stimulation treatment

The dose of gonadotrophin is decided and entered into the computer programme before randomization. The doctor and patient are blinded to the randomization until the day of hCG or GnRH agonist triggering. The study nurse is not blinded.

The ovarian stimulation with recombinant follicular stimulating hormone (rFSH) or human menopausal gonadotrophin (hMG) can start immediately after randomization in a short GnRH antagonist protocol. The gonadotrophin stimulation is performed according to the general standards in each of the clinics and can be altered according to the ovarian response. The GnRH antagonist is initiated at a daily dose of 0.25 mg at stimulation day 5 or 6 according to the clinical standards and continued throughout the rest of the gonadotropin stimulation period. The gonadotrophin dose cannot exceed a daily dose of 300 IU. Both groups are treated according to the short GnRH-antagonist protocol, where a higher dose of gonadotropin than 300 IU has been shown to be of no added value for further follicular growth. The maximum stimulation period is 20 days.

The medication for the study is bought by the patients themselves according to general prescription rules.

Ultrasound

Ultrasound examination is performed on cycle day 2-3 (Stim1), Stim 5-8 and thereafter every 2-3 days until ovulation trigger is decided. At the start of stimulation comprehensive sonography is performed with details on each ovary, including ovarian volume, number of antral follicle in the following subclasses: 2-4mm, 5-7mm and 8-10mm.

The following parameters are measured on the day of ovulation trigger or the day before: Follicular development with size and number of follicles >10 mm, endometrial thickness and echogenicity and uterine pathology.

Ovulation induction

As soon as three follicles of >= 17 mm are observed or one day after a single injection of 250 µg of human chorionic gonadotrophin (hCG) is administered in the "fresh transfer"-arm, while GnRH agonist triggering with GnRH agonist Buserelin 0.5 mg is administered in the "freeze-all" arm.

Triggering of ovulation with a GnRH agonist Buserelin 0.5 mg is allowed in the fresh embryo transfer arm in case of risk of severe OHSS after the following the criteria: In the fresh embryo arm: If > 18 follicles with a diameter > 11 mm are observed on the day of triggering, GnRH agonist triggering should be used and all blastocysts frozen (Papanikolaou et al., 2011). Coasting / surfing cannot be used.

Oocyte retrieval

Oocyte retrieval is performed 36 ± 2 hours after hCG or GnRH agonist administration.

IVF/ICSI

Oocytes are fertilised by either IVF or ICSI and embryos are cultured individually according to the normal procedure in the clinics.

Embryo transfer

I. "Fresh embryo transfer" group

Single blastocyst transfer is always performed on day five after oocyte pick-up if a blastocyst is developed. Surplus good quality blastocyst are vitrified on day five or six.

Luteal phase support is administered as vaginal progesterone (vaginal gel (Crinone) 90 mg/dose x 1 daily or vaginal tablets 100 mg x 3 daily (Lutinus)) according to the standard procedure in each of the individual clinics from day 2 after oocyte retrieval and to confirmation of pregnancy or

negative hCG 11 days post transfer. In case of a positive pregnancy test an ultrasound scan is performed three to four weeks later to confirm an intrauterine pregnancy with a live foetus.

Triggering of ovulation with a GnRH agonist Buserelin 0.5 mg is allowed in the fresh embryo transfer arm in case of risk of severe OHSS, following the criteria: If > 18 follicles with a diameter > 11 mm are observed, GnRH agonist triggering should be used and all blastocysts frozen (Papanikolaou et al., 2011). Coasting / surfing cannot be used.

II. "Freeze all and transfer later" group

For patients in this group all embryos of a good quality are vitrified at the blastocyst stage day 5 in the stimulated cycle. Criteria for freezing of blastocyst are according to the criteria in the specific clinic. The "best" embryo (i.e. of the highest quality is selected after predefined strict criteria according to the specific trial laboratory manual) is marked and is the first one to be warmed after at least one menstrual cycle that is considered as a wash out period.

In the menstrual cycle with blastocyst transfer, an hCG injection of 6500 units is given when the leading follicle is $\geq$ 17 mm. Embryo transfer is performed 6-7 days after hCG injection. No luteal phase support is needed.

Pregnancy test

A serum beta-hCG test is performed 11 days after blastocyst transfer. Clinical pregnancy is confirmed by transvaginal ultrasound 3 to 4 weeks after a positive serum-hCG.

Follow-up both groups

A follow-up of all pregnancies will be performed within three months after delivery or termination of pregnancy on predefined information sheets.

Information on background data, pregnancies and deliveries are returned to Hvidovre Fertility clinic on predefined case report forms (CRF) including pregnancy and delivery information sheets. All pregnancies resulting from blastocyst retrieved and thawed according to this study protocol will be followed from study inclusion (Stim day 1) and one year onwards.

All data are anonymized by encryption in the database with no personal identifiable data.

We will retrieve data from the patient clinical files and clinical databases with information regarding previous diseases, hospital admissions, former and current fertility treatment, pregnancy and delivery data on pregnancies related to this study. Both females and males will be informed about this in the patient information. This collected information will be used to characterize the populations and to minimize risk of bias.
We will also gain information regarding the coming child and the female and male will sign a separate informed consent regarding this.

VAS-score and physical discomfort questionnaire

Women in both arms will be requested to fill-in their level of pain and discomfort on a VAS-score scale and a physical discomfort questionnaire as well as a quality of life questionnaire at the day of oocyte pick-up +4 and the day of oocyte pick up + 16.

Visits

Every visit is registered on a standardized stimulation scheme made specifically for this study. The schemes are normally used as standards in the clinics.

Criteria for withdrawal

A patient can be withdrawn from the study at any time, if the patient wishes to do so or if there is a medical indication decided by the investigator. The patient participation in the study can be interrupted, if one of the following criteria are present:

- The patients general condition contraindicates participation
- Protocol violation, which the investigator assess to have influence on the treatment
- Safety

Patients will be carefully monitored from stimulation start, at Stim6 and thereafter every 2-3 day in the clinic. The treatment will be monitored by transvaginal ultrasound of the ovaries. After each visit the patients will receive thorough information on the drug dosage and administration. This will follow the normal procedure in the clinic. If a patient is taking the wrong dosage, it will be documented on the stimulation scheme. This is not dangerous to the patient as the treatment is monitored by ultrasound scans hence a risk of OHSS will be discovered there.

In case of risk of OHSS in the "fresh-embryo-transfer"-arm, the ovulation trigger will not be induced by hCG but with Buserelin 0.5 mg. The further treatment of this patient will be handled according to the routine of the clinic.

In case of OHSS the patient is monitored at the clinic until recovery. Overall the safety of the patients is high in both the fresh embryo transfer and the freeze all group as the gonadotrophin stimulation corresponds to the normal program for patients at risk of OHSS. Furthermore patients with irregular cycles i.e. as part of polycystic ovarian syndrome, who in general have a higher risk of OHSS, are not included in this study.

6. STATISTICS AND SAMPLE SIZE

Superiority study

In all 424 (n= 212 in each arm) patients are required to have an 80% chance of detecting, as significant at the 5% level, an increase in the primary outcome measure from 30% in the control group to 43% in the experimental group. A difference of 15% was found in a randomized controlled trial by Shapiro et al, 2011 between the "freeze-all" arm and the fresh transfer group.

The statistical analyses will be performed by investigator together with statistical experts at Hvidovre Hospital and associate professor Julie Forman, Department of Biostatistics, Faculty of Health and Medical Sciences, University of Copenhagen.

7. STUDY MEDICATION

All medicine used in this study is normally used as standard care for the patients in the short GnRH antagonist protocol. Patients will have prescriptions on all the medicine and will take all the medicine at home as is the routine in the clinics.

Dosage and administration

Treatment dose at the first day and during the ovarian stimulation is planned by the investigator and the patient is further instructed by a nurse, so that the patient is confident in self administration at home according to the normal clinical routine.

Side effects

Most side effects are mild and related to the medication during the stimulation. Unwanted OHSS is a risk in all IVF treatment but is considered low in this project as a standard IVF/ICSI protocol is used with individualized dosing. Further, in the freeze-all group the risk of OHSS is expected to be lower than in the standard care group as all blastocyst transfers are postponed to cycles without ovarian stimulation.

8. DATA SECURITY AND ETHICAL ASPECTS

Data security

One full blood and one serum sample at baseline and at the day of ovulation induction will be collected and stored according to the trial specific laboratory manual on all women included in this trial for future analyses of endocrine, immunological and protein markers. All data will be collected in a single database including all project subjects with an identification code thus data on each subject will be anonymous, when entered into the database.

Ethical aspects

The study will be performed according to the Danish Law and ethical principles in the Helsinki Declaration. This covers that study subjects receive both oral and written information and the opportunity of time for reflection and that they can discuss their participation with a third person.

The participants will be given a individualized dose of gonadotropin according to their serum AMH level, which is standard for patients at all five Fertility Clinics in Denmark and Sweden. The risk of OHSS will be similar to the standard clinical protocol and lower in the "freeze-all" group.

With a "freeze-all embryo and transfer later" protocol in ART, the risk of OHSS in women undergoing IVF/ICSI will be minimized and the embryo development will benefit from an endometrium less influenced by supra-physiological levels of estradiol and progesterone in the fresh embryo transfer cycle. This may also be beneficial for the children born after the treatments.

The study is approved by the Scientific Ethical Committee in the Capital Region (H-1600-1116) and by the Scientific Ethical Committee in Region Skäne in Sweden (Dnr. 2016/654)

The study will be approved by the Data Protection Agencies in Denmark and Sweden.

9. TIME SCHEDULE AND PUBLICATION

Protocol will be send to the Scientific Ethical Committee in the Capital Region, Denmark in January 2016 and inclusion of patients will start as soon as the approval from SEC has been obtained. The inclusion of patients will run from March 2016 to February 2018. Statistical analyses, writing and preparing manuscripts will go on from February 2018 to January 2019.

The results of the study will be presented at national as well as international scientific congresses and published in high impact international scientific journals in reproductive medicine such as Human Reproduction or Fertility and Sterility. Further results of public interest will be reported in the public press.

10.FINANCING

The project is initiated by Professor Anja Pinborg. This project is part of the Reprounion program, which has been supported by the Interreg-program for Öresund-Kattegat-Skagerak from EU, Capital Region of Denmark, Region Skäne and Ferring Pharmaceutical Company. The project has been financed with 450.000 euro (3.375.000 dkk) by a grant from Interreg/EU.

Patients included in this study and the Scientific Ethical Committee will be informed if further funding is obtained for this study. Funding will be transferred to a research account in the bank of Hvidovre Hospital, Capital Region of Denmark.

11. REFERENCES

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TITLE PAGE

PROTOCOL ARTICLE

Comparison of a "freeze all" strategy including GnRH agonist trigger versus a "fresh transfer" strategy including hCG trigger in assisted reproductive technology (ART) – A study protocol for a randomised controlled trial

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ABSTRACT

Introduction Pregnancy rates after frozen-thawed embryo transfer (FET) have improved in recent years and are now approaching or even exceeding those obtained after fresh embryo transfer. This is partly due to improved laboratory techniques, but may also be caused by a more physiological hormonal and endometrial environment in FET cycles. Furthermore, the risk of ovarian hyperstimulation syndrome (OHSS) is practically eliminated in segmentation cycles followed by FET and the use of natural cycles in frozen-thawed embryo transfers may be beneficial for the post-implantational conditions of foetal development. However, a freeze-all strategy is not yet implemented as standard care due to limitations of large randomised trials showing a benefit of such a strategy. Thus, there is a need to test the concept against standard care in a randomised controlled design. This study aims to compare ongoing pregnancy and live birth rates between a freeze-all strategy with GnRH agonist triggering versus hCG trigger and fresh embryo transfer in a multicentre randomised controlled trial.

Methods and analysis Multicentre randomised, controlled, double-blinded trial of women undergoing ART treatment including 424 normo-ovulatory women aged 18 to 39 from Denmark and Sweden. Participants will be randomised (1:1) either A. GnRH agonist trigger and single vitrified-warmed blastocyst transfer in a subsequent hCG triggered natural menstrual cycle or B. hCG trigger and single blastocyst transfer in the fresh (stimulated) cycle. The primary endpoint is to compare ongoing pregnancy rates per randomised patient in the two treatment groups after the first single blastocyst transfer.

Ethics and dissemination

The study will be performed in accordance with the ethical principles in the Helsinki Declaration. The study is approved by the Scientific Ethical Committees in Denmark and Sweden. The results of the study will be publically disseminated.

Trial registration numbers: ClinicalTrials.gov identifier: NCT02746562; Ethical Approval, Denmark: H-1600-1116, Ethical Approval, Sweden: Dnr. 2016/654

Strengths and limitations of this study

Strengths

- The design: A multicentre, randomised controlled double-blinded trial powered to identify an increase in ongoing pregnancy rate in the freeze-all group compared to the conventional fresh blastocyst transfer group
- The study includes normo-ovulatory women aged 18-39 years with a BMI < 35 thus results can be extrapolated to the majority of the normo-ovulatory infertile population
- GnRH-agonist trigger in the freeze-all group adds a concept of an OHSS-free strategy

Limitations

- As both GnRH-agonist trigger and elective freeze-all are new treatment approaches, we will not be able to distinguish the two effects from each other, but compare an OHSS-free strategy to a conventional fresh transfer strategy
- The study is powered to detect a 12 % difference in ongoing pregnancy between the two groups, thus smaller but yet clinically relevant differences may be overlooked

INTRODUCTION

The use of assisted reproductive technology (ART) is increasing and presently up to 5 % of birth cohorts in certain countries are conceived by ART.[1] In recent years, pregnancy rates following frozen embryo transfer (FET) have rapidly increased and may now be a viable and appropriate alternative to the conventional fresh embryo transfer in ART. The main reason is the introduction of vitrification, increasing post-thawing survival rates after blastocyst culture significantly as compared to previous years.[2-3] Implantation as well as clinical and ongoing pregnancy rates are correspondingly improving in frozen cycles and approaching or even exceeding those associated with fresh embryo transfer.[4-6]

A freeze-all strategy has been suggested as a way to further improve success rates in ART, arguing that the use of the best embryo in frozen cycles instead of in fresh cycles may potentially increase pregnancy rates and live birth rates.[6-7] The rationale is that transfer of a frozen-thawed embryo in a subsequent natural menstrual cycle has the advantage of an endometrium that has not been exposed to the supraphysiological levels of estradiol and progesterone following controlled ovarian stimulation (COS) in fresh cycles, which may negatively affect endometrial receptivity.[5 8] Elective FET (eFET) moreover has the benefit of essentially eliminating the risk of developing late ovarian hyperstimulation syndrome (OHSS) associated with the pregnancy-related rise in human chorionic gonadotropin (hCG) levels.[9] If ovulation is induced with a GnRH agonist instead of hCG and all embryos are frozen, even early OHSS is minimized making the overall OHSS risk extremely low.[10] Freezing and thawing of embryos additionally encourages an elective single embryo transfer policy with cumulative pregnancy rates similar to those seen after double embryo transfer.[11-12]

Despite evidence suggesting that ART outcomes may be further improved with the adaptation of a freeze-all strategy, the implementation remains a topic of ongoing debate and only one in five transfers in Europe on average was performed with frozen-thawed embryos in 2012.[1] In a large recent study, including 1508 patients with polycystic ovary syndrome comparing the freeze-all strategy with conventional fresh embryo transfer, the authors found a significantly higher frequency of live birth after the first frozen embryo transfer compared with fresh embryo transfer (49.3% vs. 42.0%).[7] Correspondingly, in a meta-analysis including three trials accounting for 633 cycles in women aged 27-33 years, eFET resulted in significantly higher clinical and ongoing pregnancy rates compared with fresh embryo transfer.[6] However, the included studies showed heterogeneity and one of the included publications was later retracted due to serious methodological flaws. In addition, the vast majority of the participants were high responders (496 out of 633) accounting for a highly selected group of patients, mostly consisting of PCOS patients or patients with and

ovarian PCO like morphology.[6] Moreover, previous studies were performed in China, US and Japan making them less generalizable to a European ART setting. According to Clinicaltrials.gov there are a few ongoing European RCT´s on the freeze-all strategy, however none of these studies investigate an almost complete OHSS-free strategy including GnRH-agonist trigger in the freeze-all group.

OHSS is one of the most severe side effects of ART and is potentially life threatening. The present protocol describes a randomised trial assessing a new ART treatment strategy, where OHSS can be almost completely avoided. The results are very important as the majority of our patients could avoid the OHSS risk by applying the "GnRH agonist and freeze-all" strategy, maybe even with a higher chance of pregnancy. This concept has not been assessed before, and should relevantly be considered when planning studies investigating the freeze-all strategy underlining the need for large multicentre randomised controlled trials exploring the GnRH agonist and freeze-all strategy in a broad population of ART patients. The present study will explore this approach in a bi-national multicentre randomised controlled trial setting providing information on the prospect of a freeze-all strategy.

Objectives

Primary objective

The primary objective of the study is to investigate if the ongoing pregnancy rate per randomised patient after the first potential single blastocyst transfer is superior in a freeze-all and transfer later-strategy compared to the conventional hCG trigger and fresh transfer strategy.

Ongoing pregnancy rate is defined as an intrauterine pregnancy with a foetal heart beat at transvaginal ultrasound in gestational week 7-8.

Ongoing pregnancy rate per first blastocyst transfer is also considered as a primary aim of the study addressing possible differences in endometrial receptivity between the two groups.

Secondary objectives

Secondary objectives include:

1. To assess cumulative live birth rates after *one complete cycle* including consecutive single blastocyst transfers of all embryos deriving from that oocyte retrieval (fresh and frozen) in the two study groups
2. To assess the transfer cancellation rate in the two study groups
3. To assess the prevalence of OHSS in the two study groups
4. To compare neonatal outcomes (preterm birth, low birth weight, SGA (small-for-gestational age), LGA (large-for-gestational age) and perinatal mortality) and the incidence of preeclampsia in the two study groups

5. To measure time-to-pregnancy from the date of start of COS to the date of the first ongoing pregnancy in the two study groups
6. To assess quality of life for both female and male partners during the two treatment protocols
7. To assess physical well-being by way of questionnaires and VAS scores regarding pain and discomfort at four and 16 days after oocyte retrieval in the two study groups

METHODS AND ANALYSIS

Study design

The study is designed as a multicentre randomised, controlled double-blinded trial with seven fertility clinics in Denmark and Sweden participating. All seven clinics are part of a University Hospital setting and perform standardized treatments according to the public health care system in Denmark and Sweden. Patient enrolment started in May 2016 and the last patients are expected to be included in the study in May 2018 with the primary outcome measure, ongoing pregnancy rate, being known for these patients approximately four months later for the patients allocated to the freeze-all group.

Study population/Participants and recruitment

The study participants will consist of women and their partners initiating ART treatment at one of the seven participating public clinics in Denmark and Sweden. Before initiating treatment patients will attend an information meeting, where they will be informed about the standard ART procedures, treatment regimens as well as ongoing clinical studies at the treatment sites. Those patients not able to participate in the information meeting will instead be informed by a doctor at an outpatient clinic consultation. Recruitment will be carried out by the doctors and study nurses at the fertility clinics. Prior to the initiation of treatment, patient files will be browsed by investigators at the clinics to assess if the patient fulfills the immediate inclusion criteria. Screening, including ultrasound examination of the uterus and ovaries is done on menstrual cycle day two or three securing that all inclusion criteria are met. Patients fulfilling the study criteria will start COS using a GnRH antagonist co-treatment in accordance with the standard routines of the trial site.

Eligibility criteria

To participate in the study, women will be required to meet the following inclusion criteria: Female age 18 to 39 years; eligibility to initiate the first, second or third ART cycle with oocyte aspiration (IVF or ICSI); AMH level > 6.28 pmol/L (Roche Elecsys assay) corresponding to the AMH threshold level used in the Bologna criteria to characterize poor responders; regular menstrual

cycle ≥ 24 days and ≤ 35 days: body mass index 18–35 kg/m^2; preservation of both ovaries and capability of signing informed consent. For specific exclusion criteria see Table 1.

Table 1. Specific exclusion criteria
Endometriosis stage III to IV
Ovarian cysts with a diameter > 30 mm at day of start of stimulation
Submucosal fibroids
Women with severe co-morbidity (IDDM (insulin dependent diabetes mellitus), NIDDM (non-insulin dependent diabetes mellitus), gastrointestinal, cardiovascular, pulmonary, liver or kidney disease)
Dysregulated thyroid disease
Non-Danish or English speaking
Contraindications or allergies to use of gonadotropins or GnRH antagonists
TESA (testicular sperm aspiration)
OD (oocyte donation)
Previous inclusion in the study

Randomisation and blinding

Patients who meet the inclusion criteria are randomised 1:1 to one of the two treatment groups: A. Freeze-all including GnRH agonist trigger, blastocyst vitrification and subsequent FET in an hCG triggered natural cycle or B. Traditional hCG trigger and fresh blastocyst transfer.

The randomisation is carried out by a study nurse or a non-treating doctor using a computerised randomisation program that runs a minimization algorithm, initially seeded using a random block sequence for the first subjects. The minimization algorithm is balancing the following variables: Female age (mean, and frequency of age ≥37 years), previously performed cycles (frequency of 0/1/2 cycles), nulliparous (frequency of yes/no), fertilisation method (frequency of IVF/ICSI), smoking (frequency of yes/no), AMH (≤ 12 pmol/L, 13-28 pmol/L, >28 pmol/L) and mean BMI. It selects with high (but less than 1.0) probability the treatment arm that provides the optimal balance between the arms. It also enforces predefined maximum allowed differences in number of subjects in each treatment arm at each study site (fertility clinic) and within the whole study. Furthermore, the starting dose of FSH is entered into the randomisation program before randomisation is performed to make sure that the FSH dose is decided upon before randomisation. Both the treating consultants and patients are blinded to the randomisation results during the controlled ovarian stimulation until the day when ovulation trigger is planned.

Treatment arms and interventions

The short GnRH antagonist protocol and blastocyst culture is applied in both treatment arms. The starting dose and type of gonadotropin is decided by the doctor on stimulation day one (cycle day two or three) and entered into the randomisation program prior to randomisation. Individualized gonadotropin dosing based on AMH, age, weight, previous COH cycles are applied. Recombinant follicular stimulating hormone (rFSH) or human menopausal gonadotropin (hMG) can be used according to the preference of the site, but the daily dose cannot exceed 300 IU. The gonadotropin stimulation will be performed according to the routine in the clinics and can be changed during the treatment according to the ovarian response to stimulation evaluated through ultrasound examination. GnRH antagonist co-treatment is initiated at a daily dose of 0.25 mg on stimulation day five or six according to the general standards in each clinic and is continued throughout the rest of the gonadotropin stimulation period.

Ultrasound examination is performed on cycle day two or three (baseline), stimulation day six or seven and subsequently every second to third day until ovulation trigger is decided according to the hCG/GnRH agonist trigger criterion: as soon as three follicles are ≥ 17 mm or one day later. At baseline a comprehensive ultrasound examination will estimate endometrial thickness, ovarian volume as well as number and size of antral follicles divided into the following three subclasses: 2-4 mm, 5-7 mm and 8-10 mm. On the day of ovulation trigger endometrial thickness and morphology as well as follicular development with number and size of follicles > 10 mm are registered.

When ovulation trigger is decided, the result of the randomisation is disclosed to both doctors and patients and ovulation and oocyte maturation is triggered with a GnRH agonist trigger injection (0.5 mg Buserelin) in the freeze-all group or a single injection of 250 μg of hCG in the fresh embryo transfer group. If >18 follicles with a diameter >11 mm are observed in the fresh embryo transfer group GnRH agonist triggering with Buserelin and vitrification of all embryos will be performed to avoid severe OHSS. All fertilised oocytes are cultured to the blastocyst stage and the embryos are scored and ranked according to standardised criteria ascribed to this study. The ranking will assure that the blastocyst with the highest implantation potential is transferred first in both groups. In the fresh transfer group, single blastocyst transfer is performed on day five after oocyte retrieval if a good quality blastocyst has developed. Surplus good quality blastocysts will be vitrified on day five or six. Luteal phase support is administered as vaginal progesterone according to the clinics standard procedures from day two after oocyte retrieval until the day of hCG test; thus luteal support is not extended into early pregnancy. In the freeze-all group all blastocysts of good quality are vitrified on day five or six depending on when the blastocyst stage is reached. The blastocyst with the highest rank is marked and will be the first one used in a subsequent hCG triggered

modified natural cycle FET. There should be *at least one completed menstrual cycle in between the stimulation and the embryo transfer*. In FET cycles a single injection of 250 µg hCG is administered, when the leading follicle is ≥ 17 mm. Blastocyst transfer is performed six or seven days after the hCG injection. No luteal phase support is given.

A plasma hCG test is performed 11 days after blastocyst transfer. Ongoing clinical pregnancy is defined as foetal heart beat at gestational age 7-8 confirmed by transvaginal ultrasound 3 to 4 weeks after a positive plasma-hCG test.

Data collection and management

Treatment related data is collected at 1) Baseline (cycle day two or three), 2) Day of ovulation trigger and 3) five days after oocyte retrieval. Data on blastocysts are collected at culture day five/six. Follow-up data on all pregnancies resulting from blastocysts transferred according to the study protocol will be followed from study inclusion and one year onwards. Data is transferred to an online eCRF system called MediCase with an underlying Microsoft SQL server database located in a guarded underground facility in Sweden. Data is backed up daily (one back-up to another computer in the same physical location as the server, and a second back-up to a physically separate location, also in Sweden). MediCase has a complete audit trail and is designed to only contain de-identified data and is entirely based on anonymous subject ID numbers used in the trial.

Sample collection

Blood samples will be collected three times during the treatment process: 1) Baseline (cycle day two or three), 2) Day of ovulation trigger and 3) 16 days after oocyte retrieval (day of pregnancy test in the fresh embryo transfer group). For overview of samples see Table 2. Furthermore one serum, plasma and fullblood sample are drawn at baseline and on the day of triggering and stored according to a trial specific laboratory manual in a project-specific biobank as back-up for analysis of endocrine and immunological factors of relevance for pregnancy. The frozen samples will be kept anonymised in the biobank with only the patient specific project ID number and collection date marked on the sample. The samples will be store in the participating fertility clinics and destroyed 5 years after the end of the study period if not analysed.

Further blood samples will be collected during the luteal phase for a smaller subgroup of 30 patients in each treatment group as part of a luteal phase subgroup analysis of differences in hormone levels in the two groups. The following blood samples will be collected at 1) Day of ovulation induction and 2) Day of ovulation trigger, day of ovulation trigger +7, +11, +14, +16 and +19: Estradiol, Inhibin-A, OH-Progesterone, Progesterone, LH and hCG.

Table 2. Blood sample collection	
Baseline (cycle day 2 or 3)	AMH FSH LH Estradiol Progesterone TSH TPO-antibodies Vitamin D CRP suPAR*
Day of ovulation induction	FSH LH Estradiol Progesterone CRP suPAR*
16 days after oocyte retrieval	CRP suPAR* hCG**

* Soluble urokinase-type plasminogen activator receptor, only measured at Hvidovre Hospital
** only fresh embryo transfer group

Questionnaires

Women as well as male partners will be asked to fill in quality of life validated questionnaires twice during the treatment process: 1) Four days after oocyte retrieval and 2) 16 days after oocyte retrieval. The questionnaires consist of standardized questions specially developed to explore emotional aspects as well as quality-of life related aspects of the treatment process. The women will at the same time be asked to fill in questionnaires regarding physical discomfort including a VAS score of physical pain in relation to the treatment.

Statistics

Sample size calculation

The trial is designed as a superiority study. Sample size calculation indicates that 424 participants (n = 212 in each arm) are required to have a 80 % chance of detecting, at a significance level at 0.05, an increase in the primary outcome measure (ongoing pregnancy rate per randomised after first potential blastocyst transfer) from 30% in the control group (fresh embryo transfer) to 43 % in the experimental group (freeze-all).

Outcome measurements (primary and secondary)

The primary endpoint is the ongoing pregnancy rate per randomised patient after the transfer of the first potential blastocyst. Ongoing pregnancy is defined as a pregnancy with a positive foetal heart beat at gestational week 7-8.

Other endpoints explored in the study contribute to the assessment of other relevant aspects of the freeze-all strategy including ongoing pregnancy rates per transfer, per started stimulation and per oocyte pick-up (percentage of participants with an ultrasound confirmation of foetal heart beat at gestational age 7-8) as well as live birth rate and cumulative live birth rates (percentage of participants with 1 live born neonate after 1 year of follow-up). The study furthermore aims to document the prevalence of OHSS assessed by the number of patients admitted to hospital under this diagnosis and the number of patients having ascites puncture. In addition, it is planned to evaluate pregnancy related complications as well as neonatal outcomes in both groups. For complete overview of all secondary endpoint measures see Table 3.

Table 3. Secondary endpoints
♦ Ongoing pregnancy rate per start of *per started ovarian stimulation and per oocyte retrieval*
♦ Live birth rate after the first blastocyst transfer calculated *per randomized patient, per started ovarian stimulation*, *per oocyte retrieval* and *per transfer*
♦ Cumulative live birth rate after one stimulated cycle with oocyte retrieval
♦ Cumulative live birth rate after use of all frozen blastocyst or after at least 1 year of follow-up
♦ Number of cycles with no embryo transfer
♦ Time-to-pregnancy (from start of ovarian stimulation to positive hCG)
♦ Time-to-delivery
♦ Cancelled embryo transfers
♦ Ovarian hyperstimulation syndrome (OHSS)
♦ Preterm birth
♦ Low birth weight
♦ Small-for-gestational age (SGA)
♦ Large-for-gestational age (LGA)
♦ Perinatal mortality
♦ Preeclampsia
♦ Placental rupture
♦ Positive hCG 11 days post embryo transfer
♦ Miscarriage, biochemical pregnancies, ectopic pregnancies
♦ Quality of life for female and male partner
♦ Cost-effectiveness
Other outcome measurements
♦ Number of good blastocysts
♦ Number of fertilized oocytes
♦ Number of high quality embryos day 2
♦ Number of grade 1 blastocysts
♦ Number of frozen blastocyst
♦ Paraclinical data: Endocrine, genetic and immunological parameters

Statistical analyses

Analyses of cumulative pregnancy rates and live birth rates after one oocyte retrieval including fresh and all frozen embryo transfer cycles will be compared by Cox-regression analyses. Comparisons between treatment groups will be performed primarily according to the intention-to-treat (ITT) principle but per-protocol analyses will also be done. Continuous data will be compared by students *t*-test or Mann-Whitney U test and Kruskal-Wallis test as appropriate. Proportions will be compared with chi-square test. Predictive factors for ongoing pregnancy in the two treatment groups will be tested with multivariate logistic regression analyses. A p-value of < 0.5 will be considered as statistically significant.

Patients in fresh embryo transfer group with GnRH agonist triggering

Patients allocated to the fresh transfer group who end up receiving GnRH agonist trigger and vitrification of all blastocysts due to risk of OHSS (>18 follicles with a diameter >11 mm on trigger day) will still be analysed as part of the fresh transfer group according to the intention-to-treat principle. Their first blastocyst transfer will derive from their first FET cycle and ongoing pregnancies from these first transfers will be included in the numerator together with ongoing pregnancies derived from the majority of patients with first blastocyst transfer in the fresh cycle. The denominator will be all randomised patients.

ETHICS, SAFETY AND DISSEMINATION

The study has been approved by the Danish regional committee on Health Research Ethics of the Capital Region and the Swedish national council on medical ethics.
Following oral and written information outlining the rationale, trial design, aims and treatment procedures written informed consent will be obtained from women and male partners prior to the enrolment in the study.

The participants are stimulated using individualised doses of gonadotropin stimulation in accordance with the clinical practice at each site. In all clinics serum AMH is considered when the FSH dose is determined. All medicine used in the study is part of standard ART care.

The overall safety of the patients is high in both treatment groups. The risk of OHSS is expected to be similar to the standard clinical protocol in the fresh embryo transfer group and lower in the freeze-all group in which GnRH agonist is used for ovulation trigger. In women in the fresh embryo transfer group with a risk of OHSS development (more than 18 follicles with a diameter over 11

mm), GnRH agonist will be used for trigger instead of hCG and all blastocysts will be vitrified and the transfer postponed.

No financial incentive exists for the participants as all couples are reimbursed for their first three ART treatments in the public health care system in the Nordic countries.

The results of the study will be publically disseminated in peer-reviewed scientific journals and presented at relevant international scientific meetings such as ESHRE (European Society of Human Reproduction and Embryology) and ASRM (American Society for Reproductive Medicine). In addition results will be published in popular science journals and other media.

DISCUSSION

The increasing interest in possible benefits of a freeze-all strategy and the limitations of existing randomised controlled trials comparing this strategy with conventional fresh embryo transfer underline the need for additional studies. The few previous RCT´s have demonstrated significantly increased pregnancy- and delivery rates with freeze-all, however these studies were performed in highly selected patient populations with poor generalizability.[6-7] Further, the treatment strategy combining GnRH agonist trigger and freeze-all minimizing the risk of severe OHSS development has not yet been investigated in a RCT setting. As GnRH agonist trigger does not hamper the yield of mature oocytes[12] and reduces the risk of OHSS to an absolutely minimum, it seems rational to include GnRH agonist trigger in the freeze-all concept. Evidently, we are unable to distinguish between the effect of the GnRH-agonist trigger and the effect of elective freeze-all, when both are included in the freeze-all treatment arm. The present study therefore compares an 'OHSS-free' freeze-all strategy including GnRH agonist trigger with a fresh transfer strategy with hCG trigger. In both treatment arms individualized gonadotropin dosing is used with the possibility of conversion to GnRH agonist trigger and segmentation in case of risk of OHSS development in the fresh embryo transfer group. Individualized gonadotropin dosing based on female age and weight, antral follicle count, AMH and results of previous COH cycles is applied, as this is the standard treatment approach used routinely in all of the participating clinics. The AMH cut-off value at 6.28 pmol/L (Roche Elecsys assay) corresponding to the Bologna criteria for poor ovarian response was chosen to have a reasonable chance of the patient ending up with at least one usable blastocyst after aspiration. It could be argued that an open randomisation, rather than a double-blinded study design, would allow a better exploration of the concept as higher gonadotropin doses and more oocytes could be safely aimed for in the freeze-all group. However, as this is the first RCT of a freeze-all strategy including GnRH agonist trigger, a double-blinded design was chosen to

minimize differences between the two treatment arms and gonadotropin dosing is decided upon independently of allocation to treatment group, as this is done prior to randomisation. In addition, even though a strategy combining GnRH agonist trigger and freeze-all is near OHSS free, increasing gonadotropin dosing would nonetheless add a potential risk of early OHSS in the patients.

The primary endpoint of this study is to investigate ongoing pregnancy rates per randomised patient after the first potential blastocyst transfer. Cumulative rates are additionally planned to be calculated, but as the number of aspirated oocytes is expected to be the same in both treatment groups due to gonadotropin dosing being decided upon independently of allocated treatment group, the effect of the freeze all strategy on the results of the first transfer may be diluted with the inclusion of additional FET´s.

The strengths of this study include the design as a multicenter randomised controlled double-blinded trial as well as preregistration and publication of the study protocol for more transparency. The investigation of several outcome measures related to different aspects of success parameters, including quality of life may furthermore add important information as regards the future potential of the freeze-all strategy in assisted reproduction.

FIGURE LEGEND Figure 1:

Figure 1. Flowchart of the Freeze-all study design

Contributor statement ANA, AP and SS participated in the conception, design, and writing of the study protocol. KL, HSN and CB contributed to the revision and editing of the study protocol. AP, SS, KL, JB, LP, ANA, HSN, CB, PH, MB, ALM and SOS will be involved in the recruitment of patients and the acquisition of data. SS wrote the first draft of this manuscript. AZ was involved in developing the laboratory criteria for the study. SS, AP, ANA, PH, CB, KL, JB, LP, HSN, MB, ALM and SOS will AP, ANA, PH, CB, KL, AZ, JB, LP, HSN, MB, ALM and SOS were all involved in critical revision of the manuscript. All authors approved the final version of the manuscript to be submitted.

Competing interests None declared

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Data sharing statement This manuscript is a study protocol. Data from the final study will be shared according to the coming ICJME guidelines.

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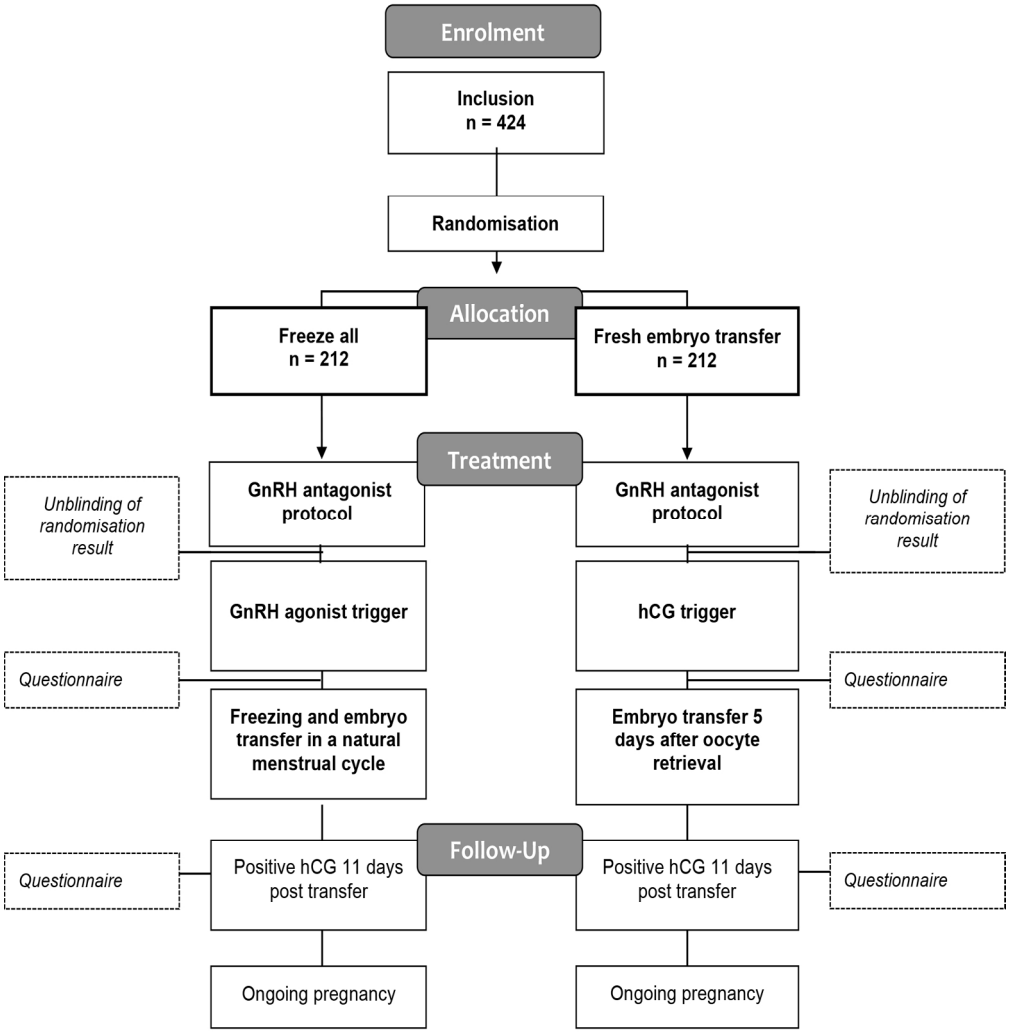

Figure 1. Flowchart of the Freeze-all study design

173x186mm (300 x 300 DPI)

A multicentre randomized controlled trial of a "Freeze all and transfer later" versus a conventional "Fresh Embryo Transfer" strategy for assisted reproductive technology (ART) in women with a regular menstrual cycle

- A Multicentre Randomized Controlled Trial of patients undergoing IVF/ICSI

Multicenter studium med otte deltagende fertilitetsklinikker i Danmark og Sverige

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1. BACKGROUND

In recent years improved cryopreservation techniques have made frozen embryo transfer (FET) a viable and promising alternative to fresh embryo transfer in assisted reproduction (ART). The optimization of cryopreservation techniques from slow-freezing to vitrification and prolonged embryo culture from cleavage to blastocyst state encourages the use of FET as the embryo survival rate following freezing and thawing is now significantly higher reaching 95-97% (Loutradi et al., 2008). Success rates including implantation as well as clinical and on-going pregnancy rates in FET are also significantly improving and approaching or even exceeding those associated with fresh embryo transfer (Kupka et al., 2014; Roque et al., 2013; Shapiro et al., 2011; Zhu et al., 2011). This is partly due the improved laboratory techniques, but may also be due to the endometrial environment in the FET cycles, which mirrors the natural cycle. In the stimulated cycle supraphysiological levels of estradiol and progesterone are present and may cause impaired endometrial receptivity (Shapiro et al., 2011). Furthermore, obstetric and perinatal outcomes after cryopreservation of embryos have been investigated and follow-up data from children born after FET have shown lower perinatal morbidity compared with fresh

embryo transfer (Kansal et al., 2011), but FET may also give rise to more large-for-gestational age babies (Pinborg et al., 2014). In addition, a recent systematic review and meta-analysis on data from 11 observational studies has shown better perinatal outcomes including lower perinatal mortality in singleton pregnancies following frozen-thawed embryo transfer compared with pregnancies after fresh embryo transfer (Maheshwari et al., 2012). Moreover, FET has the benefits of minimizing the risk of ovarian hyperstimulation syndrome (OHSS), which is the most severe side effect of ART and potentially life threatening. Finally, improved cryopreservation techniques favour an elective single embryo transfer (eSET) policy minimizing multiple pregnancies after ART (Pinborg, 2012).

Despite the noticeable advantages of embryo cryopreservation, fresh embryo transfer has persistently been the conventional in vitro fertilisation (IVF) procedure as only one in five transfers were made using frozen-thawed embryos in Denmark in 2013 (www.fertilitetsselskab.dk). This favour of a fresh embryo transfer strategy is however reflected in other European countries including Finland, Sweden and Iceland where approximately every third ART child is born after FET (Kupka et al., 2014). Some evidence suggests that IVF outcomes can be further improved with the adaptation of a `freeze-all´ or elective frozen embryo transfer (eFET) strategy with replacement of thawed embryos in natural cycles (Evans et al., 2014; Devroey et al., 2011; Maheshwari et al., 2013; Roque et al.,2013).

In a recent meta-analysis including three trials accounting for 633 cycles in women aged 27–33 years (Roque et al., 2013), FET resulted in significantly higher ongoing pregnancy rates (RR 1.32, 95% CI 1.10–1.59) and clinical pregnancy rates (RR 1.31, 95% CI 1.10–1.56). The studies showed heterogeneity and only 137 of the participants were normal responders, while the rest was high responders. Moreover one study included only cleavage stage embryo transfer while the other two included blastocyst transfers only. The studies were performed in Japan and in the US, while no European RCT has been published yet. Further, one of the included papers (Aflatoonian et al., 2010) was later retracted based on findings of serious methodological flaws in the study. This accentuates the need for a large multicentre, randomized controlled trial to

evaluate the prospect and clinical consequences of a “freeze all embryos and transfer later” policy compared with conventional fresh embryo transfer.

The aim of this multicentre randomized controlled trial is to compare a “freeze-all” embryo strategy with a conventional single fresh embryo transfer strategy in women 18 to 40 years of age undergoing their first to third IVF/ICSI cycle women with regard to treatment outcomes, risks for mother and child, quality of life and cost-effectiveness aspects of the two treatment modalities in a short GnRH antagonist protocol with blastocyst transfer and vitrification as the freezing method.

2. STUDY AIMS

1. The primary aim is to compare ongoing pregnancy rates per randomized patient and ongoing pregnancy rates per transfer in the “freeze-all” versus “fresh embryo transfer” group.

2. To assess live birth rates per randomized patient and per transfer in the “freeze-all” versus “fresh embryo transfer” group

3. To assess cumulative live birth rates after one stimulated cycle with oocyte retrieval in the two study arms.

4. To compare perinatal outcomes (preterm birth, low birth weight, small-for-gestational age, large-for-gestational-age, preeclampsia and perinatal mortality) in the two groups.

5. To measure time to pregnancy from start of ovarian stimulation and quality of life in both females and males in the two groups.

6. To explore VAS scores regarding pain and discomfort at the day of embryo transfer and 11 days post transfer in the two study arms

7. To assess female physical well-being during the two treatment modalities and to assess quality of life for both female and male partners during the two treatment protocols.

3. ENDPOINTS

Primary endpoints

- Ongoing pregnancy rate *per randomized patient*
  (pregnancy with positive fetal heart beat in gestational week 7-8)
- Ongoing pregnancy rate *per transfer* of the first blastocyst
  (pregnancy with positive fetal heart beat in gestational week 7-8)
- Ongoing pregnancy rate *per oocyte pick-up*
  (pregnancy with positive fetal heart beat in gestational week 7-8)
- Ongoing pregnancy rate *per start of ovarian stimulation*
  (pregnancy with positive fetal heart beat in gestational week 7-8)

Secondary endpoints

- Live birth rates calculated per randomized patient, per started ovarian stimulation, per oocyte pick-up and per transfer
- Cumulative live birth rate after one stimulated cycle with oocyte retrieval
- Cumulative live birth rate after use of all frozen blastocysts or after at least 1 year of follow-up.
- Number of cycles with no embryo transfer
- Time-to-pregnancy (from start of ovarian stimulation to positive hCG)
- Time-to-delivery
- Cancelled embryo transfers
- OHSS
- Preterm birth
- Low birth weight
- Small-for-gestational age (SGA)
- Large-for-gestational age (LGA)
- Perinatal mortality

- Preeclampsia
- Placental rupture
- Positive hCG 11 days or according to the local routine post embryo transfer
- Miscarriage, biochemical pregnancies, ectopic pregnancies
- Quality of life for female and ~~male~~ partner
- Cost-effectiveness

Other outcomes

- Number of good quality blastocyst
- Number of fertilized oocytes
- Number of high quality embryos day 2 (defined by the study laboratory manual)
- Number of grade 1 blastocysts (defined by the study laboratory manual)
- Number of frozen blastocysts
- Para-clinical data: Endocrine, genetic and immunological parameters influencing pregnancy

4. STUDYPOPULATION

Inclusion criteria

- Women > 6.28 pmol/L with the Roche Elecsys assay* (AMH > 1.1 ng/ml ~ 7.85 pmol/L old assay). This is according to the Bologna criteria for POR; AMH < 0.5–1.1 ng/ml (3.57-7,85 pmol/l (old assay) ~ 2,86 – 6,28 pmol/l Elecsys)(Ferraretti et al., 2011)
- Female age 18 year to less than 40 years
- 1.-3. IVF/ICSI cycle with oocyte aspiration
- Regular menstrual cycle $\geq$ 24 days and $\leq$ 35 days
- BMI $\geq$ 18 or < 35 kg/m^2
- Two ovaries
- Can and will sign the informed content

- **Exclusion criteria**
- Women who do not fulfil the inclusion criteria

- Endometriosis stage III to IV
- Ovarian cysts with diameter > 30 mm at day of start of stimulation
- Submucosal fibroids
- Women with severe co-morbidity (i.e Insulin Dependent Diabetes Mellitus (IDDM), Non-Insulin Dependent Diabetes Mellitus (NIDDM), gastrointestinal, cardio-vascular, pulmonary, liver or kidney disease)
- Dysregulation of thyroid disease
- Not Danish, Swedish or English speaking women
- Contraindications or allergies to use of gonadotrophins or GnRH antagonists
- TESA (testicular sperm aspiration)
- OD (oocyte donation)
- Previous inclusion in the study

5. METHODS

Inclusion of patients

- All couples or single/lesbian women starting IVF/ICSI treatment participate in a standard information meeting arranged by the clinic. During this 2 hour meeting patients and their partners are informed about the normal IVF/ICSI procedures, treatments and research in the clinic as well as this study. If patients are not attending the information meeting, the will be informed about the study at their first outpatient visit at the clinic.
- Few patients do not participate in the information meeting and they will have an appointment at the outpatient clinic in the Fertility clinic, where they will receive information about the IVF/ICSI treatment and be informed about this study.
- Patient files are browsed by one of the investigators, who decide if the patient is eligible. After the information meeting all patients receives a phone call from a doctor/study nurse, where they are informed about the treatment plan. If the inclusion criteria are fulfilled, the couples will receive oral information about the study and asked if they are interested in participating in the study, if so, the written patient information is sent to the couples by

email. If the couples are interested, a visit is planned on menstrual cycle day 2-4. The couple is informed that they can bring an assessor to the oral information visit. The written information is send by email, which leaves possibility of reading and reflection.

Informed consent

At the fertility clinic the patient will be seen by one of the investigators. Patients will be informed about the aim of the project and risks in accordance with the guidelines from the Scientific Ethical Committee. In case of questions, these will be answered. If the patients need more time for reflection, a new visit will be arranged. After signing the informed consent, the patient will be screened.

Screening – cycle day 2-4

- Medical and gynaecological history inclusive reproductive history including menstrual cycle length, smoking (yes/no), years of infertility
- Transvaginal ultrasound examination including ovarian volume, antral follicle count (AFC), and endometrial thickness and morphology and exclusion of pathology
- Height and weight
- Blood samples: AMH, FSH, LH, estradiol, progesterone, TSH, TPO antibodies, vitamin D, CRP and suPAR* (*only done at Hvidovre Hospital)
- One full blood, one plasma and one serum sample is cryopreserved as back-up and for analysis of endocrine and immunological factors of relevance for pregnancy

Screening should be performed no later than 3 months before randomization.

Randomization

When the patient has signed the informed consent, has been screened and it is confirmed that the inclusion criteria are meet, the patient is randomized to one of the two arms:

I. hCG arm with traditional hCG triggering and fresh blastocyst transfer

II. GnRH agonist triggering arm with blastocyst cryopreservation and subsequent transfer in a natural cycle.

Computerized randomization is performed according to the 1) Trial site and to 2) Female age <= 37 years or >37 years. The gonadotrophin stimulation dose is decided upon before randomization and entered into the database before randomization. The doctor and patient are blinded to the randomization until the day of hCG or GnRH agonist triggering.

Blood samples

Blood samples are collected at

- Baseline before the first gonadotrophin injection (cycle day 2-4): AMH, FSH, LH, estradiol, progesterone, TSH, TPO-antibodies, vitamin D CRP and suPAR* (*only done at Hvidovre Hospital)
- Day of trigger-injection: FSH, LH, estradiol, progesterone, CRP and suPAR* (*only done at Hvidovre Hospital)
- Day 16 after oocyte pick-up: hCG (only in the fresh embryo transfer group), CRP and suPAR* (*only done at Hvidovre Hospital)

At baseline and at day of trigger an extra full blood, plasma and serum sample is collected and stored according to a trail-specific laboratory manual. This will be stored in the freezer as back-up and for analysis of endocrine and immunological factors of relevance for this study.

Subgroup analyses in the luteal phase

During the lutealphase with embryo transfer of the stimulated fresh cycle and non-stimulated FER cycle blood samples are taken on day of hCG injection, hCG injection day+7, +11, +14, +16 and +19 for patients included at Hvidovre Hospital until 30 patient in each arm has been achieved. The following blood samples are collected; Estradiol, Inhibin-A, OH-progesterone, Progesterone, LH and hCG.

All blood samples are confidential. The frozen samples are anonymous, so no person identifiable date is left on the sample. Only the patient project ID number and the collection date identifies the sample. The study will be approved by the Danish Data Protection Agency and the Scientific Ethical Committee of the Capital Region, Region Zeeland and the Region Skåne in Sweden. The blood samples will be stored in the participating fertility clinics and if not used then five years after end of the study, at December 1st, 2023 blood samples will be definitively destroyed.

Gonadotropin stimulation treatment

The dose of gonadotrophin is decided and entered into the computer programme before randomization. The doctor and patient are blinded to the randomization until the day of hCG or GnRH agonist triggering. The study nurse is not blinded.

The ovarian stimulation with recombinant follicular stimulating hormone (rFSH) or human menopausal gonadotrophin (hMG) can start immediately after randomization in a short GnRH antagonist protocol. The gonadotrophin stimulation is performed according to the general standards in each of the clinics and can be altered according to the ovarian response. The GnRH antagonist is initiated at a daily dose of 0.25 mg at stimulation day 5 or 6 according to the clinical standards and continued throughout the rest of the gonadotropin stimulation period. The gonadotrophin dose cannot exceed a daily dose of 300 IU. Both groups are treated according to the short GnRH-antagonist protocol, where a higher dose of gonadotropin than 300 IU has been shown to be of no added value for further follicular growth. The maximum stimulation period is 20 days.

The medication for the study is bought by the patients themselves according to general prescription rules.

Ultrasound

Ultrasound examination is performed on cycle day 2-3 (Stim1), Stim 5-8 and thereafter every 2-3 days until ovulation trigger is decided. At the start of stimulation comprehensive sonography is

performed with details on each ovary, including ovarian volume, number of antral follicle in the following subclasses: 2-4mm, 5-7mm and 8-10mm.

The following parameters are measured on the day of ovulation trigger or the day before: Follicular development with size and number of follicles >10 mm, endometrial thickness and echogenicity and uterine pathology.

Ovulation induction

As soon as three follicles of >= 17 mm are observed or one day after a single injection of 250 µg of human chorionic gonadotrophin (hCG) is administered in the "fresh transfer"-arm, while GnRH agonist triggering with GnRH agonist Buserelin 0.5 mg is administered in the "freeze-all" arm.

Triggering of ovulation with a GnRH agonist Buserelin 0.5 mg is allowed in the fresh embryo transfer arm in case of risk of severe OHSS after the following the criteria: In the fresh embryo arm: If > 18 follicles with a diameter > 11 mm are observed on the day of triggering, GnRH agonist triggering should be used and all blastocysts frozen (Papanikolaou et al., 2011). Coasting / surfing cannot be used.

Oocyte retrieval

Oocyte retrieval is performed 36 ± 2 hours after hCG or GnRH agonist administration.

IVF/ICSI

Oocytes are fertilised by either IVF or ICSI and embryos are cultured individually according to the normal procedure in the clinics.

Embryo transfer

I. "Fresh embryo transfer" group

Single blastocyst transfer is always performed on day five after oocyte pick-up if a blastocyst is developed. Surplus good quality blastocyst are vitrified on day five or six.

Luteal phase support is administered as vaginal progesterone (vaginal gel (Crinone) 90 mg/dose x 1 daily or vaginal tablets 100 mg x 3 daily (Lutinus)) according to the standard procedure in each of the individual clinics from day 2 after oocyte retrieval and to confirmation of pregnancy or negative hCG 11-15 days post transfer. In case of a positive pregnancy test an ultrasound scan is performed three to four weeks later to confirm an intrauterine pregnancy with a live foetus.

Triggering of ovulation with a GnRH agonist Buserelin 0.5 mg is allowed in the fresh embryo transfer arm in case of risk of severe OHSS, following the criteria: If > 18 follicles with a diameter > 11 mm are observed, GnRH agonist triggering should be used and all blastocysts frozen (Papanikolaou et al., 2011). Coasting / surfing cannot be used.

II. "Freeze all and transfer later" group

For patients in this group all embryos of a good quality are vitrified at the blastocyst stage day 5 in the stimulated cycle. Criteria for freezing of blastocyst are according to the criteria in the specific clinic. The "best" embryo (i.e. of the highest quality is selected after predefined strict criteria according to the specific trial laboratory manual) is marked and is the first one to be warmed after at least one menstrual cycle that is considered as a wash out period.

In the menstrual cycle with blastocyst transfer, an hCG injection of 6500 units is given when the leading follicle is $\geq$ 17 mm. Embryo transfer is performed 6-7 days after hCG injection. No luteal phase support is needed.

Pregnancy test

A serum beta-hCG test is performed 11 days after blastocyst transfer or according to local routine. Clinical pregnancy is confirmed by transvaginal ultrasound 3 to 4 weeks after a positive serum-hCG.

Follow-up both groups

A follow-up of all pregnancies will be performed within three months after delivery or termination of pregnancy on predefined information sheets.

Information on background data, pregnancies and deliveries are returned to Hvidovre Fertility clinic on predefined case report forms (CRF) including pregnancy and delivery information sheets. All pregnancies resulting from blastocyst retrieved and thawed according to this study protocol will be followed from study inclusion (Stim day 1) and one year onwards.

All data are anonymized by encryption in the database with no personal identifiable data.

We will retrieve data from the patient clinical files and clinical databases with information regarding previous diseases, hospital admissions, former and current fertility treatment, pregnancy and delivery data on pregnancies related to this study. Both females and males will be informed about this in the patient information. This collected information will be used to characterize the populations and to minimize risk of bias.
We will also gain information regarding the coming child and the female and partner will sign a separate informed consent in Denmark and in Sweden a single consent form for the couple regarding this.

VAS-score and physical discomfort questionnaire

Women in both arms will be requested to fill-in their level of pain and discomfort on a VAS-score scale and a physical discomfort questionnaire as well as a quality of life questionnaire at the day of oocyte pick-up +4 and the day of oocyte pick up + 16.

Visits

Every visit is registered on a standardized stimulation scheme made specifically for this study. The schemes are normally used as standards in the clinics.

Criteria for withdrawal

A patient can be withdrawn from the study at any time, if the patient wishes to do so or if there is a medical indication decided by the investigator. The patient participation in the study can be interrupted, if one of the following criteria is present:

- The patients general condition contraindicates participation
- Protocol violation, which the investigator assess to have influence on the treatment
- Safety

Patients will be carefully monitored from stimulation start, at Stim6 and thereafter every 2-3 day in the clinic. The treatment will be monitored by transvaginal ultrasound of the ovaries. After each visit the patients will receive thorough information on the drug dosage and administration. This will follow the normal procedure in the clinic. If a patient is taking the wrong dosage, it will be documented on the stimulation scheme. This is not dangerous to the patient as the treatment is monitored by ultrasound scans hence a risk of OHSS will be discovered there.

In case of risk of OHSS in the “fresh-embryo-transfer”-arm, the ovulation trigger will not be induced by hCG but with Buserelin 0.5 mg. The further treatment of this patient will be handled according to the routine of the clinic.

In case of OHSS the patient is monitored at the clinic until recovery. Overall the safety of the patients is high in both the fresh embryo transfer and the freeze all group as the gonadotrophin stimulation corresponds to the normal program for patients at risk of OHSS. Furthermore patients with irregular cycles i.e. as part of polycystic ovarian syndrome, who in general have a higher risk of OHSS, are not included in this study.

6. STATISTICS AND SAMPLE SIZE

Superiority study

In all 424 (n= 212 in each arm) patients are required to have an 80% chance of detecting, as significant at the 5% level, an increase in the primary outcome measure ongoing pregnancy rate per randomised patient and per transfer from 30% in the control group to 43% in the experimental group. A difference of 15% was found in a randomized controlled trial by Shapiro et al, 2011 between the "freeze-all" arm and the fresh transfer group.

The statistical analyses will be performed by investigator together with statistical experts at Hvidovre Hospital and associate professor Julie Forman, Department of Biostatistics, Faculty of Health and Medical Sciences, University of Copenhagen.

A Statistical Analysis Plan (SAP) will be presented before closing of the database and before any statistical analyses are performed.

7. STUDY MEDICATION

All medicine used in this study is normally used as standard care for the patients in the short GnRH antagonist protocol. Patients will have prescriptions on all the medicine and will take all the medicine at home as is the routine in the clinics.

Dosage and administration

Treatment dose at the first day and during the ovarian stimulation is planned by the investigator and the patient is further instructed by a nurse, so that the patient is confident in self administration at home according to the normal clinical routine.

Side effects

Most side effects are mild and related to the medication during the stimulation. Unwanted OHSS is a risk in all IVF treatment but is considered low in this project as a standard IVF/ICSI protocol is used with individualized dosing. Further, in the freeze-all group the risk of OHSS is expected to be lower than in the standard care group as all blastocyst transfers are postponed to cycles without ovarian stimulation.

8. DATA SECURITY AND ETHICAL ASPECTS

Data security

One full blood and one serum sample at baseline and at the day of ovulation induction will be collected and stored according to the trial specific laboratory manual on all women included in this trial for future analyses of endocrine, immunological and protein markers. All data will be collected in a single database including all project subjects with an identification code thus data on each subject will be anonymous, when entered into the database.

Ethical aspects

The study will be performed according to the Danish Law and ethical principles in the Helsinki Declaration. This covers that study subjects receive both oral and written information and the opportunity of time for reflection and that they can discuss their participation with a third person.

The participants will be given a individualized dose of gonadotropin according to their serum AMH level, which is standard for patients at all five Fertility Clinics in Denmark and Sweden. The risk of OHSS will be similar to the standard clinical protocol and lower in the "freeze-all" group.

With a "freeze-all embryo and transfer later" protocol in ART, the risk of OHSS in women undergoing IVF/ICSI will be minimized and the embryo development will benefit from an endometrium less influenced by supra-physiological levels of estradiol and progesterone in the fresh embryo transfer cycle. This may also be beneficial for the children born after the treatments.

The study is approved by the Scientific Ethical Committee in the Capital Region (H-1600-1116) and by the Scientific Ethical Committee in Region Skåne in Sweden (Dnr. 2016/654)

The study will be approved by the Data Protection Agencies in Denmark and Sweden.

9. TIME SCHEDULE AND PUBLICATION

Protocol will be send to the Scientific Ethical Committee in the Capital Region, Denmark in January 2016 and inclusion of patients will start as soon as the approval from SEC has been obtained. The inclusion of patients will run from March 2016 to February 2018. Statistical analyses, writing and preparing manuscripts will go on from February 2018 to January 2019.

The results of the study will be presented at national as well as international scientific congresses and published in high impact international scientific journals in reproductive medicine such as Human Reproduction or Fertility and Sterility. Further results of public interest will be reported in the public press.

10.FINANCING

The project is initiated by Professor Anja Pinborg. This project is part of the Reprounion program, which has been supported by the Interreg-program for Öresund-Kattegat-Skagerak from EU, Capital Region of Denmark, Region Skåne and Ferring Pharmaceutical Company. The project has been financed with 450.000 euro (3.375.000 dkk) by a grant from Interreg/EU.

Patients included in this study and the Scientific Ethical Committee will be informed if further funding is obtained for this study. Funding will be transferred to a research account in the bank of Hvidovre Hospital, Capital Region of Denmark.

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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			Page 1 of protocol
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	_____
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	Page 17 of protocol _____
	2b	All items from the World Health Organization Trial Registration Data Set	All accounted for see items below _____
Protocol version	3	Date and version identifier	Page 1 of protocol (header) _____
Funding	4	Sources and types of financial, material, and other support	Page 18 of protocol _____
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	Page 1, 2, 3 of protocol _____

	5b	Name and contact information for the trial sponsor	Page 1 of protocol
	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	Not applicable
	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)	Not applicable
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	Page 3, 4 5 of protocol
	6b	Explanation for choice of comparators	Page 3, 4 5 of protocol
Objectives	7	Specific objectives or hypotheses	Page 5 of protocol
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)	Page 8-10 of protocol
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	Page 8 List of study sites; page 1-3
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)	Page 7-8
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	Page 11-13
	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)	Page 14-15
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	Page 11 (visits)
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	Not applicable
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	Page 5-7
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)	Page 5-13
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	Page 15
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	Page 8

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	Page 9
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	Page 9
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	Page 9, 11
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	Page 9
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	No circumstances
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	Page 9, 10, 11 and 14
	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	Page 13-14

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	Page 13,14, Page 16 Details data management can be obtained through contact to primary investigator ("projektansvarlig læge" of trial
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	Page 15 Details of planned statistical analysed can be obtained through contact to primary investigator ("projektansvarlig læge" of trial
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	Same as item 20a
	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)	Same as item 20a
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	No data monitoring committee used

	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	No interim analyses will be performed
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	Not applicable
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	Not available in protocol
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	Page 17
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)	Not available in protocol
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	Page 8 of protocol
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	Not 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	Page 14, 16
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	Page 18 No competing interests exists

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	Not available in protocol
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	Not applicable
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	Not available in protocol
	31b	Authorship eligibility guidelines and any intended use of professional writers	Not available in protocol
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	No current plans
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Not available in protocol – Standard consent forms from the Danish etichal committee is used as well s participant information approved by the Ethical Committee
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	Page 10

*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.

BMJ Open

Comparison of a "freeze all" strategy including GnRH agonist trigger versus a "fresh transfer" strategy including hCG trigger in assisted reproductive technology (ART) – A study protocol for a randomised controlled trial

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Primary Subject Heading:	Reproductive medicine
Secondary Subject Heading:	Obstetrics and gynaecology

Keywords:	ART, FET, Freeze-all, RCT, Ongoing pregnancy rate, OPR

TITLE PAGE

PROTOCOL ARTICLE

Comparison of a "freeze all" strategy including GnRH agonist trigger versus a "fresh transfer" strategy including hCG trigger in assisted reproductive technology (ART) – A study protocol for a randomised controlled trial

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ABSTRACT

Introduction Pregnancy rates after frozen-thawed embryo transfer (FET) have improved in recent years and are now approaching or even exceeding those obtained after fresh embryo transfer. This is partly due to improved laboratory techniques, but may also be caused by a more physiological hormonal and endometrial environment in FET cycles. Furthermore, the risk of ovarian hyperstimulation syndrome (OHSS) is practically eliminated in segmentation cycles followed by FET and the use of natural cycles in frozen-thawed embryo transfers may be beneficial for the post-implantational conditions of foetal development. However, a freeze-all strategy is not yet implemented as standard care due to limitations of large randomised trials showing a benefit of such a strategy. Thus, there is a need to test the concept against standard care in a randomised controlled design. This study aims to compare ongoing pregnancy and live birth rates between a freeze-all strategy with GnRH agonist triggering versus hCG trigger and fresh embryo transfer in a multicentre randomised controlled trial.

Methods and analysis Multicentre randomised, controlled, double-blinded trial of women undergoing ART treatment including 424 normo-ovulatory women aged 18 to 39 from Denmark and Sweden. Participants will be randomised (1:1) either A. GnRH agonist trigger and single vitrified-warmed blastocyst transfer in a subsequent hCG triggered natural menstrual cycle or B. hCG trigger and single blastocyst transfer in the fresh (stimulated) cycle. The primary endpoint is to compare ongoing pregnancy rates per randomised patient in the two treatment groups after the first single blastocyst transfer.

Ethics and dissemination

The study will be performed in accordance with the ethical principles in the Helsinki Declaration. The study is approved by the Scientific Ethical Committees in Denmark and Sweden. The results of the study will be publically disseminated.

Trial registration numbers: ClinicalTrials.gov identifier: NCT02746562; Ethical Approval, Denmark: H-1600-1116, Ethical Approval, Sweden: Dnr. 2016/654

Strengths and limitations of this study

Strengths

- The design: A multicentre, randomised controlled double-blinded trial powered to identify an increase in ongoing pregnancy rate in the freeze-all group compared to the conventional fresh blastocyst transfer group
- The study includes normo-ovulatory women aged 18-39 years with a BMI < 35 thus results can be extrapolated to the majority of the normo-ovulatory infertile population
- GnRH-agonist trigger in the freeze-all group adds a concept of an OHSS-free strategy

Limitations

- As both GnRH-agonist trigger and elective freeze-all are new treatment approaches, we will not be able to distinguish the two effects from each other, but compare an OHSS-free strategy to a conventional fresh transfer strategy
- The study is powered to detect a 12 % difference in ongoing pregnancy between the two groups, thus smaller but yet clinically relevant differences may be overlooked

INTRODUCTION

The use of assisted reproductive technology (ART) is increasing and presently up to 5 % of birth cohorts in certain countries are conceived by ART.[1] In recent years, pregnancy rates following frozen embryo transfer (FET) have rapidly increased and may now be a viable and appropriate alternative to the conventional fresh embryo transfer in ART. The main reason is the introduction of vitrification, increasing post-thawing survival rates after blastocyst culture significantly as compared to previous years.[2-3] Implantation as well as clinical and ongoing pregnancy rates are correspondingly improving in frozen cycles and approaching or even exceeding those associated with fresh embryo transfer.[4-6]

A freeze-all strategy has been suggested as a way to further improve success rates in ART, arguing that the use of the best embryo in frozen cycles instead of in fresh cycles may potentially increase pregnancy rates and live birth rates.[6-7] The rationale is that transfer of a frozen-thawed embryo in a subsequent natural menstrual cycle has the advantage of an endometrium that has not been exposed to the supraphysiological levels of estradiol and progesterone following controlled ovarian stimulation (COS) in fresh cycles, which may negatively affect endometrial receptivity.[5 8] Elective FET (eFET) moreover has the benefit of essentially eliminating the risk of developing late ovarian hyperstimulation syndrome (OHSS) associated with the pregnancy-related rise in human chorionic gonadotropin (hCG) levels.[9] If ovulation is induced with a GnRH agonist instead of hCG and all embryos are frozen, even early OHSS is minimized making the overall OHSS risk extremely low.[10] Freezing and thawing of embryos additionally encourages an elective single embryo transfer policy with cumulative pregnancy rates similar to those seen after double embryo transfer.[11-12]

Despite evidence suggesting that ART outcomes may be further improved with the adaptation of a freeze-all strategy, the implementation remains a topic of ongoing debate and only one in five transfers in Europe on average was performed with frozen-thawed embryos in 2012.[1] In a large recent study, including 1508 patients with polycystic ovary syndrome comparing the freeze-all strategy with conventional fresh embryo transfer, the authors found a significantly higher frequency of live birth after the first frozen embryo transfer compared with fresh embryo transfer (49.3% vs. 42.0%).[7] Correspondingly, in a meta-analysis including three trials accounting for 633 cycles in women aged 27-33 years, eFET resulted in significantly higher clinical and ongoing pregnancy rates compared with fresh embryo transfer.[6] However, the included studies showed heterogeneity and one of the included publications was later retracted due to serious methodological flaws. In addition, the vast majority of the participants were high responders (496 out of 633) accounting for a highly selected group of patients, mostly consisting of PCOS patients or patients with and

ovarian PCO like morphology.[6] Moreover, previous studies were performed in China, US and Japan making them less generalizable to a European ART setting. According to Clinicaltrials.gov there are a few ongoing European RCT´s on the freeze-all strategy, however none of these studies investigate an almost complete OHSS-free strategy including GnRH-agonist trigger in the freeze-all group.

OHSS is one of the most severe side effects of ART and is potentially life threatening. The present protocol describes a randomised trial assessing a new ART treatment strategy, where OHSS can be almost completely avoided. The results are very important as the majority of our patients could avoid the OHSS risk by applying the "GnRH agonist and freeze-all" strategy, maybe even with a higher chance of pregnancy. This concept has not been assessed before, and should relevantly be considered when planning studies investigating the freeze-all strategy underlining the need for large multicentre randomised controlled trials exploring the GnRH agonist and freeze-all strategy in a broad population of ART patients. The present study will explore this approach in a bi-national multicentre randomised controlled trial setting providing information on the prospect of a freeze-all strategy.

Objectives

Primary objective

The primary objective of the study is to investigate if the ongoing pregnancy rate per randomised patient after the first potential single blastocyst transfer is superior in a freeze-all and transfer later-strategy compared to the conventional hCG trigger and fresh transfer strategy.

Ongoing pregnancy rate is defined as an intrauterine pregnancy with a foetal heart beat at transvaginal ultrasound in gestational week 7-8.

Ongoing pregnancy rate per first blastocyst transfer is also considered as a primary aim of the study addressing possible differences in endometrial receptivity between the two groups.

Secondary objectives

Secondary objectives include:

1. To assess cumulative live birth rates after *one complete treatment cycle* including consecutive single blastocyst transfers of all embryos deriving from that oocyte retrieval (fresh and frozen) in the two study groups
2. To assess the transfer cancellation rate in the two study groups
3. To assess the prevalence of OHSS in the two study groups
4. To compare neonatal outcomes (preterm birth, low birth weight, SGA (small-for-gestational age), LGA (large-for-gestational age) and perinatal mortality) and the incidence of preeclampsia in the two study groups

5. To measure time-to-pregnancy from the date of start of COS to the date of the first ongoing pregnancy in the two study groups
6. To assess quality of life for both female and male partners during the two treatment protocols
7. To assess physical well-being by way of questionnaires and VAS scores regarding pain and discomfort at four and 16 days after oocyte retrieval in the two study groups

METHODS AND ANALYSIS

Study design

The study is designed as a multicentre randomised, controlled double-blinded trial with seven fertility clinics in Denmark and Sweden participating. All seven clinics are part of a University Hospital setting and perform standardized treatments according to the public health care system in Denmark and Sweden. Patient enrolment started in May 2016 and the last patients are expected to be included in the study in May 2018 with the primary outcome measure, ongoing pregnancy rate, being known for these patients approximately four months later for the patients allocated to the freeze-all group.

Study population/Participants and recruitment

The study participants will consist of women and their partners initiating ART treatment at one of the seven participating public clinics in Denmark and Sweden. Before initiating treatment patients will attend an information meeting, where they will be informed about the standard ART procedures, treatment regimens as well as ongoing clinical studies at the treatment sites. Those patients not able to participate in the information meeting will instead be informed by a doctor at an outpatient clinic consultation. Recruitment will be carried out by the doctors and study nurses at the fertility clinics. Prior to the initiation of treatment, patient files will be browsed by investigators at the clinics to assess if the patient fulfills the immediate inclusion criteria. Screening, including ultrasound examination of the uterus and ovaries is done on menstrual cycle day two or three securing that all inclusion criteria are met. Patients fulfilling the study criteria will start COS using a GnRH antagonist co-treatment in accordance with the standard routines of the trial site.

Eligibility criteria

To participate in the study, women will be required to meet the following inclusion criteria: Female age 18 to 39 years; eligibility to initiate the first, second or third ART cycle with oocyte aspiration (IVF or ICSI); AMH level > 6.28 pmol/L (Roche Elecsys assay) corresponding to the AMH threshold level used in the Bologna criteria to characterize poor responders; regular menstrual

cycle ≥ 24 days and ≤ 35 days: body mass index 18–35 kg/m^2; preservation of both ovaries and capability of signing informed consent. For specific exclusion criteria see Table 1.

Table 1. Specific exclusion criteria
Endometriosis stage III to IV
Ovarian cysts with a diameter > 30 mm at day of start of stimulation
Submucosal fibroids
Women with severe co-morbidity (IDDM (insulin dependent diabetes mellitus), NIDDM (non-insulin dependent diabetes mellitus), gastrointestinal, cardiovascular, pulmonary, liver or kidney disease)
Dysregulated thyroid disease
Non-Danish or English speaking
Contraindications or allergies to use of gonadotropins or GnRH antagonists
TESA (testicular sperm aspiration)
OD (oocyte donation)
Previous inclusion in the study

Randomisation and blinding

Patients who meet the inclusion criteria are randomised 1:1 to one of the two treatment groups: A. Freeze-all including GnRH agonist trigger, blastocyst vitrification and subsequent FET in an hCG triggered natural cycle or B. Traditional hCG trigger and fresh blastocyst transfer.

The randomisation is carried out by a study nurse or a non-treating doctor using a computerised randomisation program that runs a minimization algorithm, initially seeded using a random block sequence for the first subjects. The minimization algorithm is balancing the following variables: Female age (mean, and frequency of age ≥37 years), previously performed cycles (frequency of 0/1/2 cycles), nulliparous (frequency of yes/no), fertilisation method (frequency of IVF/ICSI), smoking (frequency of yes/no), AMH (≤ 12 pmol/L, 13-28 pmol/L, >28 pmol/L) and mean BMI. It selects with high (but less than 1.0) probability the treatment arm that provides the optimal balance between the arms. It also enforces predefined maximum allowed differences in number of subjects in each treatment arm at each study site (fertility clinic) and within the whole study.

Furthermore, the starting dose of FSH is entered into the randomisation program before randomisation is performed to make sure that the FSH dose is decided upon before randomisation.

Both the treating consultants and patients are blinded to the randomisation results during the controlled ovarian stimulation until the day when ovulation trigger is planned.

Treatment arms and interventions

The short GnRH antagonist protocol and blastocyst culture is applied in both treatment arms. The starting dose and type of gonadotropin is decided by the doctor on stimulation day one (cycle day two or three) and entered into the randomisation program prior to randomisation. Individualized gonadotropin dosing based on AMH, age, weight, previous COH cycles are applied. Recombinant follicular stimulating hormone (rFSH) or human menopausal gonadotropin (hMG) can be used according to the preference of the site, but the daily dose cannot exceed 300 IU. The gonadotropin stimulation will be performed according to the routine in the clinics and can be changed during the treatment according to the ovarian response to stimulation evaluated through ultrasound examination. GnRH antagonist co-treatment is initiated at a daily dose of 0.25 mg on stimulation day five or six according to the general standards in each clinic and is continued throughout the rest of the gonadotropin stimulation period.

Ultrasound examination is performed on cycle day two or three (baseline), stimulation day six or seven and subsequently every second to third day until ovulation trigger is decided according to the hCG/GnRH agonist trigger criterion: as soon as three follicles are ≥ 17 mm or one day later. At baseline a comprehensive ultrasound examination will estimate endometrial thickness, ovarian volume as well as number and size of antral follicles divided into the following three subclasses: 2-4 mm, 5-7 mm and 8-10 mm. On the day of ovulation trigger endometrial thickness and morphology as well as follicular development with number and size of follicles > 10 mm are registered.

When ovulation trigger is decided, the result of the randomisation is disclosed to both doctors and patients and ovulation and oocyte maturation is triggered with a GnRH agonist trigger injection (0.5 mg Buserelin) in the freeze-all group or a single injection of 250 μg of hCG in the fresh embryo transfer group. If > 18 follicles with a diameter > 11 mm are observed in the fresh embryo transfer group GnRH agonist triggering with Buserelin and vitrification of all embryos will be performed to avoid severe OHSS. All fertilised oocytes are cultured to the blastocyst stage and the embryos are scored and ranked according to standardised criteria ascribed to this study. The ranking will assure that the blastocyst with the highest implantation potential is transferred first in both groups. In the fresh transfer group, single blastocyst transfer is performed on day five after oocyte retrieval if a good quality blastocyst has developed. Surplus good quality blastocysts will be vitrified on day five or six. Luteal phase support is administered as vaginal progesterone according to the clinics standard procedures from day two after oocyte retrieval until the day of hCG test; thus luteal support is not extended into early pregnancy. In the freeze-all group all blastocysts of good quality are vitrified on day five or six depending on when the blastocyst stage is reached. The blastocyst with the highest rank is marked and will be the first one used in a subsequent hCG triggered

modified natural cycle FET. There should be *at least one completed menstrual cycle in between the stimulation and the embryo transfer*. In FET cycles a single injection of 250 µg hCG is administered, when the leading follicle is ≥ 17 mm. Blastocyst transfer is performed six or seven days after the hCG injection. No luteal phase support is given.

A plasma hCG test is performed 11 days after blastocyst transfer. Ongoing clinical pregnancy is defined as foetal heart beat at gestational age 7-8 confirmed by transvaginal ultrasound 3 to 4 weeks after a positive plasma-hCG test. For overview of study design see figure 1.

Data collection and management

Treatment related data is collected at 1) Baseline (cycle day two or three), 2) Day of ovulation trigger and 3) five days after oocyte retrieval. Data on blastocysts are collected at culture day five/six. Follow-up data on all pregnancies resulting from blastocysts transferred according to the study protocol will be followed from study inclusion and one year onwards. Data is transferred to an online eCRF system called MediCase with an underlying Microsoft SQL server database located in a guarded underground facility in Sweden. Data is backed up daily (one back-up to another computer in the same physical location as the server, and a second back-up to a physically separate location, also in Sweden). MediCase has a complete audit trail and is designed to only contain de-identified data and is entirely based on anonymous subject ID numbers used in the trial.

Sample collection

Blood samples will be collected three times during the treatment process: 1) Baseline (cycle day two or three), 2) Day of ovulation trigger and 3) 16 days after oocyte retrieval (day of pregnancy test in the fresh embryo transfer group). For overview of samples see Table 2. Furthermore one serum, plasma and fullblood sample are drawn at baseline and on the day of triggering and stored according to a trial specific laboratory manual in a project-specific biobank as back-up for analysis of endocrine and immunological factors of relevance for pregnancy. The frozen samples will be kept anonymised in the biobank with only the patient specific project ID number and collection date marked on the sample. The samples will be store in the participating fertility clinics and destroyed 5 years after the end of the study period if not analysed.

Further blood samples will be collected during the luteal phase for a smaller subgroup of 30 patients in each treatment group as part of a luteal phase subgroup analysis of differences in hormone levels in the two groups. The following blood samples will be collected at 1) Day of ovulation induction and 2) Day of ovulation trigger, day of ovulation trigger +7, +11, +14, +16 and +19: Estradiol, Inhibin-A, OH-Progesterone, Progesterone, LH and hCG.

Table 2. Blood sample collection	
Baseline (cycle day 2 or 3)	AMH FSH LH Estradiol Progesterone TSH TPO-antibodies Vitamin D CRP suPAR*
Day of ovulation induction	FSH LH Estradiol Progesterone CRP suPAR*
16 days after oocyte retrieval	CRP suPAR* hCG**

* Soluble urokinase-type plasminogen activator receptor, only measured at Hvidovre Hospital
** only fresh embryo transfer group

Questionnaires

Women as well as male partners will be asked to fill in quality of life validated questionnaires twice during the treatment process: 1) Four days after oocyte retrieval and 2) 16 days after oocyte retrieval. The questionnaires consist of standardized questions specially developed to explore emotional aspects as well as quality-of life related aspects of the treatment process. The women will at the same time be asked to fill in questionnaires regarding physical discomfort including a VAS score of physical pain in relation to the treatment.

Statistics

Sample size calculation

The trial is designed as a superiority study. Sample size calculation indicates that 424 participants (n = 212 in each arm) are required to have a 80 % chance of detecting, at a significance level at 0.05, an increase in the primary outcome measure (ongoing pregnancy rate per randomised after first potential blastocyst transfer) from 30% in the control group (fresh embryo transfer) to 43 % in the experimental group (freeze-all).

Outcome measurements (primary and secondary)

The primary endpoint is the ongoing pregnancy rate per randomised patient after the transfer of the first potential blastocyst. Ongoing pregnancy is defined as a pregnancy with a positive foetal heart beat at gestational week 7-8.

Other endpoints explored in the study contribute to the assessment of other relevant aspects of the freeze-all strategy including ongoing pregnancy rates per transfer, per started stimulation and per oocyte pick-up (percentage of participants with an ultrasound confirmation of foetal heart beat at gestational age 7-8) as well as live birth rate and cumulative live birth rates (percentage of participants with 1 live born neonate after 1 year of follow-up). The study furthermore aims to document the prevalence of OHSS assessed by the number of patients admitted to hospital under this diagnosis and the number of patients having ascites puncture. In addition, it is planned to evaluate pregnancy related complications as well as neonatal outcomes in both groups. For complete overview of all secondary endpoint measures see Table 3.

Table 3. Secondary endpoints
♦ Ongoing pregnancy rate per start of *per started ovarian stimulation and per oocyte retrieval*
♦ Live birth rate after the first blastocyst transfer calculated *per randomized patient, per started ovarian stimulation*, *per oocyte retrieval* and *per transfer*
♦ Cumulative live birth rate after one stimulated cycle with oocyte retrieval
♦ Cumulative live birth rate after use of all frozen blastocyst or after at least 1 year of follow-up
♦ Number of cycles with no embryo transfer
♦ Time-to-pregnancy (from start of ovarian stimulation to positive hCG)
♦ Time-to-delivery
♦ Cancelled embryo transfers
♦ Ovarian hyperstimulation syndrome (OHSS)
♦ Preterm birth
♦ Low birth weight
♦ Small-for-gestational age (SGA)
♦ Large-for-gestational age (LGA)
♦ Perinatal mortality
♦ Preeclampsia
♦ Placental rupture
♦ Positive hCG 11 days post embryo transfer
♦ Miscarriage, biochemical pregnancies, ectopic pregnancies
♦ Quality of life for female and male partner
♦ Cost-effectiveness
Other outcome measurements
♦ Number of good blastocysts
♦ Number of fertilized oocytes
♦ Number of high quality embryos day 2
♦ Number of grade 1 blastocysts
♦ Number of frozen blastocyst
♦ Paraclinical data: Endocrine, genetic and immunological parameters

Statistical analyses

Analyses of cumulative pregnancy rates and live birth rates after one oocyte retrieval including fresh and all frozen embryo transfer cycles will be compared by Cox-regression analyses. Comparisons between treatment groups will be performed primarily according to the intention-to-treat (ITT) principle but per-protocol analyses will also be done. Continuous data will be compared by students *t*-test or Mann-Whitney U test and Kruskal-Wallis test as appropriate. Proportions will be compared with chi-square test. Predictive factors for ongoing pregnancy in the two treatment groups will be tested with multivariate logistic regression analyses. A p-value of < 0.5 will be considered as statistically significant.

Patients in fresh embryo transfer group with GnRH agonist triggering

Patients allocated to the fresh transfer group who end up receiving GnRH agonist trigger and vitrification of all blastocysts due to risk of OHSS (> 18 follicles with a diameter > 11 mm on trigger day) will still be analysed as part of the fresh transfer group according to the intention-to-treat principle. Their first blastocyst transfer will derive from their first FET cycle and ongoing pregnancies from these first transfers will be included in the numerator together with ongoing pregnancies derived from the majority of patients with first blastocyst transfer in the fresh cycle. The denominator will be all randomised patients.

ETHICS, SAFETY AND DISSEMINATION

The study has been approved by the Danish regional committee on Health Research Ethics of the Capital Region and the Swedish national council on medical ethics.
Following oral and written information outlining the rationale, trial design, aims and treatment procedures written informed consent will be obtained from women and male partners prior to the enrolment in the study.

The participants are stimulated using individualised doses of gonadotropin stimulation in accordance with the clinical practice at each site. In all clinics serum AMH is considered when the FSH dose is determined. All medicine used in the study is part of standard ART care.

The overall safety of the patients is high in both treatment groups. The risk of OHSS is expected to be similar to the standard clinical protocol in the fresh embryo transfer group and lower in the freeze-all group in which GnRH agonist is used for ovulation trigger. In women in the fresh embryo transfer group with a risk of OHSS development (more than 18 follicles with a diameter over 11

mm), GnRH agonist will be used for trigger instead of hCG and all blastocysts will be vitrified and the transfer postponed.

No financial incentive exists for the participants as all couples are reimbursed for their first three ART treatments in the public health care system in the Nordic countries.

The results of the study will be publically disseminated in peer-reviewed scientific journals and presented at relevant international scientific meetings such as ESHRE (European Society of Human Reproduction and Embryology) and ASRM (American Society for Reproductive Medicine). In addition results will be published in popular science journals and other media.

DISCUSSION

The increasing interest in possible benefits of a freeze-all strategy and the limitations of existing randomised controlled trials comparing this strategy with conventional fresh embryo transfer underline the need for additional studies. The few previous RCT´s have demonstrated significantly increased pregnancy- and delivery rates with freeze-all, however these studies were performed in highly selected patient populations with poor generalizability.[6-7] Further, the treatment strategy combining GnRH agonist trigger and freeze-all minimizing the risk of severe OHSS development has not yet been investigated in a RCT setting. As GnRH agonist trigger does not hamper the yield of mature oocytes[12] and reduces the risk of OHSS to an absolutely minimum, it seems rational to include GnRH agonist trigger in the freeze-all concept. Evidently, we are unable to distinguish between the effect of the GnRH-agonist trigger and the effect of elective freeze-all, when both are included in the freeze-all treatment arm. The present study therefore compares an 'OHSS-free' freeze-all strategy including GnRH agonist trigger with a fresh transfer strategy with hCG trigger. In both treatment arms individualized gonadotropin dosing is used with the possibility of conversion to GnRH agonist trigger and segmentation in case of risk of OHSS development in the fresh embryo transfer group. Individualized gonadotropin dosing based on female age and weight, antral follicle count, AMH and results of previous COH cycles is applied, as this is the standard treatment approach used routinely in all of the participating clinics. The AMH cut-off value at 6.28 pmol/L (Roche Elecsys assay) corresponding to the Bologna criteria for poor ovarian response was chosen to have a reasonable chance of the patient ending up with at least one usable blastocyst after aspiration. It could be argued that an open randomisation, rather than a double-blinded study design, would allow a better exploration of the concept as higher gonadotropin doses and more oocytes could be safely aimed for in the freeze-all group. However, as this is the first RCT of a freeze-all strategy including GnRH agonist trigger, a double-blinded design was chosen to

minimize differences between the two treatment arms and gonadotropin dosing is decided upon independently of allocation to treatment group, as this is done prior to randomisation. In addition, even though a strategy combining GnRH agonist trigger and freeze-all is near OHSS free, increasing gonadotropin dosing would nonetheless add a potential risk of early OHSS in the patients.

The primary endpoint of this study is to investigate ongoing pregnancy rates per randomised patient after the first potential blastocyst transfer. Cumulative rates are additionally planned to be calculated, but as the number of aspirated oocytes is expected to be the same in both treatment groups due to gonadotropin dosing being decided upon independently of allocated treatment group, the effect of the freeze all strategy on the results of the first transfer may be diluted with the inclusion of additional FET´s.

The strengths of this study include the design as a multicenter randomised controlled double-blinded trial as well as preregistration and publication of the study protocol for more transparency. The investigation of several outcome measures related to different aspects of success parameters, including quality of life may furthermore add important information as regards the future potential of the freeze-all strategy in assisted reproduction.

FIGURE LEGEND Figure 1:

Figure 1. Flowchart of the Freeze-all study design

Contributor statement ANA, AP and SS participated in the conception, design, and writing of the study protocol. KL, HSN and CB contributed to the revision and editing of the study protocol. AP, SS, KL, JB, LP, ANA, HSN, CB, PH, MB, ALM and SOS will be involved in the recruitment of patients and the acquisition of data. SS wrote the first draft of this manuscript. AZ was involved in developing the laboratory criteria for the study. SS, AP, ANA, PH, CB, KL, JB, LP, HSN, MB, ALM and SOS will AP, ANA, PH, CB, KL, AZ, JB, LP, HSN, MB, ALM and SOS were all involved in critical revision of the manuscript. All authors approved the final version of the manuscript to be submitted.

Competing interests None declared

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Data sharing statement This manuscript is a study protocol. Data from the final study will be shared according to the coming ICJME guidelines.

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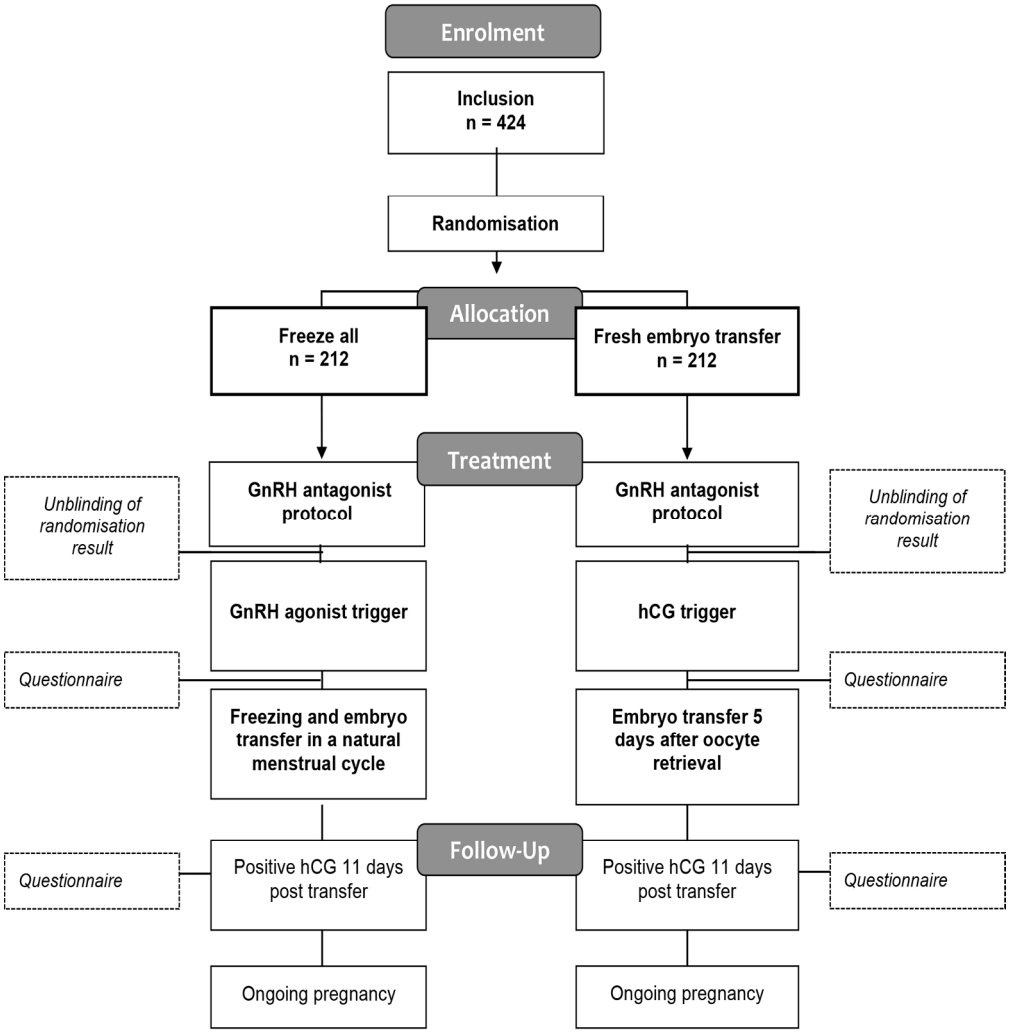

Figure 1. Flowchart of the Freeze-all study design

173x186mm (300 x 300 DPI)

A multicentre randomized controlled trial of a "Freeze all and transfer later" versus a conventional "Fresh Embryo Transfer" strategy for assisted reproductive technology (ART) in women with a regular menstrual cycle

- A Multicentre Randomized Controlled Trial of patients undergoing IVF/ICSI

Multicenter studium med otte deltagende fertilitetsklinikker i Danmark og Sverige

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1. BACKGROUND

In recent years improved cryopreservation techniques have made frozen embryo transfer (FET) a viable and promising alternative to fresh embryo transfer in assisted reproduction (ART). The optimization of cryopreservation techniques from slow-freezing to vitrification and prolonged embryo culture from cleavage to blastocyst state encourages the use of FET as the embryo survival rate following freezing and thawing is now significantly higher reaching 95-97% (Loutradi et al., 2008). Success rates including implantation as well as clinical and on-going pregnancy rates in FET are also significantly improving and approaching or even exceeding those associated with fresh embryo transfer (Kupka et al., 2014; Roque et al., 2013; Shapiro et al., 2011; Zhu et al., 2011). This is partly due the improved laboratory techniques, but may also be due to the endometrial environment in the FET cycles, which mirrors the natural cycle. In the stimulated cycle supraphysiological levels of estradiol and progesterone are present and may cause impaired endometrial receptivity (Shapiro et al., 2011). Furthermore, obstetric and perinatal outcomes after cryopreservation of embryos have been investigated and follow-up data from children born after FET have shown lower perinatal morbidity compared with fresh

embryo transfer (Kansal et al., 2011), but FET may also give rise to more large-for-gestational age babies (Pinborg et al., 2014). In addition, a recent systematic review and meta-analysis on data from 11 observational studies has shown better perinatal outcomes including lower perinatal mortality in singleton pregnancies following frozen-thawed embryo transfer compared with pregnancies after fresh embryo transfer (Maheshwari et al., 2012). Moreover, FET has the benefits of minimizing the risk of ovarian hyperstimulation syndrome (OHSS), which is the most severe side effect of ART and potentially life threatening. Finally, improved cryopreservation techniques favour an elective single embryo transfer (eSET) policy minimizing multiple pregnancies after ART (Pinborg, 2012).

Despite the noticeable advantages of embryo cryopreservation, fresh embryo transfer has persistently been the conventional in vitro fertilisation (IVF) procedure as only one in five transfers were made using frozen-thawed embryos in Denmark in 2013 (www.fertilitetsselskab.dk). This favour of a fresh embryo transfer strategy is however reflected in other European countries including Finland, Sweden and Iceland where approximately every third ART child is born after FET (Kupka et al., 2014). Some evidence suggests that IVF outcomes can be further improved with the adaptation of a `freeze-all´ or elective frozen embryo transfer (eFET) strategy with replacement of thawed embryos in natural cycles (Evans et al., 2014; Devroey et al., 2011; Maheshwari et al., 2013; Roque et al.,2013).

In a recent meta-analysis including three trials accounting for 633 cycles in women aged 27–33 years (Roque et al., 2013), FET resulted in significantly higher ongoing pregnancy rates (RR 1.32, 95% CI 1.10–1.59) and clinical pregnancy rates (RR 1.31, 95% CI 1.10–1.56). The studies showed heterogeneity and only 137 of the participants were normal responders, while the rest was high responders. Moreover one study included only cleavage stage embryo transfer while the other two included blastocyst transfers only. The studies were performed in Japan and in the US, while no European RCT has been published yet. Further, one of the included papers (Aflatoonian et al., 2010) was later retracted based on findings of serious methodological flaws in the study. This accentuates the need for a large multicentre, randomized controlled trial to

evaluate the prospect and clinical consequences of a “freeze all embryos and transfer later” policy compared with conventional fresh embryo transfer.

The aim of this multicentre randomized controlled trial is to compare a “freeze-all” embryo strategy with a conventional single fresh embryo transfer strategy in women 18 to 40 years of age undergoing their first to third IVF/ICSI cycle women with regard to treatment outcomes, risks for mother and child, quality of life and cost-effectiveness aspects of the two treatment modalities in a short GnRH antagonist protocol with blastocyst transfer and vitrification as the freezing method.

2. STUDY AIMS

1. The primary aim is to compare ongoing pregnancy rates per randomized patient and ongoing pregnancy rates per transfer in the “freeze-all” versus “fresh embryo transfer” group.

2. To assess live birth rates per randomized patient and per transfer in the “freeze-all” versus “fresh embryo transfer” group

3. To assess cumulative live birth rates after one stimulated cycle with oocyte retrieval in the two study arms.

4. To compare perinatal outcomes (preterm birth, low birth weight, small-for-gestational age, large-for-gestational-age, preeclampsia and perinatal mortality) in the two groups.

5. To measure time to pregnancy from start of ovarian stimulation and quality of life in both females and males in the two groups.

6. To explore VAS scores regarding pain and discomfort at the day of embryo transfer and 11 days post transfer in the two study arms

7. To assess female physical well-being during the two treatment modalities and to assess quality of life for both female and male partners during the two treatment protocols.

3. ENDPOINTS

Primary endpoints

- Ongoing pregnancy rate *per randomized patient*
  (pregnancy with positive fetal heart beat in gestational week 7-8)
- Ongoing pregnancy rate *per transfer* of the first blastocyst
  (pregnancy with positive fetal heart beat in gestational week 7-8)
- Ongoing pregnancy rate *per oocyte pick-up*
  (pregnancy with positive fetal heart beat in gestational week 7-8)
- Ongoing pregnancy rate *per start of ovarian stimulation*
  (pregnancy with positive fetal heart beat in gestational week 7-8)

Secondary endpoints

- Live birth rates calculated per randomized patient, per started ovarian stimulation, per oocyte pick-up and per transfer
- Cumulative live birth rate after one stimulated cycle with oocyte retrieval
- Cumulative live birth rate after use of all frozen blastocysts or after at least 1 year of follow-up.
- Number of cycles with no embryo transfer
- Time-to-pregnancy (from start of ovarian stimulation to positive hCG)
- Time-to-delivery
- Cancelled embryo transfers
- OHSS
- Preterm birth
- Low birth weight
- Small-for-gestational age (SGA)
- Large-for-gestational age (LGA)
- Perinatal mortality

- Preeclampsia
- Placental rupture
- Positive hCG 11 days or according to the local routine post embryo transfer
- Miscarriage, biochemical pregnancies, ectopic pregnancies
- Quality of life for female and ~~male~~ partner
- Cost-effectiveness

Other outcomes

- Number of good quality blastocyst
- Number of fertilized oocytes
- Number of high quality embryos day 2 (defined by the study laboratory manual)
- Number of grade 1 blastocysts (defined by the study laboratory manual)
- Number of frozen blastocysts
- Para-clinical data: Endocrine, genetic and immunological parameters influencing pregnancy

4. STUDYPOPULATION

Inclusion criteria

- Women > 6.28 pmol/L with the Roche Elecsys assay* (AMH > 1.1 ng/ml ~ 7.85 pmol/L old assay). This is according to the Bologna criteria for POR; AMH < 0.5–1.1 ng/ml (3.57-7,85 pmol/l (old assay) ~ 2,86 – 6,28 pmol/l Elecsys)(Ferraretti et al., 2011)
- Female age 18 year to less than 40 years
- 1.-3. IVF/ICSI cycle with oocyte aspiration
- Regular menstrual cycle $\geq$ 24 days and $\leq$ 35 days
- BMI $\geq$ 18 or < 35 kg/m^2
- Two ovaries
- Can and will sign the informed content

- **Exclusion criteria**
- Women who do not fulfil the inclusion criteria

- Endometriosis stage III to IV
- Ovarian cysts with diameter > 30 mm at day of start of stimulation
- Submucosal fibroids
- Women with severe co-morbidity (i.e Insulin Dependent Diabetes Mellitus (IDDM), Non-Insulin Dependent Diabetes Mellitus (NIDDM), gastrointestinal, cardio-vascular, pulmonary, liver or kidney disease)
- Dysregulation of thyroid disease
- Not Danish, Swedish or English speaking women
- Contraindications or allergies to use of gonadotrophins or GnRH antagonists
- TESA (testicular sperm aspiration)
- OD (oocyte donation)
- Previous inclusion in the study

5. METHODS

Inclusion of patients

- All couples or single/lesbian women starting IVF/ICSI treatment participate in a standard information meeting arranged by the clinic. During this 2 hour meeting patients and their partners are informed about the normal IVF/ICSI procedures, treatments and research in the clinic as well as this study. If patients are not attending the information meeting, the will be informed about the study at their first outpatient visit at the clinic.
- Few patients do not participate in the information meeting and they will have an appointment at the outpatient clinic in the Fertility clinic, where they will receive information about the IVF/ICSI treatment and be informed about this study.
- Patient files are browsed by one of the investigators, who decide if the patient is eligible. After the information meeting all patients receives a phone call from a doctor/study nurse, where they are informed about the treatment plan. If the inclusion criteria are fulfilled, the couples will receive oral information about the study and asked if they are interested in participating in the study, if so, the written patient information is sent to the couples by

email. If the couples are interested, a visit is planned on menstrual cycle day 2-4. The couple is informed that they can bring an assessor to the oral information visit. The written information is send by email, which leaves possibility of reading and reflection.

Informed consent

At the fertility clinic the patient will be seen by one of the investigators. Patients will be informed about the aim of the project and risks in accordance with the guidelines from the Scientific Ethical Committee. In case of questions, these will be answered. If the patients need more time for reflection, a new visit will be arranged. After signing the informed consent, the patient will be screened.

Screening – cycle day 2-4

- Medical and gynaecological history inclusive reproductive history including menstrual cycle length, smoking (yes/no), years of infertility
- Transvaginal ultrasound examination including ovarian volume, antral follicle count (AFC), and endometrial thickness and morphology and exclusion of pathology
- Height and weight
- Blood samples: AMH, FSH, LH, estradiol, progesterone, TSH, TPO antibodies, vitamin D, CRP and suPAR* (*only done at Hvidovre Hospital)
- One full blood, one plasma and one serum sample is cryopreserved as back-up and for analysis of endocrine and immunological factors of relevance for pregnancy

Screening should be performed no later than 3 months before randomization.

Randomization

When the patient has signed the informed consent, has been screened and it is confirmed that the inclusion criteria are meet, the patient is randomized to one of the two arms:

I. hCG arm with traditional hCG triggering and fresh blastocyst transfer

II. GnRH agonist triggering arm with blastocyst cryopreservation and subsequent transfer in a natural cycle.

Computerized randomization is performed according to the 1) Trial site and to 2) Female age <= 37 years or >37 years. The gonadotrophin stimulation dose is decided upon before randomization and entered into the database before randomization. The doctor and patient are blinded to the randomization until the day of hCG or GnRH agonist triggering.

Blood samples

Blood samples are collected at

- Baseline before the first gonadotrophin injection (cycle day 2-4): AMH, FSH, LH, estradiol, progesterone, TSH, TPO-antibodies, vitamin D CRP and suPAR* (*only done at Hvidovre Hospital)
- Day of trigger-injection: FSH, LH, estradiol, progesterone, CRP and suPAR* (*only done at Hvidovre Hospital)
- Day 16 after oocyte pick-up: hCG (only in the fresh embryo transfer group), CRP and suPAR* (*only done at Hvidovre Hospital)

At baseline and at day of trigger an extra full blood, plasma and serum sample is collected and stored according to a trail-specific laboratory manual. This will be stored in the freezer as back-up and for analysis of endocrine and immunological factors of relevance for this study.

Subgroup analyses in the luteal phase

During the lutealphase with embryo transfer of the stimulated fresh cycle and non-stimulated FER cycle blood samples are taken on day of hCG injection, hCG injection day+7, +11, +14, +16 and +19 for patients included at Hvidovre Hospital until 30 patient in each arm has been achieved. The following blood samples are collected; Estradiol, Inhibin-A, OH-progesterone, Progesterone, LH and hCG.

All blood samples are confidential. The frozen samples are anonymous, so no person identifiable date is left on the sample. Only the patient project ID number and the collection date identifies the sample. The study will be approved by the Danish Data Protection Agency and the Scientific Ethical Committee of the Capital Region, Region Zeeland and the Region Skåne in Sweden. The blood samples will be stored in the participating fertility clinics and if not used then five years after end of the study, at December 1st, 2023 blood samples will be definitively destroyed.

Gonadotropin stimulation treatment

The dose of gonadotrophin is decided and entered into the computer programme before randomization. The doctor and patient are blinded to the randomization until the day of hCG or GnRH agonist triggering. The study nurse is not blinded.

The ovarian stimulation with recombinant follicular stimulating hormone (rFSH) or human menopausal gonadotrophin (hMG) can start immediately after randomization in a short GnRH antagonist protocol. The gonadotrophin stimulation is performed according to the general standards in each of the clinics and can be altered according to the ovarian response. The GnRH antagonist is initiated at a daily dose of 0.25 mg at stimulation day 5 or 6 according to the clinical standards and continued throughout the rest of the gonadotropin stimulation period. The gonadotrophin dose cannot exceed a daily dose of 300 IU. Both groups are treated according to the short GnRH-antagonist protocol, where a higher dose of gonadotropin than 300 IU has been shown to be of no added value for further follicular growth. The maximum stimulation period is 20 days.

The medication for the study is bought by the patients themselves according to general prescription rules.

Ultrasound

Ultrasound examination is performed on cycle day 2-3 (Stim1), Stim 5-8 and thereafter every 2-3 days until ovulation trigger is decided. At the start of stimulation comprehensive sonography is

performed with details on each ovary, including ovarian volume, number of antral follicle in the following subclasses: 2-4mm, 5-7mm and 8-10mm.

The following parameters are measured on the day of ovulation trigger or the day before: Follicular development with size and number of follicles >10 mm, endometrial thickness and echogenicity and uterine pathology.

Ovulation induction

As soon as three follicles of >= 17 mm are observed or one day after a single injection of 250 µg of human chorionic gonadotrophin (hCG) is administered in the "fresh transfer"-arm, while GnRH agonist triggering with GnRH agonist Buserelin 0.5 mg is administered in the "freeze-all" arm.

Triggering of ovulation with a GnRH agonist Buserelin 0.5 mg is allowed in the fresh embryo transfer arm in case of risk of severe OHSS after the following the criteria: In the fresh embryo arm: If > 18 follicles with a diameter > 11 mm are observed on the day of triggering, GnRH agonist triggering should be used and all blastocysts frozen (Papanikolaou et al., 2011). Coasting / surfing cannot be used.

Oocyte retrieval

Oocyte retrieval is performed 36 ± 2 hours after hCG or GnRH agonist administration.

IVF/ICSI

Oocytes are fertilised by either IVF or ICSI and embryos are cultured individually according to the normal procedure in the clinics.

Embryo transfer

I. "Fresh embryo transfer" group

Single blastocyst transfer is always performed on day five after oocyte pick-up if a blastocyst is developed. Surplus good quality blastocyst are vitrified on day five or six.

Luteal phase support is administered as vaginal progesterone (vaginal gel (Crinone) 90 mg/dose x 1 daily or vaginal tablets 100 mg x 3 daily (Lutinus)) according to the standard procedure in each of the individual clinics from day 2 after oocyte retrieval and to confirmation of pregnancy or negative hCG 11-15 days post transfer. In case of a positive pregnancy test an ultrasound scan is performed three to four weeks later to confirm an intrauterine pregnancy with a live foetus.

Triggering of ovulation with a GnRH agonist Buserelin 0.5 mg is allowed in the fresh embryo transfer arm in case of risk of severe OHSS, following the criteria: If > 18 follicles with a diameter > 11 mm are observed, GnRH agonist triggering should be used and all blastocysts frozen (Papanikolaou et al., 2011). Coasting / surfing cannot be used.

II. "Freeze all and transfer later" group

For patients in this group all embryos of a good quality are vitrified at the blastocyst stage day 5 in the stimulated cycle. Criteria for freezing of blastocyst are according to the criteria in the specific clinic. The "best" embryo (i.e. of the highest quality is selected after predefined strict criteria according to the specific trial laboratory manual) is marked and is the first one to be warmed after at least one menstrual cycle that is considered as a wash out period.

In the menstrual cycle with blastocyst transfer, an hCG injection of 6500 units is given when the leading follicle is $\geq$ 17 mm. Embryo transfer is performed 6-7 days after hCG injection. No luteal phase support is needed.

Pregnancy test

A serum beta-hCG test is performed 11 days after blastocyst transfer or according to local routine. Clinical pregnancy is confirmed by transvaginal ultrasound 3 to 4 weeks after a positive serum-hCG.

Follow-up both groups

A follow-up of all pregnancies will be performed within three months after delivery or termination of pregnancy on predefined information sheets.

Information on background data, pregnancies and deliveries are returned to Hvidovre Fertility clinic on predefined case report forms (CRF) including pregnancy and delivery information sheets. All pregnancies resulting from blastocyst retrieved and thawed according to this study protocol will be followed from study inclusion (Stim day 1) and one year onwards.

All data are anonymized by encryption in the database with no personal identifiable data.

We will retrieve data from the patient clinical files and clinical databases with information regarding previous diseases, hospital admissions, former and current fertility treatment, pregnancy and delivery data on pregnancies related to this study. Both females and males will be informed about this in the patient information. This collected information will be used to characterize the populations and to minimize risk of bias.
We will also gain information regarding the coming child and the female and partner will sign a separate informed consent in Denmark and in Sweden a single consent form for the couple regarding this.

VAS-score and physical discomfort questionnaire

Women in both arms will be requested to fill-in their level of pain and discomfort on a VAS-score scale and a physical discomfort questionnaire as well as a quality of life questionnaire at the day of oocyte pick-up +4 and the day of oocyte pick up + 16.

Visits

Every visit is registered on a standardized stimulation scheme made specifically for this study. The schemes are normally used as standards in the clinics.

Criteria for withdrawal

A patient can be withdrawn from the study at any time, if the patient wishes to do so or if there is a medical indication decided by the investigator. The patient participation in the study can be interrupted, if one of the following criteria is present:

- The patients general condition contraindicates participation
- Protocol violation, which the investigator assess to have influence on the treatment
- Safety

Patients will be carefully monitored from stimulation start, at Stim6 and thereafter every 2-3 day in the clinic. The treatment will be monitored by transvaginal ultrasound of the ovaries. After each visit the patients will receive thorough information on the drug dosage and administration. This will follow the normal procedure in the clinic. If a patient is taking the wrong dosage, it will be documented on the stimulation scheme. This is not dangerous to the patient as the treatment is monitored by ultrasound scans hence a risk of OHSS will be discovered there.

In case of risk of OHSS in the “fresh-embryo-transfer”-arm, the ovulation trigger will not be induced by hCG but with Buserelin 0.5 mg. The further treatment of this patient will be handled according to the routine of the clinic.

In case of OHSS the patient is monitored at the clinic until recovery. Overall the safety of the patients is high in both the fresh embryo transfer and the freeze all group as the gonadotrophin stimulation corresponds to the normal program for patients at risk of OHSS. Furthermore patients with irregular cycles i.e. as part of polycystic ovarian syndrome, who in general have a higher risk of OHSS, are not included in this study.

6. STATISTICS AND SAMPLE SIZE

Superiority study

In all 424 (n= 212 in each arm) patients are required to have an 80% chance of detecting, as significant at the 5% level, an increase in the primary outcome measure ongoing pregnancy rate per randomised patient and per transfer from 30% in the control group to 43% in the experimental group. A difference of 15% was found in a randomized controlled trial by Shapiro et al, 2011 between the "freeze-all" arm and the fresh transfer group.

The statistical analyses will be performed by investigator together with statistical experts at Hvidovre Hospital and associate professor Julie Forman, Department of Biostatistics, Faculty of Health and Medical Sciences, University of Copenhagen.

A Statistical Analysis Plan (SAP) will be presented before closing of the database and before any statistical analyses are performed.

7. STUDY MEDICATION

All medicine used in this study is normally used as standard care for the patients in the short GnRH antagonist protocol. Patients will have prescriptions on all the medicine and will take all the medicine at home as is the routine in the clinics.

Dosage and administration

Treatment dose at the first day and during the ovarian stimulation is planned by the investigator and the patient is further instructed by a nurse, so that the patient is confident in self administration at home according to the normal clinical routine.

Side effects

Most side effects are mild and related to the medication during the stimulation. Unwanted OHSS is a risk in all IVF treatment but is considered low in this project as a standard IVF/ICSI protocol is used with individualized dosing. Further, in the freeze-all group the risk of OHSS is expected to be lower than in the standard care group as all blastocyst transfers are postponed to cycles without ovarian stimulation.

8. DATA SECURITY AND ETHICAL ASPECTS

Data security

One full blood and one serum sample at baseline and at the day of ovulation induction will be collected and stored according to the trial specific laboratory manual on all women included in this trial for future analyses of endocrine, immunological and protein markers. All data will be collected in a single database including all project subjects with an identification code thus data on each subject will be anonymous, when entered into the database.

Ethical aspects

The study will be performed according to the Danish Law and ethical principles in the Helsinki Declaration. This covers that study subjects receive both oral and written information and the opportunity of time for reflection and that they can discuss their participation with a third person.

The participants will be given a individualized dose of gonadotropin according to their serum AMH level, which is standard for patients at all five Fertility Clinics in Denmark and Sweden. The risk of OHSS will be similar to the standard clinical protocol and lower in the "freeze-all" group.

With a "freeze-all embryo and transfer later" protocol in ART, the risk of OHSS in women undergoing IVF/ICSI will be minimized and the embryo development will benefit from an endometrium less influenced by supra-physiological levels of estradiol and progesterone in the fresh embryo transfer cycle. This may also be beneficial for the children born after the treatments.

The study is approved by the Scientific Ethical Committee in the Capital Region (H-1600-1116) and by the Scientific Ethical Committee in Region Skåne in Sweden (Dnr. 2016/654)

The study will be approved by the Data Protection Agencies in Denmark and Sweden.

9. TIME SCHEDULE AND PUBLICATION

Protocol will be send to the Scientific Ethical Committee in the Capital Region, Denmark in January 2016 and inclusion of patients will start as soon as the approval from SEC has been obtained. The inclusion of patients will run from March 2016 to February 2018. Statistical analyses, writing and preparing manuscripts will go on from February 2018 to January 2019.

The results of the study will be presented at national as well as international scientific congresses and published in high impact international scientific journals in reproductive medicine such as Human Reproduction or Fertility and Sterility. Further results of public interest will be reported in the public press.

10.FINANCING

The project is initiated by Professor Anja Pinborg. This project is part of the Reprounion program, which has been supported by the Interreg-program for Öresund-Kattegat-Skagerak from EU, Capital Region of Denmark, Region Skåne and Ferring Pharmaceutical Company. The project has been financed with 450.000 euro (3.375.000 dkk) by a grant from Interreg/EU.

Patients included in this study and the Scientific Ethical Committee will be informed if further funding is obtained for this study. Funding will be transferred to a research account in the bank of Hvidovre Hospital, Capital Region of Denmark.

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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			Page 1 of protocol
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	_____
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	Page 17 of protocol _____
	2b	All items from the World Health Organization Trial Registration Data Set	All accounted for see items below _____
Protocol version	3	Date and version identifier	Page 1 of protocol (header) _____
Funding	4	Sources and types of financial, material, and other support	Page 18 of protocol _____
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	Page 1, 2, 3 of protocol _____

	5b	Name and contact information for the trial sponsor	Page 1 of protocol
	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	Not applicable
	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)	Not applicable
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	Page 3, 4 5 of protocol
	6b	Explanation for choice of comparators	Page 3, 4 5 of protocol
Objectives	7	Specific objectives or hypotheses	Page 5 of protocol
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)	Page 8-10 of protocol
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	Page 8 List of study sites; page 1-3
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)	Page 7-8
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	Page 11-13
	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)	Page 14-15
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	Page 11 (visits)
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	Not applicable
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	Page 5-7
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)	Page 5-13
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	Page 15
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	Page 8

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	Page 9
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	Page 9
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	Page 9, 11
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	Page 9
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	No circumstances
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	Page 9, 10, 11 and 14
	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	Page 13-14

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	Page 13,14, Page 16 Details data management can be obtained through contact to primary investigator ("projektansvarlig læge" of trial
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	Page 15 Details of planned statistical analysed can be obtained through contact to primary investigator ("projektansvarlig læge" of trial
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	Same as item 20a
	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)	Same as item 20a
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	No data monitoring committee used

	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	No interim analyses will be performed
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	Not applicable
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	Not available in protocol
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	Page 17
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)	Not available in protocol
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	Page 8 of protocol
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	Not 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	Page 14, 16
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	Page 18 No competing interests exists

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	Not available in protocol
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	Not applicable
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	Not available in protocol
	31b	Authorship eligibility guidelines and any intended use of professional writers	Not available in protocol
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	No current plans
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Not available in protocol – Standard consent forms from the Danish etichal committee is used as well s participant information approved by the Ethical Committee
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	Page 10

*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.