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Title: A protocol for a double-blind, placebo-controlled multicenter trial on the effect of clindamycin and a live biotherapeutic on the reproductive outcomes of IVF patients with abnormal vaginal microbiota

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Abstract:

Introduction

Recent studies in IVF patients have associated abnormal vaginal microbiota with poor clinical pregnancy rates of 6-9% per embryo transfer. The biological plausibility for this finding is hypothesized to be ascending infection to the endometrium which in turn hampers embryo implantation. The prevalence ranges from 9-28%. The important question is whether screening and treatment of abnormal vaginal microbiota would improve reproductive outcomes in IVF patients. Herein, we describe a protocol for an ongoing double-blind, placebo-controlled multicenter trial of IVF patients with abnormal vaginal microbiota randomized in three parallel groups 1:1:1.

Methods and analysis

This is a drug intervention study where IVF patients will be screened for abnormal vaginal microbiota, using a qPCR assay targeting *Atopobium vaginae* and *Gardnerella vaginalis*. If positive, patients will be randomized to one of the three study arms. The first arm consists of clindamycin 300mg x2 daily for 7 days followed by topical *Lactobacillus crispatus* CTV-05 until clinical pregnancy scan week 7-9. The second arm consists of clindamycin and placebo *Lactobacillus crispatus* CTV-05, whereas patients in the third arm will be treated with placebo/placebo. We used a superiority design to estimate that active treatment in both arms will increase the primary outcome, clinical pregnancy rate per transfer, from 20% to 40%. A potential difference between the two active arms was considered exploratory. With a power of 80% and an alpha at 5%, the sample size is estimated to be 333 patients randomized. A pre-planned interim analysis is scheduled at 167 patients randomized.

Ethics and dissemination

All patients have to give informed consent. Dissemination of results is ensured in clinical trial agreements whether they be positive or not.

Registration

This trial is registered in all relevant national agencies as well as in EudraCT with registration number 2016-002385-31. Moreover, the trial is ICH-GCP monitored.

Keywords: IVF, Microbiota, Bacterial vaginosis, Clindamycin, Gardnerella, RCT

Article summary: 'Strengths and limitations of this study'

- Diagnosis of abnormal vaginal microbiota was validated in pilot studies
- The first RCT in IVF patients with abnormal vaginal microbiota investigating treatment effect on reproductive outcome of clindamycin and live lactobacillus treatment
- The *Lactobacillus crispatus* CTV-05 treatment is an investigational live biotherapeutic product regulated by the US FDA
- Inclusion criteria are relatively broad

Introduction

Bacterial Vaginosis (BV) is a common vaginal dysbiosis in reproductive age women with a prevalence of 29% (95% CI 27-31%) as reported in a US population based survey, N=3739 (1). It is well-known that there is higher BV rates among African-Americans compared to Caucasian women (1). However, this finding could be affected by the fact that asymptomatic African-Americans seem to have a more diverse vaginal microbiota as compared to Caucasians (2,3). Other risk factors include frequent vaginal douching and number of lifetime sex partners (1). In the *in vitro* fertilization (IVF) population, a recent meta-analysis (N-patients=2980) reported that the prevalence of BV exhibited huge interstudy heterogeneity ranging from 4-38% (4). In this study, BV was clearly associated with tubal factor infertility, but not endometriosis. The most recent studies using a molecular based analysis to determine an abnormal vaginal microbiota observed a prevalence of 17% and 28%, respectively (5,6). It is known that despite diagnosed with BV by the gold standard Nugent method (7), more than 80% of BV positives remain asymptomatic (1). Hence, the important question is whether the many asymptomatic BV cases should be screened and treated. Screening and treatment for BV is recommended in patients undergoing gynecological surgery or invasive diagnostic procedures through the vagina to minimize infection (8). However, most clinical guidelines do not support screening and treatment for asymptomatic BV to optimize reproductive outcome – a topic which has been thoroughly investigated in obstetric populations for preterm birth prevention (9,10). Today, a new frontier is emerging with optimized molecular based diagnosis and new treatment possibilities including well-studied and well-characterized probiotics – that have been designated "live biotherapeutic products" by FDA (11,12). Haahr et al. (2016) reported the advantages of a molecular based diagnosis of vaginal dysbiosis in IVF patients (5). The main advantages were i) a more objective diagnosis as microscopists had significant interrater variability with the prior gold standard, Nugent score ii) dichotomization of the Nugent intermediate group which was difficult to

interpret clinically and iii) the establishment of quantitative thresholds using key vaginal bacteria to detect IVF patients at risk of a poor reproductive outcome. Hence, a new terminology termed abnormal vaginal microbiota (AVM) was proposed for IVF patients (5). AVM was significantly associated with poor clinical pregnancy rates as compared to normal vaginal microbiota patients, 9% (2/22) versus 44% (27/62) (5). Later, these findings were corroborated by Koedooder et al. (6) who found clinical pregnancy rates of 6% (2/34) versus 42% (65/154) in patients with unfavorable and favorable vaginal microbiota, respectively.

In the field of reproductive medicine, there have been two different approaches to investigate the potential influence of the genital tract microbiota on IVF outcomes: either i) to directly investigate the endometrial microbiota by transcervical swabs/suctions (13–15) or ii) to investigate the vaginal microbiota as a proxy for the endometrial microbiota (5,6,16). The bacterial load in the uterus as compared to the vagina is very low (17), and for this reason the studies on endometrial microbiota have been criticized for reporting contamination from the transcervical sampling approach - and not a genuine endometrial microbiota. Nevertheless, endometrial samples from women undergoing hysterectomy provide evidence for a genuine endometrial microbiota (17,18) that seems to be highly influenced by the vaginal microbiota (18,19). Several groups are developing or further optimizing molecular based approaches to diagnose IVF women at risk of poor reproductive outcomes caused by genital tract dysbiosis. However, only one study validated a molecular diagnostic approach in IVF women against the gold standard for vaginal dysbiosis – Nugent score of Gram stained vaginal smears (5). Two other studies applied arbitrary cut-offs for *Lactobacillus* dominance in the vaginal microbiota (6,16). Subsequently, these studies were criticized for insufficient methods (20,21), including the application of arbitrary thresholds based on relative abundances which does not sufficiently take into account differences in the total abundance bacteria (20–22).

The recommended first-line treatments for BV are antibiotic therapy with either metronidazole or clindamycin as reported by the 2015 CDC (Center for Disease Prevention and Control) Sexually Transmitted Disease Guideline and the 2018 European IUSTI/WHO (International Union against Sexually Transmitted Infections) guideline. Clindamycin was reported to effectively eradicate BV-related bacteria in the endometrium of patients with endometritis (23), while it was also proven to enter the endometrial tissue in high concentrations if administered orally (24). In contrast, metronidazole was less effective against *Gardnerella vaginalis* both *in vivo* (25) and *in vitro* (23). Finally, a recent systematic review and meta-analysis reported that the use of additional probiotic treatment alongside standard treatment of bacterial vaginosis could improve BV cure rates, RR = 1.28, 95% CI (1.05, 1.56) (26). However, currently there is no consensus on which vaginal *Lactobacillus* product should be recommended.

The pioneering work by Ravel and colleagues (2011) established that the vaginal microbiota is heterogeneous and four *Lactobacillus* dominated community state types (CSTs) can be identified using taxonomic stratification at the species level, with each CST dominated by a different *Lactobacillus* species or being a diversity CST not dominated by *Lactobacillus* (2). Although such stratification was based on hierarchical clustering and relative abundance – in contrast to absolute abundance, these CSTs have been adopted by the majority of researchers in the vaginal microbiome field. Consistently, publications have reported the *L. crispatus* CST to be associated with optimal genital health and reproductive outcomes (16,27–30). Abundant *in vitro* evidence point towards a beneficial production of lactic acid isomers that seem to be unique for *L. crispatus* as compared to other common vaginal lactobacilli (31,32). At the time of planning the present study, only one *L. crispatus* product, LACTIN-V, existed as an investigational live biotherapeutic product regulated by FDA – at that time in Phase 2 development (11).

Taking into consideration the abovementioned evidence, the research question of the present study is: does antibiotic alone or in combination with live biotherapeutic treatment of an abnormal vaginal microbiota improve the reproductive outcomes of IVF patients? The intervention is clindamycin either alone or in combination with LACTIN-V, a live biotherapeutic product containing *L.crispatus* CTV-05 (11). The study is designed as a double-blind, placebo-controlled multicenter trial of three parallel groups randomized 1:1:1. Randomization is by computer generated code and allocation concealment is performed by the pharmacy who will send out medication to the participating clinics with identical appearance and randomization numbers. The randomization code is with the pharmacy and can only be opened in case of emergency by the principal investigators or as planned by the sponsor-investigator. The benefit of the intervention would potentially lead to increased pregnancy rates and, for those suffering from symptomatic BV, also relief of BV symptoms. In contrast, the expected adverse reactions of concern are especially gastrointestinal symptoms caused by clindamycin, whereas LACTIN-V might cause increased vaginal discharge but is otherwise not expected to cause adverse reactions as based on prior studies (11,33).

Methods and analysis

Setting and eligibility criteria

The present trial is conducted at four University affiliated clinics and one private fertility clinic in Denmark. The list of study sites is available with EudraCT clinical trial identifier: 2016-002385-31, first registration day 2016-07-11. The current version of the protocol is 7, 2019-10-10. Patients are enrolled in a cohort study (Clinicaltrials.gov NCT03420859) from which we will recruit patients for the randomized trial (EudraCT: 2016-002385-31). Eligibility criteria are described in Table 1.

In brief, IVF patients attending their first, second or third IVF stimulation cycle or embryo transfer will be approached for informed consent by the study nurse or treating physician. Patients are told

about the project in a private room with the right to have an assessor, allowing time to reflect whether they will participate. They are handed out written information material with a link to the study website with full information about the project – www.reproflor.dk. The vaginal swab can be taken by the treating physician or the patient herself after careful instruction. In this case, patients are instructed to place the swab at least 8 cm into the vaginal cavity for 5 seconds and rotate clockwise. This is to ensure that the vaginal bacteria in the fornix or in its close proximity will be caught by the flocked swab. Subsequently, the vaginal swab will be sent to a central laboratory at Statens Serum Institut, Copenhagen to be analyzed for AVM within 7 days as determined in a previous study (5). If AVM positive, patients are asked to provide informed consent that they are willing to participate in the randomized controlled trial. Patients should ideally be randomized on the first day of ovarian stimulation with exogenous gonadotropins, allowing a minimum of 12 days of study medication to be acceptable for inclusion in the study. If elective frozen embryo transfer (FET) is planned, patients should be randomized during the first days of the FET cycle allowing for at least 12 days of study medication.

Interventions

Active Treatment 1: **Oral Clindamycin** 300 mg 2 times per day for 7 days followed by **LACTIN-V** (Osel, Inc.) until completion of the clinical pregnancy scan at week 7-9. LACTIN-V (2 x 10^9 CFU/dose, 200 mg, delivered with pre-filled, single use vaginal applicator) regimen is once daily from the clindamycin stop for 7 consecutive days.

Active Treatment 2: **Oral Clindamycin** 300 mg 2 times per day for 7 days followed by **LACTIN-V placebo** (Osel, Inc.) until completion of the clinical pregnancy scan at week 7-9. The LACTIN-V placebo regimen is once daily from the clindamycin stop for 7 consecutive days.

Inactive treatment (placebo): Matching **clindamycin placebo** 2 times per day for 7 days followed by **LACTIN-V placebo** (Osel Inc.) until completion of the clinical pregnancy scan at week 7-9. LACTIN-V placebo regimen is once daily from clindamycin stop and the following 7 days.

If there are embryos to transfer (90% of patients), then LACTIN-V/placebo treatment is continued twice weekly until clinical pregnancy scan, however with a maximum of 21 applicators per patient. If the patient has no embryos to transfer or is confirmed not pregnant (negative hCG test), then LACTIN-V treatment can be stopped, albeit at least 7 days of LACTIN-V administration need to be administered. An overview of the study medication and allocation can be seen in Table 2. Patients are not allowed to take other antibiotics (unless medically indicated), probiotics, neuromuscular blocking drugs, immunosuppressive medication or investigational drug preparations other than the study product.

Labelling and packaging

Labelling and packaging of the medication are performed by Glostrup Pharmacy, Denmark in accordance with ICH-GCP guideline and EU GMP Annex 13. Patients are informed that it is important not to have penile-vaginal intercourse within 12 hours after LACTIN V application. Patient compliance will be measured by tablet counting of the medication packs (clindamycin). Any unused LACTIN-V applicators should be delivered to the clinics, otherwise they are considered used.

Patients can withdraw their informed consent at any given time and without any reason according to Danish law. The reason for discontinuation has to be stated in the electronic Case Report Form (eCRF). Moreover, in case of protocol deviations, this also has to be stated in the eCRF and the principal investigator should decide whether trial medication can continue or not. Furthermore, trial medication is stopped, should the patient develop hypersensitivity, allergy or severe diarrhea that a primary investigator suspects may be trial medication related. Vaginal swabs will be taken alongside treatment to monitor the vaginal microbiota and its response to treatment, see Table 3 and appendix

1. Specifically, vaginal swabs will be taken on the day of randomization immediately before study medication, after clindamycin treatment, on the day of embryo transfer and again on the day of clinical pregnancy scan. A total of 20 patients will be asked to deliver vaginal samples for each day they take medication and the swab should be taken immediately before the medication on that specific day.

Outcomes

The primary outcome is the clinical pregnancy rate per first embryo transfer defined as ultrasound proven fetal heartbeat in gestational week 7-9. The secondary outcome are the live birth rate per embryo transfer, biochemical pregnancy rate (hCG positive at 9-11 days after embryo transfer according to local laboratory standards), implantation rate, early miscarriage, late miscarriage, preterm birth rates, birth weight, and adverse effects of the medication through a safety analysis. As part of the cohort study (Clinicaltrials.gov, NCT03420859), the effect of treatment on the vaginal microbiota of the mother throughout pregnancy will be determined using qPCR and next generation sequencing methods. Later, we plan to investigate cumulative live birth results of subsequent transfer of spare frozen thawed embryos of patients attending the study in a fresh cycle.

Sample size

In 2014, the average clinical pregnancy rate per embryo transfer in our fertility clinic was approximately 40% for an IVF cycle. In our pilot study (5), the unadjusted risk difference between the AVM group and the normal group was 34% (95%CI 17-52%). This was a relatively large confidence interval which was not biologically meaningful. Taken together, we estimated a superiority design were women in each AVM arm treated with active medication will have at least a 40% chance for clinical pregnancy per embryo transfer as compared to the placebo arm which was estimated to have a maximum of 20% chance of clinical pregnancy/transfer. By two samples proportion test with a power of 80% and an alpha at 5%, the aim was to randomize 92 patients in each

group. A potential difference between the two active arms was considered exploratory and consequently this was not part of the power calculation, but we decided to include the same number of patients in the active/active arm to investigate a potential added benefit of live biotherapeutic treatment.

An interim analysis will be performed, and to adjust for this, we add 10% to the 92 randomized patients as suggested in Wittes et al (34). Approximately 10% of couples will have no embryos for transfer; we adjusted for this by adding another 10% to each randomized group, i.e. 19 + 92 =111 (see Figure 1). Considering an estimated 20% AVM rate, a total of 1850 IVF patients will be screened to randomize 333 patients (three arms). It was estimated that inclusion will be distributed according to the size of the centers. Furthermore, we make the following assumptions: i) very limited loss to follow-up, ii) near full compliance to study medication and iii) homogeneity in the treatment effect.

Allocation

Randomization is performed by Glostrup pharmacy by a computer-generated code. The medication packs labelled with the randomization number are received at the IVF centers from the pharmacy in blocks of 15, five of each of the three treatments, to secure equal distribution of treatment arms at the centers. The medication have identical appearance and only the randomization number differ, hence both patients and study personnel are blinded for the intervention. A block of 15 medication packs will be sent from the pharmacy from start of study and new blocks can be requested when 5 medication packs are left. The 15 medication packs are mixed and appear identical to both personnel and patients. The randomization number is continuous and unique for each patient, starting from 1 to 333 and the number is prelabelled from the pharmacy before distribution to the clinics.

The randomization list is secured by the pharmacy throughout the trial, and only the sponsor has the authority to unblind the trial. However, in case of medical emergency the principal investigator (PI)

can call the pharmacy to unblind. Each participant's medication package is labeled with a randomization number that is linked to their study ID number in the eCRF. Although both patients and clinicians will be blinded to allocation, they may suspect active medication in case of side-effects. This small risk of bias seems to be unavoidable. However, to investigate such an effect, patients are asked if they believed that they received active or inactive medication.

Data collection methods

Study data are collected and managed using REDCap (Research Electronic Data Capture) electronic data capture tools hosted at Aarhus University (35,36). REDCap is a secure, web-based software platform designed to support data capture for research studies, providing 1) an intuitive interface for validated data capture; 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data downloads to common statistical packages; and 4) procedures for data integration and interoperability with external sources. All data collectors of the study have to be trained in Good Clinical Practice (GCP) procedures and as minimum to have passed the course provided by the Danish GCP institution. All inclusion and exclusion criteria as well as outcome data will be monitored by external GCP monitors to ensure optimal data quality. Data collection forms and other data entry related information can be requested from the corresponding author.

Protocol deviations have to be stated in the eCRF. Loss to follow-up is unlikely for patients in IVF treatment who will be highly motivated to come to the clinic. However, patients who are not pregnant may opt to go to other clinics for further treatment and, thus will be lost to follow-up. Patients who are not pregnant (negative HCG-test) and continue LACTIN-V treatment are informed to contact the respective clinics in case of adverse events and these will be captured in the eCRF. If patients decide to end study product treatment, they are informed to contact the clinics and to deliver the unused

LACTIN-V to the clinic at which point they would be asked about any adverse events. The eCRF instruments have range checks and other data rules that have to be passed to ensure optimal data input.

Statistical methods

The total significance level of the study was set to be 5%. Based on the O'Brien-Fleming method, the total significance was split into 0.1% for the interim and 4.9% for the final analysis (37). Therefore, a p-value with 99.9% confidence interval is calculated in the interim analysis to test the possible effect of one or both active treatment arms (combined or separately) on clinical pregnancy rate per embryo transfer (primary outcome) compared to placebo. An analysis between the two active treatment arms will also be conducted. All four analyses: 1) active/active vs active/placebo, 2) active/placebo vs placebo/placebo, 3) active/active vs placebo/placebo, 4) active/active AND active/placebo vs placebo/placebo will be done as first a crude estimate and then secondly adjustment with confounders for double embryo transfer, quality of the embryo (Cleavage/blastocyst), female age (continous variable) and center effect (public/private). If the trial is discontinued according to the criteria stated under the paragraph "interim analysis", a full statistical analysis will be made as described below. The final statistical analysis includes crude odds ratios (ORs) and 94.9% confidence intervals (CI's), that are calculated from Chi-square test for possible effect of active treatment on study outcomes (e.g. clinical pregnancy rate [primary outcome]) compared with placebo. Analyses will be conducted at intention-to-treat level and for those completing the treatment protocol without violation. Specific analyses will also include regression analyses, e.g. logistic and linear regressions, taking confounding factors into account.

Interim analysis

An interim analysis of the four analyses mentioned above will be performed to evaluate the clinical pregnancy rate per embryo transfer when 167 patients have been randomized. If study medication is affecting the clinical pregnancy rate statistically significant in either of the groups, the trial will discontinue. Furthermore, the drop-out rate will be evaluated considering both the number of positive AVM declining to participate and the number of patients who drop-out after randomization. A drop-out rate above 20% will lead to discontinuation. External statisticians from Aarhus University, Denmark will conduct the interim analysis. Only a small study board, including sponsor and principal investigators will know the result of the interim analysis. Sponsor-investigator make the decision to continue or discontinue the trial. The study will continue in case there is no statistical difference in either of the 4 tests, drop-out rate is acceptable, and the logistical requirements to finish the study can be met within reasonable time considering e.g. expiry of study medication and time to recruit all patients. The time to undertake the interim analysis and the decision to continue or discontinue is approximately 3 weeks. The trial is on hold during these 3 weeks.

Data monitoring

Investigator(s)/institution(s) will be permitted direct access to source data/documents for trial-related monitoring, audits, IRB/IEC review, and regulatory inspection(s). Primary investigators only have access to patients from their own center. This study will be monitored by the Danish GCP units, primarily the GCP-unit at Aarhus University and GCP-unit Copenhagen University Hospital. Furthermore, this trial is open for audit and quality assurance by the Danish Medicines Agency as specified by Danish law.

Adverse events and reactions

Adverse events and adverse reactions will be registered in a questionnaire handed out by study personnel to the patient on the day of embryo transfer and on the day of the clinical pregnancy scan. In case there is no embryos for transfer patients will be approached to answer the questionnaire either by email or at oocyte retrieval day. Patients who enter luteal phase stimulation (Duostim) or segmentation (freeze-all) will use the same questionnaire on the oocyte retrieval day of the cycle where they have study medication, corresponding to approximately 14 days of study medication. In the questionnaire, patients will also be asked to answer questions regarding gastrointestinal symptoms that might be related to the treatment with antibiotic clindamycin. There will be at least 8 weeks of follow-up to register any late-occurring adverse events/reactions. Patients will be asked the same questionnaire concerning potential late occurring LACTIN-V related side-effects on the day of the clinical pregnancy scan. Moreover, patients are asked if they have symptoms at all study visits and these symptoms are recorded in the eCRF, including an adverse reaction judgement from the treating physician.

Serious adverse events (SAE)

At each center, primary investigators will report serious adverse events (SAE) to sponsor within 24 hours by email or phone. Sponsor ensures that all Suspected Unexpected Serious Adverse Reactions (SUSARs) that are fatal or life-threatening are recorded and reported to the Danish Medicines Agency and the scientific Ethics Committee as soon as possible and no later than 7 days after the sponsor became aware of such possible side effect. Within 8 days after a SUSAR has been reported, the sponsor must notify the Danish Medicines Agency and the Ethics Committee with all relevant information on the follow-up of any SUSAR that may occur. All other unexpected serious or suspected serious adverse reactions will be reported to the Danish Medicines Agency and the scientific Ethics Committee within 15 days after the sponsor become aware of these. An annual safety

report regarding the trial participants will be performed, consisting of serious adverse event suspected to be related to the investigational drug will be submitted to Danish Medicines Agency and the Ethics Committee. At end of study, all AEs and SAEs will be reported according to regulations in Denmark.

Ethics

Approvals from the Regional Scientific Ethical Committee (M-2017-157-17), the Danish Data Protection Agency (1-16-02-790-17) and Danish Medicines Agency (2016-002385-31) were obtained prior to trial initiation December 7th, 2017. Danish law will be complied with regarding the handling of personal information. Protocol amendments will be provided to the relevant parties, including the Regional Scientific Ethical Committees and Danish Medicines Agency. All protocol amendments have to be approved by the Danish Medicines Agency and the scientific ethical committee before taken into use. Logging of trial amendments is secured at both these institutions, the sponsor-investigator as well as updated at EudraCT. Patient confidentiality is ensured by data capture in REDCap™. All patients are covered by a public insurance in Denmark.

Access to data

Only the sponsor-investigator has full access to the dataset. The interim analysis will be performed by external statisticians at the local university according to the pre-set plan explained above. Primary investigators and statisticians may have access to data at the discretion of the sponsor-investigator. External parties can only gain access to trial data following establishment of a data handling agreement.

Dissemination

Positive, negative as well as inconclusive results will be published, aiming for high impact journals with full data transparency. Dissemination of results is ensured in clinical trial agreements between the participating institutions and Sponsors institution, Aarhus University, Denmark. The Vancouver guidelines for authorship will be followed.

Trial status

The first patient was screened December 7th, 2017. By September 7th, 2019 we had screened 533 patients and randomized 119 patients. Interim analysis is expected by March 2020. End of trial is expected to be summer 2021.

Author Contributions

PH, JSJ, NU and TH were the primary writers and inventors of this protocol. PH is the sponsor-investigator. TP provided information on LACTIN-V and contributed to the study design and protocol development. TH, NlCF, AP, VH, ALM and HSN are primary investigators at the involved clinics and contributed to the protocol and amendments during the initiation phase of the study.

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Competing interests' statement

PH, JSJ, NU, TP and TH are listed as inventors in an international patent application (PCT/UK2018/040882), involving the therapeutic use of vaginal lactobacilli to improve IVF outcomes. TP is an employee of Osel, Inc. Not related to this trial, TH received honoraria for lectures from Ferring, IBSA, Besins and Merck. PH received unrestricted research grants from MSD, Merck, and Ferring as well as honoraria for lectures from MSD, Merck, Gedeon-Richter, Theramex, and IBSA. JSJ received speaker's fee from Hologic, BD, SpeeDx, and Cepheid and serves scientific advisory board of Roche Molecular Systems, Abbott Molecular, and Cepheid. NF received unrestricted research grant from Gedeon Richter and honoraria for lectures from Merck. HSN received unrestricted research grant from Ferring and honoraria for lectures from Merck, IBSA and Ferring.

Patients and public involvement

Neither patients nor the public was directly involved in the planning of this trial.

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Table 1: In- and exclusion criteria

Inclusion criteria:	**Exclusion criteria:**
Abnormal vaginal microbiota as described above. The screening swab should be repeated if more than 3 months old at randomization day	HIV, Hepatitis B or C positivity.
First, second or third IVF stimulation cycle or embryo transfer therefrom.	HPV CIN 2 or higher.
BMI<35	Known or suspected hypersensitivity to clindamycin.
Informed consent.	Former or current inflammatory bowel disease
18-42 years old	Severe concomitant disease, including diabetes.
A maximum of 2 embryos to be transferred	Artificial heart valve

	Intrauterine malformations with operation indication as determined by treating physician (Polyps, Septum, fibroma)

Table 2: Study medication scheme

	Clindamycin "Alternova"	LACTIN-V™
Dose	300mg	200mg/2x109 CFU/applicator
Dose schedule	Two times per day minimum 6 hours interval. Max. 14 tablets	Before sleeping Max. 21 applicators
Allocation	Patients start medication at least 12 days prior to embryo transfer in a fresh or a frozen cycle	Patients start medication at least 12 days prior to embryo transfer in a fresh or a frozen cycle
Route of administration	Oral	Vaginal/topical
Treatment period	7 days	Once per day until embryo transfer followed by administration twice weekly until clinical pregnancy scan or confirmed not pregnant. In the event of negative hCG test (not pregnant), patients are, however, allowed to continue

		LACTIN-V treatment until all applicators have been used*.
Follow-up period in the present RCT	Clinical pregnancy scan 7-9 weeks later	Clinical pregnancy scan 7-9 weeks later
Medication permitted	All other than the below mentioned	All other than the below mentioned
Medication not permitted	Other antibiotics (unless medically indicated), probiotics, neuromuscular blocking drugs, immunosuppressive medication. Investigational drug preparations other than the study product.	Antibiotics (unless medically indicated), other probiotics and investigational drug preparations other than the study product.

* Patients not pregnant are informed to contact the department in case of any LACTIN-V related side-effect.

Table 3: Study timeline

	Enrolment	**Allocation**				
TIMEPOINT	**Max 3 months prior to allocation day**	**Minimum 12 days prior to embryo transfer**	***7 days later***	***Embryo transfer***	***Pregnancy scan***	***Gestional week 22, 37, after birth***
ENROLMENT:						

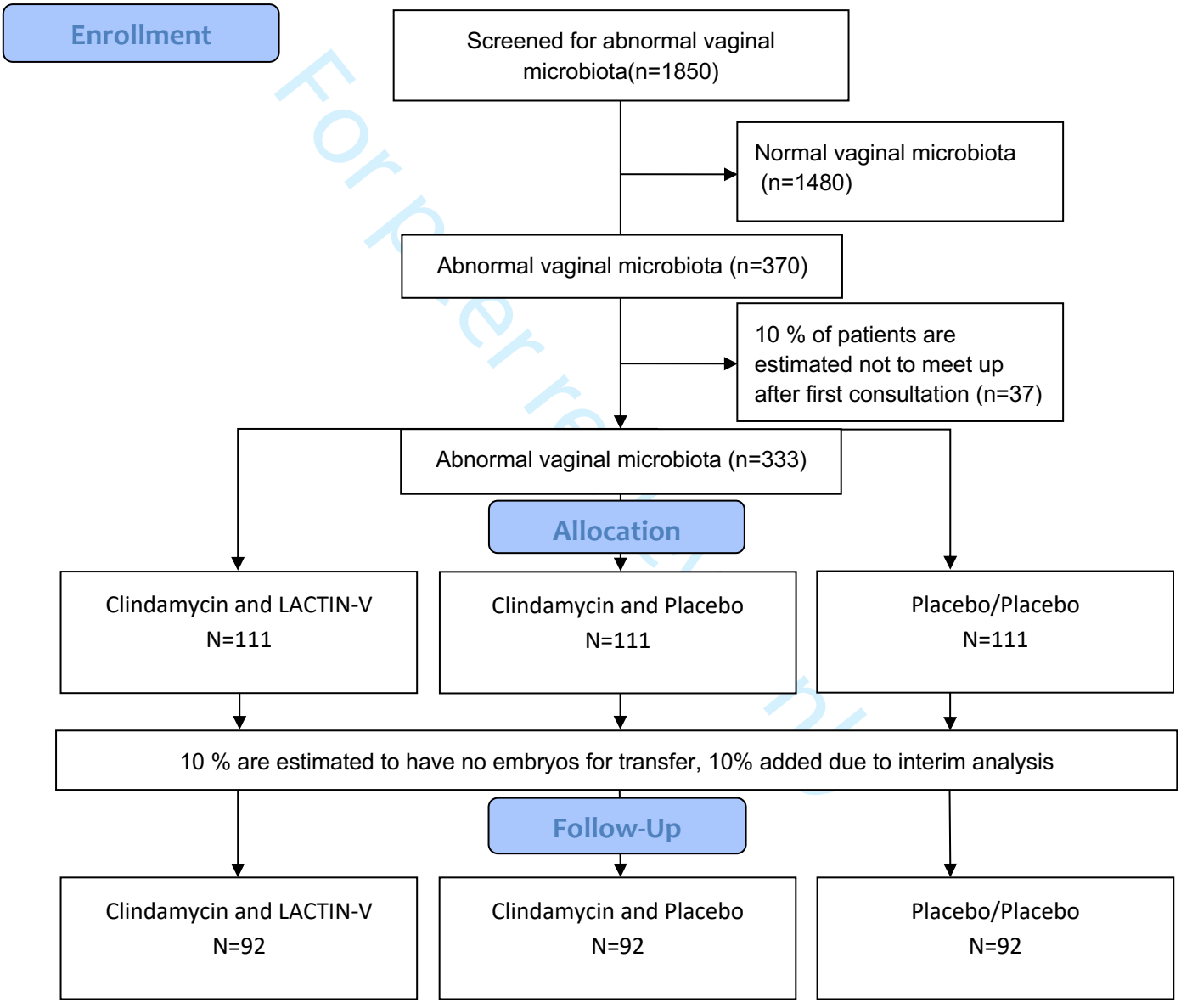
Eligibility screen	X					
Informed consent	X					
Vaginal swab	X					
Allocation		X				
INTERVENTIONS:						
Clindamycin		X ⟷				
Lactin-V			X ⟷			
IVF treatment		X ⟷				
ASSESSMENTS:						
Vaginal swab		X	X	X	X	X

Figure 1: Study Flowchart

See figure attached.

Figure legend: We add 20% more patients to the 92 randomized patients to adjust for couples who have no embryos for transfer and to adjust for the interim analysis, i.e. 19 + 92 =111. Considering an estimated 20% AVM rate, a total of 1850 IVF patients will be screened to randomize 333 patients (three arms).

CONSORT 2010 Flow Diagram



Appendix 1

Samples will be collected from the vagina as outlined in table 3 and in the protocol. Moreover, we collect seminal samples from the partner at oocyte retrieval day and a fecal specimen from the newborn diaper within 3 weeks from birth. Vaginal swabs that are taken at the clinic are taken with a flocked swab and placed in Eswab (Copan™) except for the seminal sample, which is kept in a 1.8 mL cryotube, Nunc™, cryotube™, Thermo Scientific. The vaginal swabs taken at home in pregnancy after birth and the fecal swab are taken by the patient herself using flocked swabs and placed in an eNAT tube (Copan™). All the vaginal samples are taken after these instructions: the vaginal swab has to be the placed at least 8 cm into the vaginal cavity for 5 seconds and rotated clockwise. The fecal swab has to be a deep sample and has to be rotated in the feces for 5 seconds. The semen sample is collected after homogenization and then 200 microL are transferred to the cryotube using a pipette.

Only the vaginal swab at screening visit is sent immediately to the central laboratory at Statens Serum Institute for analysis within 7 days. All vaginal swabs taken during study visits and the seminal sample are immediately frozen at minus 80 degrees at the respective clinics. All samples are stored at the clinics until they are to be transferred by dry ice shipment to the central laboratory. Finally, the home-samples (vaginal samples and the fecal sample) are sent with return envelopes to the patients when they are supposed to take the sample and samples are received back at the Fertility Clinic in Skive, Denmark where they are stored at -80 degrees. For the substudy explained in the protocol concerning the 20 patients taking daily vaginal home-samples (Copan ESwab™), these are kept in the patient's own freezer (-20 degrees) until embryo transfer day where the first 12-16 samples will be delivered to the clinic and stored at minus 80 degrees. The remaining 5-9 samples are kept in the patient's own freezer (-20) until her next visit which may be either hCG test day, clinical pregnancy scan or a visit during the subsequent cycle due to no pregnancy.

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

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	3
	2b	All items from the World Health Organization Trial Registration Data Set	2,8-15
Protocol version	3	Date and version identifier	8
Funding	4	Sources and types of financial, material, and other support	18
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	1,18
	5b	Name and contact information for the trial sponsor	1
	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	3,17,18
	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)	15

Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	5-8
	6b	Explanation for choice of comparators	7
Objectives	7	Specific objectives or hypotheses	8
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)	8
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	8
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)	8-9
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	9
	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)	14
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	10,15
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	10
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	11

Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	table 3 p.24
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	11
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	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	8,12
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	8,12
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	8, 12
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	8,12
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	12,13
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	13,15

	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	13
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	13
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	15
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	15
	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)	15
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	15
	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	14,15
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	15,16
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	15
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	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)	17
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	8
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	N/A
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	17
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	19
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	17
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	17
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	17
	31b	Authorship eligibility guidelines and any intended use of professional writers	17
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	17
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	www.reproflor.dk
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	See appendix 1

*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

A protocol for a double-blind, placebo-controlled multicenter trial on the effect of clindamycin and a live biotherapeutic on the reproductive outcomes of IVF patients with abnormal vaginal microbiota

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Title: A protocol for a double-blind, placebo-controlled multicenter trial on the effect of clindamycin and a live biotherapeutic on the reproductive outcomes of IVF patients with abnormal vaginal microbiota

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Abstract:

Introduction

Recent studies in *in vitro* fertilization (IVF) patients have associated abnormal vaginal microbiota (AVM) with poor clinical pregnancy rates of 6-9% per embryo transfer. The biological plausibility for this finding is hypothesized to be ascending infection to the endometrium which in turn hampers embryo implantation. The prevalence of AVM ranges from 4-38%. New molecular diagnosis may offer advantages compared to microscopical diagnosis; however, the important question is whether screening and treatment of AVM would improve reproductive outcomes in IVF patients. Herein, we describe a protocol for an ongoing double-blind, placebo-controlled multicenter trial of IVF patients with molecular defined AVM randomized in three parallel groups 1:1:1.

Methods and analysis

This is a drug intervention study where IVF patients will be screened for AVM, using a qPCR assay targeting *Atopobium vaginae* and *Gardnerella vaginalis*. If positive, patients will be randomized to one of the three study arms. The first arm consists of clindamycin 300mg x2 daily for 7 days followed by topical *Lactobacillus crispatus* CTV-05 until clinical pregnancy scan week 7-9. The second arm consists of clindamycin and placebo *Lactobacillus crispatus* CTV-05, whereas patients in the third arm will be treated with placebo/placebo. We used a superiority design to estimate that active treatment in both arms will increase the primary outcome, clinical pregnancy rate per embryo transfer, from 20% to 40%. A potential difference between the two active arms was considered exploratory. With a power of 80% and an alpha at 5%, the sample size is estimated to be 333 patients randomized. A pre-planned interim analysis is scheduled at 167 patients randomized.

Ethics and dissemination

All patients have to give informed consent. Dissemination of results is ensured in clinical trial agreements whether they be positive or not.

Registration

This ICH-GCP monitored trial is registered in relevant national agencies. EudraCT number 2016-002385-31.

Keywords: IVF, Microbiota, Bacterial vaginosis, Clindamycin, Gardnerella, RCT, Lactobacillus

Article summary: 'Strengths and limitations of this study'

- Molecular based diagnosis of abnormal vaginal microbiota was validated in pilot studies
- The first RCT in IVF patients with abnormal vaginal microbiota investigating treatment effect on reproductive outcome of clindamycin and live lactobacillus treatment
- The *Lactobacillus crispatus* CTV-05 treatment is an investigational live biotherapeutic product regulated by the US FDA
- Inclusion criteria are relatively broad

Introduction

Bacterial Vaginosis (BV) is a common vaginal dysbiosis in reproductive age women with a prevalence of 29% (95% CI 27-31%) as reported in a US population based survey, N=3739 (1). It is well-known that there is higher BV rates among African-Americans compared to Caucasian women (1). However, this finding could be affected by the fact that asymptomatic African-Americans seem to have a more diverse vaginal microbiota as compared to Caucasians(2,3). Other risk factors include frequent vaginal douching and number of lifetime sex partners(1). In the *in vitro* fertilization (IVF) population, a recent meta-analysis (N=2980) reported that the prevalence of BV exhibited huge interstudy heterogeneity ranging from 4-38% (4). In this study, BV was clearly associated with tubal factor infertility, but not endometriosis. The most recent studies using a molecular based analysis to determine an abnormal vaginal microbiota observed a prevalence of 17% and 28%, respectively (5,6). It is known that despite diagnosed with BV by the gold standard Nugent method (7), more than 80% of BV positives remain asymptomatic(1). Hence, the important question is whether the many asymptomatic BV cases should be screened and treated. Screening and treatment for BV is recommended in patients undergoing gynecological surgery or invasive diagnostic procedures through the vagina to minimize infection(8). However, most clinical guidelines do not support screening and treatment for asymptomatic BV to optimize reproductive outcome – a topic which has been thoroughly investigated in obstetric populations for preterm birth prevention (9,10). Today, a new frontier is emerging with optimized molecular based diagnosis and new treatment possibilities including well-studied and well-characterized probiotics – that have been designated "live biotherapeutic products" by FDA(11,12).

Haahr et al. (2016) reported the advantages of a molecular based diagnosis of vaginal dysbiosis in IVF patients(5). The main advantages were i) a more objective diagnosis as microscopists had significant interrater variability with the prior gold standard, Nugent score ii) dichotomization of the

Nugent intermediate group which was difficult to interpret clinically and iii) the establishment of quantitative thresholds using key vaginal bacteria to detect IVF patients at risk of a poor reproductive outcome. Hence, a new terminology termed abnormal vaginal microbiota (AVM) was proposed for IVF patients (5,13). AVM was significantly associated with poor clinical pregnancy rates as compared to normal vaginal microbiota patients, 9% (2/22) versus 44% (27/62)(5). Later, these findings were corroborated by Koedooder et al. (6) who found clinical pregnancy rates of 6% (2/34) versus 42% (65/154) in patients with unfavorable and favorable vaginal microbiota, respectively.

In the field of reproductive medicine, there have been two different approaches to investigate the potential influence of the genital tract microbiota on IVF outcomes: either i) to directly investigate the endometrial microbiota by transcervical swabs/suctions (14–16) or ii) to investigate the vaginal microbiota as a proxy for the endometrial microbiota (5,6,17). The bacterial load in the uterus as compared to the vagina is very low (18), and for this reason the studies on endometrial microbiota have been criticized for reporting contamination from the transcervical sampling approach - and not a genuine endometrial microbiota. Nevertheless, endometrial samples from women undergoing hysterectomy provide evidence for a genuine endometrial microbiota (18,19) that seems to be highly influenced by the vaginal microbiota (19), especially in the case of BV where the odds of having endometrial colonization, including *Gardnerella vaginalis* biofilm infection, was significant as compared to normal vaginal microbiota patients: OR 5.7 (95% CI, 1.8–18.3, P = 0.002) (20). Several groups are developing or further optimizing molecular based approaches to diagnose IVF women at risk of poor reproductive outcomes caused by genital tract dysbiosis. However, only one study validated a molecular diagnostic approach in IVF women against the gold standard for vaginal dysbiosis – Nugent score of Gram stained vaginal smears(5). Two other studies applied arbitrary cut-offs for *Lactobacillus* dominance in the vaginal microbiota (6,17). Subsequently, these studies were criticized for insufficient methods (21,22), including the application of arbitrary thresholds based on

relative abundances which does not sufficiently take into account differences in the total abundance (21–23).

The recommended first-line treatments for BV are antibiotic therapy with either metronidazole or clindamycin as reported by the 2015 CDC (Center for Disease Prevention and Control) Sexually Transmitted Disease Guideline and the 2018 European IUSTI/WHO (International Union against Sexually Transmitted Infections) guideline. Clindamycin was reported to effectively eradicate BV-related bacteria in the endometrium of patients with endometritis (24), while it was also proven to enter the endometrial tissue in high concentrations if administered orally (25). In contrast, metronidazole was less effective against *Gardnerella vaginalis* both *in vivo* (26) and *in vitro* (24).

Finally, a recent systematic review and meta-analysis reported that the use of additional probiotic treatment alongside standard treatment of bacterial vaginosis could improve BV cure rates, RR = 1.28, 95% CI (1.05, 1.56) (27). However, due to primarily poor study quality(28), there is currently no consensus on which vaginal *Lactobacillus* product, if any, should be recommended (29).

The pioneering work by Ravel and colleagues (2011) established that the vaginal microbiota is heterogeneous and four *Lactobacillus* dominated community state types (CSTs) can be identified using taxonomic stratification at the species level, with each CST dominated by a different vaginal *Lactobacillus* species or a diverse CST not dominated by *Lactobacillus (2)*. Although such stratification was based on hierarchical clustering and relative abundance – in contrast to absolute abundance, these CSTs have been adopted by the majority of researchers in the vaginal microbiome field. Consistently, publications have reported the *L. crispatus* CST to be associated with optimal genital health and reproductive outcomes (13,17,30–32). Abundant *in vitro* evidence point towards a beneficial production of both D and L lactic acid isomers by *L. crispatus* that not all other common vaginal lactobacilli produce (33,34). At the time of planning the present study, only one *L. crispatus* product, LACTIN-V, existed as an investigational live biotherapeutic product regulated by FDA – at

that time in Phase 2 development(11). Recently, adjuvant LACTIN-V after vaginal metronidazole was reported to lower BV recurrence rates in a phase 2b trial, RR 0.66 (95%CI 0.44-0.87)(35).

Taking into consideration the abovementioned evidence, the research question of the present study is: does antibiotic alone or in combination with live biotherapeutic treatment of an abnormal vaginal microbiota improve the reproductive outcomes of IVF patients? The intervention is clindamycin either alone or in combination with LACTIN-V, a live biotherapeutic product containing *L.crispatus* CTV-05 (11). The study is designed as a double-blind, placebo-controlled multicenter trial of three parallel groups randomized 1:1:1. Randomization is by computer generated code and allocation concealment is performed by the pharmacy who will send out medication to the participating clinics with identical appearance and randomization numbers. The randomization code is with the pharmacy and can only be opened in case of emergency by the principal investigators or as planned by the sponsor-investigator. The benefit of the intervention would potentially lead to increased pregnancy rates and, for those suffering from symptomatic BV, also relief of BV symptoms. In contrast, the expected adverse reactions of concern are especially gastrointestinal symptoms caused by clindamycin, whereas LACTIN-V might cause increased vaginal discharge but is otherwise not expected to cause adverse reactions as based on prior studies(11,36).

Methods and analysis

Setting and eligibility criteria

The present trial is conducted at four University affiliated clinics and one private fertility clinic in Denmark. The list of study sites is available with EudraCT clinical trial identifier: 2016-002385-31, first registration day 2016-07-11. The current version of the protocol is 9, 2020-02-07. Patients are

enrolled in a cohort study (Clinicaltrials.gov NCT03420859) from which we will recruit patients for the randomized trial (EudraCT: 2016-002385-31). Eligibility criteria are described in Table 1.

In brief, IVF patients attending their first, second or third IVF stimulation cycle or embryo transfer therefrom will be approached for informed consent by the study nurse or treating physician. Patients are told about the project in a private room with the right to have an assessor, allowing time to reflect whether they will participate. They are handed out written information material with a link to the study website with full information about the project – www.reproflor.dk. The vaginal swab can be taken by the treating physician or the patient herself after careful instruction. In this case, patients are instructed to place the swab at least 8 cm into the vaginal cavity for 5 seconds and rotate clockwise. This is to ensure that the vaginal bacteria in the fornix or in its close proximity will be caught by the flocked swab. Subsequently, the vaginal swab will be sent to a central laboratory at Statens Serum Institut, Copenhagen to be analyzed for AVM within 7 days as determined in a previous study(5). If AVM positive, patients are asked to provide informed consent that they are willing to participate in the randomized controlled trial. Patients should ideally be randomized on the first day of ovarian stimulation with exogenous gonadotropins, allowing a minimum of 12 days of study medication to be acceptable for inclusion in the study. If elective frozen embryo transfer (FET) is planned, patients should be randomized during the first days of the FET cycle allowing for at least 12 days of study medication. If patients enter the trial and have less than 12 days of study medication despite the abovementioned inclusion criteria (e.g. when hormonal stimulation is shortened due to an unexpected ovarian response), it is considered a protocol violation and they will be excluded from the per protocol analysis, i.e. not from ITT-analysis.

Interventions

Active Treatment 1: **Oral Clindamycin** 300 mg 2 times per day for 7 days followed by **LACTIN-V** (Osel, Inc.) until completion of the clinical pregnancy scan at week 7-9. LACTIN-V containing *L.*

crispatus CTV-05 (2 x 10^9 CFU/dose, 200 mg, delivered with pre-filled, single use vaginal applicators) regimen is once daily from the clindamycin stop for 7 consecutive days.

Active Treatment 2: **Oral Clindamycin** 300 mg 2 times per day for 7 days followed by **LACTIN-V placebo** (Osel, Inc.) until completion of the clinical pregnancy scan at week 7-9. The LACTIN-V placebo regimen is once daily from the clindamycin stop for 7 consecutive days.

Inactive treatment (placebo): Matching **clindamycin placebo** 2 times per day for 7 days followed by **LACTIN-V placebo** (Osel Inc.) until completion of the clinical pregnancy scan at week 7-9. LACTIN-V placebo regimen is once daily from clindamycin stop and the following 7 days.

If there are embryos to transfer (90% of patients), then LACTIN-V/placebo treatment is continued twice weekly until clinical pregnancy scan, however with a maximum of 21 applicators per patient. If the patient has no embryos to transfer or is confirmed not pregnant (negative hCG test), then LACTIN-V treatment can be stopped, albeit at least 7 days of LACTIN-V administration need to be administered. An overview of the study medication and allocation can be seen in Table 2. Patients are not allowed to take other antibiotics (unless medically indicated), probiotics, neuromuscular blocking drugs, immunosuppressive medication or investigational drug preparations other than the study product. Placebo clindamycin consists of encapsulated Mannitolum. The placebo LACTIN-V formulation contains the same inactive ingredients as LACTIN-V, without *Lactobacillus crispatus* CTV-05.

Labelling and packaging

Labelling and packaging of the medication are performed by Glostrup Pharmacy, Denmark in accordance with ICH-GCP guideline and EU GMP Annex 13. Patients are informed that it is important not to have penile-vaginal intercourse within 12 hours after LACTIN V application. Patient compliance will be measured by tablet counting of the medication packs (clindamycin). Any unused LACTIN-V applicators should be delivered to the clinics, otherwise they are considered used.

Patients can withdraw their informed consent at any given time and without any reason according to Danish law. If available, the reason for discontinuation has to be stated in the electronic Case Report Form (eCRF). Moreover, in case of protocol deviations, this also has to be stated in the eCRF and the principal investigator should decide whether trial medication can continue or not. Furthermore, trial medication is stopped, should the patient develop hypersensitivity, allergy or severe diarrhea that a primary investigator suspects may be trial medication related. Vaginal swabs will be taken alongside treatment to monitor the vaginal microbiota and its response to treatment, see Table 3 and appendix 1. Specifically, vaginal swabs will be taken on the day of randomization immediately before study medication, after clindamycin treatment, on the day of embryo transfer and again on the day of clinical pregnancy scan. A total of 20 patients will be asked to deliver vaginal samples for each day they take medication and the swab should be taken immediately before the medication on that specific day.

Outcomes

The primary outcome is the clinical pregnancy rate per first embryo transfer defined as ultrasound proven fetal heartbeat in gestational week 7-9. The secondary outcomes are the live birth rate per embryo transfer, biochemical pregnancy rate (hCG positive at 9-11 days after embryo transfer according to local laboratory standards), implantation rate, early miscarriage, late miscarriage, preterm birth rates, birth weight, and adverse effects of the medication through a safety analysis. As part of the cohort study (Clinicaltrials.gov, NCT03420859), the effect of treatment on the vaginal microbiota of the mother throughout study participation and potential pregnancy will be determined using quantitative PCR (qPCR) and next generation sequencing methods. The colonization of the *L. crispatus* CTV-05 strain will also be investigated using qPCR. Later, we plan to investigate cumulative live birth results of subsequent transfer of spare frozen thawed embryos of patients attending the study in a fresh cycle.

Sample size

In 2014, the average clinical pregnancy rate per embryo transfer in our fertility clinic was approximately 40% for an IVF cycle. In our pilot study(5), the adjusted odds ratio between the AVM group and the normal group was 0.06, 95% CI (0.01–0.47) for clinical pregnancy per embryo transfer. Taken together, we estimated a superiority design were women in each AVM arm treated with active medication will have at least a 40% chance for clinical pregnancy per embryo transfer as compared to the placebo arm which was estimated to have a maximum of 20% chance of clinical pregnancy/transfer. By two samples proportion test with a power of 80% and an alpha at 5%, the aim was to randomize 92 patients in each group. A potential difference between the two active arms was considered exploratory and consequently this was not part of the power calculation, but we decided to include the same number of patients in the active/active arm to investigate a potential added benefit of live biotherapeutic treatment.

An interim analysis will be performed, and to adjust for this, we add 10% to the 92 randomized patients as suggested in Wittes et al (37). Approximately 10% of couples will have no embryos for transfer; we adjusted for this by adding another 10% to each randomized group, i.e. 19 + 92 =111 (see Figure 1). Considering an estimated 20% AVM rate, a total of 1850 IVF patients will be screened to randomize 333 patients (three arms). It was estimated that inclusion will be distributed according to the size of the centers. Furthermore, we make the following assumptions: i) very limited loss to follow-up, ii) near full compliance to study medication and iii) homogeneity in the treatment effect.

Allocation

Randomization is performed by Glostrup pharmacy by a computer-generated code (www.randomization.com). The medication packs labelled with the randomization number are received at the IVF centers from the pharmacy in blocks of 15, five of each of the three treatments, to secure equal distribution of treatment arms at the centers. The medication has identical appearance

and only the randomization number differ, hence both patients and study personnel are blinded for the intervention. A block of 15 medication packs will be sent from the pharmacy from start of study and new blocks can be requested when 5 medication packs are left. The 15 medication packs are mixed and appear identical to both personnel and patients. The randomization number is continuous and unique for each patient, starting from 1 to 333 and the number is prelabelled from the pharmacy before distribution to the clinics. The last 3 medication packs from 330-333 is also one block.

The randomization list is secured by the pharmacy throughout the trial, and only the sponsor has the authority to unblind the trial. However, in case of medical emergency the principal investigator (PI) can call the pharmacy to unblind. Each participant's medication package is labeled with a randomization number that is linked to their study ID number in the eCRF. Although both patients and clinicians will be blinded to allocation, they may suspect active medication in case of side-effects. This small risk of bias seems to be unavoidable. However, to investigate such an effect, patients are asked if they believed that they received active or inactive medication.

Data collection methods

Study data are collected and managed using REDCap (Research Electronic Data Capture) electronic data capture tools hosted at Aarhus University, Denmark(38,39). REDCap is a secure, web-based software platform designed to support data capture for research studies, providing 1) an intuitive interface for validated data capture; 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data downloads to common statistical packages; and 4) procedures for data integration and interoperability with external sources. All data collectors of the study have to be trained in Good Clinical Practice (GCP) procedures and as minimum to have passed the course provided by the Danish GCP institution. All inclusion and exclusion criteria as well as outcome data will be monitored by external GCP monitors to ensure optimal data quality.

Data collection forms and other data entry related information can be requested from the corresponding author.

Protocol deviations have to be stated in the eCRF. Loss to follow-up is unlikely for patients in IVF treatment who will be highly motivated to come to the clinic. However, patients who are not pregnant may opt to go to other clinics for further treatment and, thus will be lost to follow-up. Patients who are not pregnant (negative HCG-test) and continue LACTIN-V treatment are informed to contact the respective clinics in case of adverse events and these will be captured in the eCRF. If patients decide to end study product treatment, they are informed to contact the clinics and to deliver the unused LACTIN-V to the clinic at which point they would be asked about any adverse events. The eCRF instruments have range checks and other data rules that have to be passed to ensure optimal data input. In case of missing outcome data we plan to use the framework proposed by White et al (2011)(40).

Statistical methods

The total significance level of the study was set to be 5%. Based on the O'Brien-Fleming method, the total significance was split into 0.1% for the interim and 4.9% for the final analysis (37). Therefore, a p-value with 99.9% confidence interval is calculated in the interim analysis to test the possible effect of one or both active treatment arms (combined or separately) on clinical pregnancy rate per embryo transfer (primary outcome) compared to placebo. A Walds Chi-square test for possible effect will be conducted comparing all 3 arms. Moreover, four analyses: 1) active/active vs active/placebo, 2) active/placebo vs placebo/placebo, 3) active/active vs placebo/placebo, 4) average effect of active/active AND active/placebo vs placebo/placebo will be done as first a crude estimate and then secondly adjustment with confounders for double embryo transfer, quality of the embryo (Cleavage/blastocyst), female age (continous variable) and center effect (public/private). If the trial

is discontinued according to the criteria stated under the paragraph "interim analysis", a full statistical analysis will be made as described below. First, a Walds Chi-square test for possible effect of active treatment on clinical pregnancy rate [primary outcome] will be made across all 3 groups. Moreover, pairwise comparisons for the abovementioned 4 tests will be made with odds ratios (OR) and risk ratios (RR) and 94.9% confidence intervals (CI's) calculated from logistic and linear regressions models, taking abovementioned confounding factors into account. Analyses will be conducted at intention-to-treat level (all randomized patients) and for those completing the treatment protocol without violation. Intention-to-treat is considered the primary analysis.

Interim analysis

An interim analysis of the four analyses mentioned above will be performed to evaluate the clinical pregnancy rate per embryo transfer when 167 patients have been randomized and completed the study for primary outcome evaluation. If study medication is affecting the clinical pregnancy rate statistically significant in either of the analyses, the trial will discontinue. Furthermore, the drop-out rate will be evaluated considering both the number of positive AVM declining to participate and the number of patients who drop-out after randomization. A drop-out rate above 20% will lead to discontinuation. External statisticians from Aarhus University, Denmark will conduct the interim analysis. Only a small study board, including sponsor and principal investigators will know the result of the interim analysis. Sponsor-investigator make the decision to continue or discontinue the trial. The study will continue in case there is no statistical difference in either of the tests, drop-out rate is acceptable, and the logistical requirements to finish the study can be met within reasonable time considering e.g. expiry of study medication and time to recruit all patients. The time to undertake the interim analysis and the decision to continue or discontinue is approximately 3 weeks.

Data monitoring

Investigator(s)/institution(s) will be permitted direct access to source data/documents for trial-related monitoring, audits, IRB/IEC review, and regulatory inspection(s). Primary investigators only have access to patients from their own center. This study will be monitored by the Danish GCP units, primarily the GCP-unit at Aarhus University and GCP-unit Copenhagen University Hospital. Furthermore, this trial is open for audit and quality assurance by the Danish Medicines Agency as specified by Danish law.

Adverse events and reactions

Adverse events and adverse reactions will be registered in a questionnaire handed out by study personnel to the patient on the day of embryo transfer and on the day of the clinical pregnancy scan. In case there is no embryos for transfer patients will be approached to answer the questionnaire either by email or at oocyte retrieval day. Patients who enter luteal phase stimulation (Duostim) or segmentation ("freeze-all") will use the same questionnaire on the oocyte retrieval day of the cycle where they have started study medication, corresponding to approximately 14 days of study medication. In the questionnaire, patients will also be asked to answer questions regarding gastrointestinal symptoms that might be related to the treatment with antibiotic clindamycin. There will be at least 8 weeks of follow-up to register any late-occurring adverse events/reactions. Patients will be asked the same questionnaire concerning potential late occurring LACTIN-V related side-effects on the day of the clinical pregnancy scan. Moreover, patients are asked if they have symptoms at all study visits and these symptoms are recorded in the eCRF, including an adverse reaction judgement from the treating physician.

Serious adverse events (SAE)

At each center, primary investigators will report serious adverse events (SAE) to sponsor within 24 hours by email or phone. Sponsor ensures that all Suspected Unexpected Serious Adverse Reactions (SUSARs) that are fatal or life-threatening are recorded and reported to the Danish Medicines Agency and the scientific Ethics Committee as soon as possible and no later than 7 days after the sponsor became aware of such possible side effect. Within 8 days after a SUSAR has been reported, the sponsor must notify the Danish Medicines Agency and the Ethics Committee with all relevant information on the follow-up of any SUSAR that may occur. All other unexpected serious or suspected serious adverse reactions will be reported to the Danish Medicines Agency and the scientific Ethics Committee within 15 days after the sponsor become aware of these. An annual safety report regarding the trial participants will be performed, consisting of serious adverse event suspected to be related to the investigational drug will be submitted to Danish Medicines Agency and the Ethics Committee. At end of study, all AEs and SAEs will be reported according to regulations in Denmark.

Ethics

Approvals from the Regional Scientific Ethical Committee (M-2017-157-17), the Danish Data Protection Agency (1-16-02-790-17) and Danish Medicines Agency (2016-002385-31) were obtained prior to trial initiation December 7th, 2017. Danish law will be complied with regarding the handling of personal information. Protocol amendments will be provided to the relevant parties, including the Regional Scientific Ethical Committees and Danish Medicines Agency. All protocol amendments have to be approved by the Danish Medicines Agency and the scientific ethical committee before taken into use. Logging of trial amendments is secured at both these institutions, the sponsor-investigator as well as updated at EudraCT. Patient confidentiality is ensured by data capture in REDCap™. All patients are covered by a public insurance in Denmark.

Access to data

Only the sponsor-investigator has full access to the dataset. The interim analysis will be performed by external statisticians at the local university according to the pre-set plan explained above. Primary investigators and statisticians may have access to data at the discretion of the sponsor-investigator. External parties can only gain access to trial data following establishment of a data handling agreement.

Dissemination

Positive, negative as well as inconclusive results will be published, aiming for high impact journals with full data transparency. Dissemination of results is ensured in clinical trial agreements between the participating institutions and Sponsors institution, Aarhus University, Denmark. The Vancouver guidelines for authorship will be followed.

Trial status

The first patient was screened December 7th, 2017. By September 7th, 2019 we had screened 533 patients and randomized 119 patients. Interim analysis is expected by March 2020. End of trial is expected to be summer 2021.

Author Contributions

PH, JSJ, NU and TH were the primary writers and inventors of this protocol. PH is the sponsor-investigator. TP provided information on LACTIN-V and contributed to the study design and protocol development. TH, NlCF, AP, VH, ALM and HSN are primary investigators at the involved clinics and contributed to the protocol and amendments during the initiation phase of the study.

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Competing interests statement

PH, JSJ, NU, TP and TH are listed as inventors in an international patent application (PCT/UK2018/040882), involving the therapeutic use of vaginal lactobacilli to improve IVF outcomes. TP is an employee of Osel, Inc. Not related to this trial, TH received honoraria for lectures from Ferring, IBSA, Besins and Merck. PH received unrestricted research grants from MSD, Merck, and Ferring as well as honoraria for lectures from MSD, Merck, Gedeon-Richter, Theramex, and IBSA. JSJ received speaker's fee from Hologic, BD, SpeeDx, and Cepheid and serves scientific advisory board of Roche Molecular Systems, Abbott Molecular, and Cepheid. NF received unrestricted research grant from Gedeon Richter and honoraria for lectures from Merck. HSN

received unrestricted research grant from Ferring and honoraria for lectures from Merck, IBSA and Ferring.

Patients and public involvement

Neither patients nor the public was directly involved in the planning of this trial.

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Table 1: In- and exclusion criteria

Inclusion criteria:	**Exclusion criteria:**

Abnormal vaginal microbiota as described above. The screening swab should be repeated if more than 3 months old at randomization day	HIV, Hepatitis B or C positivity.
First, second or third IVF stimulation cycle or embryo transfer therefrom.	HPV CIN 2 or higher.
BMI<35	Known or suspected hypersensitivity to clindamycin.
Informed consent.	Former or current inflammatory bowel disease
18-42 years old	Severe concomitant disease, including diabetes.
A maximum of 2 embryos to be transferred	Artificial heart valve
	Intrauterine malformations with operation indication as determined by treating physician (Polyps, Septum, fibroma)

Table 2: Study medication scheme

	Clindamycin "Alternova"	LACTIN-V™
Dose	300mg	200mg/2x10^9 CFU/applicator
Dose schedule	Two times per day minimum 6 hours interval. Max. 14 tablets	Before sleeping Max. 21 applicators

Allocation	Patients start medication at least 12 days prior to embryo transfer in a fresh or a frozen cycle	Patients start medication at least 12 days prior to embryo transfer in a fresh or a frozen cycle
Route of administration	Oral	Vaginal/topical
Treatment period	7 days	Once per day until embryo transfer followed by administration twice weekly until clinical pregnancy scan or confirmed not pregnant. In the event of negative hCG test (not pregnant), patients are, however, allowed to continue LACTIN-V treatment until all applicators have been used*.
Follow-up period in the present RCT	Clinical pregnancy scan 7-9 weeks later	Clinical pregnancy scan 7-9 weeks later
Medication permitted	All other than the below mentioned	All other than the below mentioned
Medication not permitted	Other antibiotics (unless medically indicated), probiotics, neuromuscular blocking drugs, immunosuppressive	Antibiotics (unless medically indicated), other probiotics and investigational drug preparations other than the study product.

	medication. Investigational drug preparations other than the study product.	

* Patients not pregnant are informed to contact the department in case of any LACTIN-V related side-effect.

Table 3: Study timeline

	Enrolment	**Allocation**				
TIMEPOINT	**Max 3 months prior to allocation day**	**Minimum 12 days prior to embryo transfer**	***7 days later***	***Embryo transfer***	***Pregnancy scan***	***Gestional week 22, 37, after birth***
ENROLMENT:						
Eligibility screen	X					
Informed consent	X					
Vaginal swab	X					
Allocation		X				
INTERVENTIONS:						
Clindamycin		X ←	→			
Lactin-V			X	→	→	
IVF treatment		X ←	→	→	→	

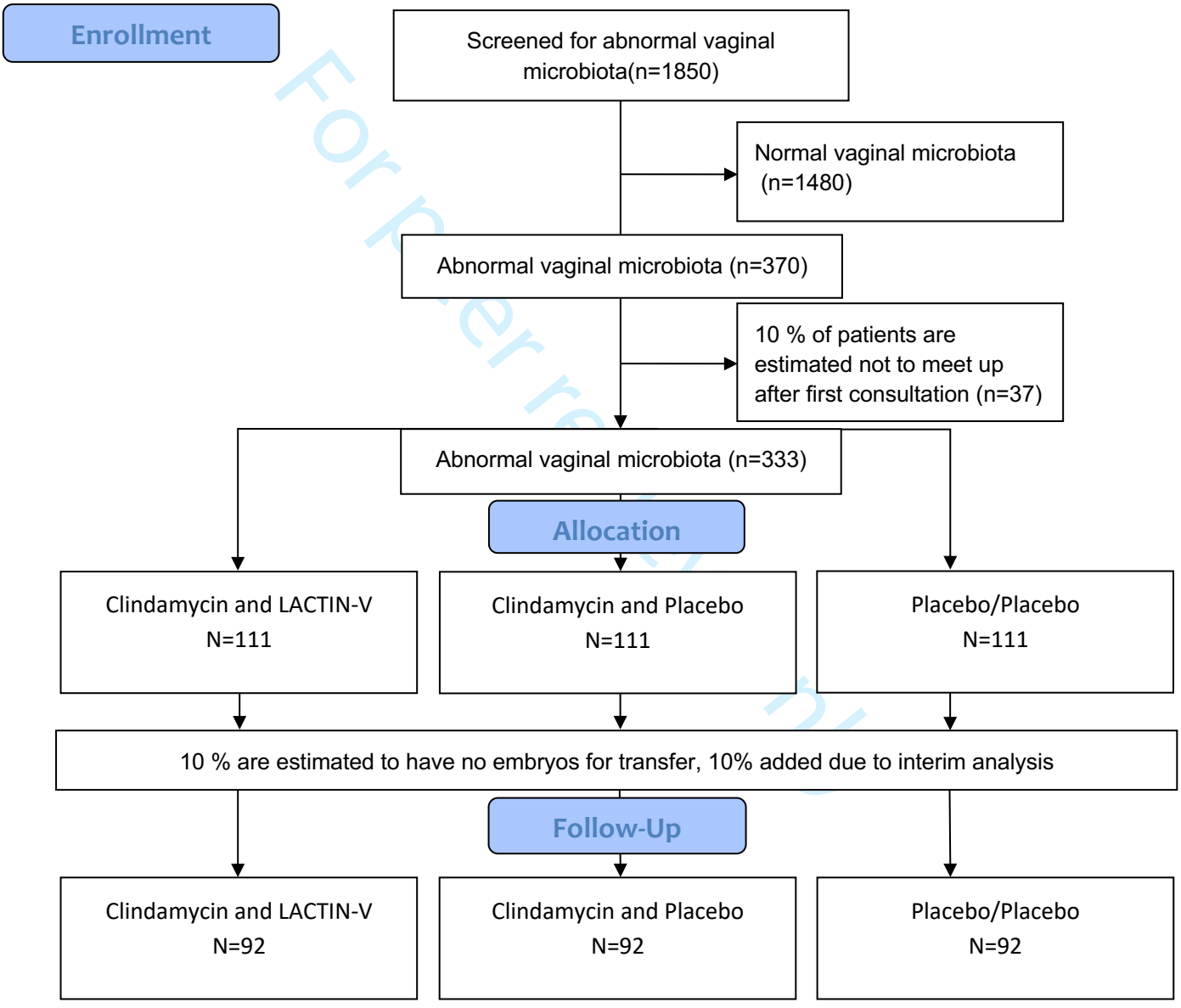
ASSESSMENTS:						
Vaginal swab		X	X	X	X	X

Figure 1: Study Flowchart

See figure attached.

Figure legend: We add 20% more patients to the 92 randomized patients to adjust for couples who have no embryos for transfer and to adjust for the interim analysis, i.e. 19 + 92 =111. Considering an estimated 20% AVM rate, a total of 1850 IVF patients will be screened to randomize 333 patients (three arms).

CONSORT 2010 Flow Diagram



Appendix 1

Samples will be collected from the vagina as outlined in table 3 and in the protocol. Moreover, we collect seminal samples from the partner at oocyte retrieval day and a fecal specimen from the newborn diaper within 3 weeks from birth. Vaginal swabs that are taken at the clinic are taken with a flocked swab and placed in Eswab (Copan™) except for the seminal sample, which is kept in a 1.8 mL cryotube, Nunc™, cryotube™, Thermo Scientific. The vaginal swabs taken at home in pregnancy after birth and the fecal swab are taken by the patient herself using flocked swabs and placed in an eNAT tube (Copan™). All the vaginal samples are taken after these instructions: the vaginal swab has to be the placed at least 8 cm into the vaginal cavity for 5 seconds and rotated clockwise. The fecal swab has to be a deep sample and has to be rotated in the feces for 5 seconds. The semen sample is collected after homogenization and then 200 microL are transferred to the cryotube using a pipette.

Only the vaginal swab at screening visit is sent immediately to the central laboratory at Statens Serum Institute for analysis within 7 days. All vaginal swabs taken during study visits and the seminal sample are immediately frozen at minus 80 degrees at the respective clinics. All samples are stored at the clinics until they are to be transferred by dry ice shipment to the central laboratory. Finally, the home-samples (vaginal samples and the fecal sample) are sent with return envelopes to the patients when they are supposed to take the sample and samples are received back at the Fertility Clinic in Skive, Denmark where they are stored at -80 degrees. For the sub-study explained in the protocol concerning the 20 patients taking daily vaginal home-samples (Copan ESwab™), these are kept in the patient's own freezer (-20 degrees) until embryo transfer day where the first 12-16 samples will be delivered to the clinic and stored at minus 80 degrees. The remaining 5-9 samples are kept in the patient's own freezer (-20) until her next visit which may be either hCG test day, clinical pregnancy scan or a visit during the subsequent cycle due to no pregnancy.

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

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	3
	2b	All items from the World Health Organization Trial Registration Data Set	2,8-15
Protocol version	3	Date and version identifier	8
Funding	4	Sources and types of financial, material, and other support	18
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	1,18
	5b	Name and contact information for the trial sponsor	1
	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	3,17,18
	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)	15

Introduction

Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	5-8
	6b	Explanation for choice of comparators	7
Objectives	7	Specific objectives or hypotheses	8
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)	8

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	8
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)	8-9
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	9
	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)	14
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	10,15
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	10
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	11

Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	table 3 p.24
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	11
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	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	8,12
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	8,12
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	8, 12
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	8,12
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	12,13
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	13,15

	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	13
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	13
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	15
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	15
	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)	15
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	15
	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	14,15
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	15,16
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	15
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	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)	17
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	8
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	N/A
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	17
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	19
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	17
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	17
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	17
	31b	Authorship eligibility guidelines and any intended use of professional writers	17
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	17
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	www.reproflor.dk
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	See appendix 1

*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

A protocol for a double-blind, placebo-controlled multicenter trial on the effect of clindamycin and a live biotherapeutic on the reproductive outcomes of IVF patients with abnormal vaginal microbiota

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Title: A protocol for a double-blind, placebo-controlled multicenter trial on the effect of clindamycin and a live biotherapeutic on the reproductive outcomes of IVF patients with abnormal vaginal microbiota

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Abstract:

Introduction

Recent studies in *in vitro* fertilization (IVF) patients have associated abnormal vaginal microbiota (AVM) with poor clinical pregnancy rates of 6-9% per embryo transfer. The biological plausibility for this finding is hypothesized to be ascending infection to the endometrium which in turn hampers embryo implantation. The prevalence of AVM ranges from 4-38%. New molecular diagnosis may offer advantages compared to microscopical diagnosis; however, the important question is whether screening and treatment of AVM would improve reproductive outcomes in IVF patients. Herein, we describe a protocol for an ongoing double-blind, placebo-controlled multicenter trial of IVF patients with molecular defined AVM randomized in three parallel groups 1:1:1.

Methods and analysis

This is a drug intervention study where IVF patients will be screened for AVM, using a qPCR assay targeting *Atopobium vaginae* and *Gardnerella vaginalis*. If positive, patients will be randomized to one of the three study arms. The first arm consists of clindamycin 300mg x2 daily for 7 days followed by topical *Lactobacillus crispatus* CTV-05 until clinical pregnancy scan week 7-9. The second arm consists of clindamycin and placebo *Lactobacillus crispatus* CTV-05, whereas patients in the third arm will be treated with placebo/placebo. We used a superiority design to estimate that active treatment in both arms will increase the primary outcome, clinical pregnancy rate per embryo transfer, from 20% to 40%. A potential difference between the two active arms was considered exploratory. With a power of 80% and an alpha at 5%, the sample size is estimated to be 333 patients randomized. A pre-planned interim analysis is scheduled at 167 patients randomized.

Ethics and dissemination

All patients have to give informed consent. Dissemination of results is ensured in clinical trial agreements whether they be positive or not. Ethics committee, Central Denmark Region approved this protocol.

Registration

ICH-GCP monitored trial, EudraCT: 2016-002385-31.

Keywords: IVF, Microbiota, Bacterial vaginosis, Clindamycin, Gardnerella, RCT, Lactobacillus

Article summary: 'Strengths and limitations of this study'

- Molecular based diagnosis of abnormal vaginal microbiota was validated in pilot studies
- The first RCT in IVF patients with abnormal vaginal microbiota investigating treatment effect on reproductive outcome of clindamycin and live lactobacillus treatment
- The *Lactobacillus crispatus* CTV-05 treatment is an investigational live biotherapeutic product regulated by the US FDA
- ICH-GCP monitored trial
- Inclusion criteria are relatively broad

Introduction

Bacterial Vaginosis (BV) is a common vaginal dysbiosis in reproductive age women with a prevalence of 29% (95% CI 27-31%) as reported in a US population based survey, N=3739 (1). It is well-known that there is a higher BV rate among African-Americans compared to Caucasian women (1). However, this finding could be affected by the fact that asymptomatic African-Americans seem to have a more diverse physiological vaginal microbiota as compared to Caucasians(2,3). Other risk factors include frequent vaginal douching and number of lifetime sex partners(1). In the *in vitro* fertilization (IVF) population, a recent meta-analysis (N=2980) reported that the prevalence of BV exhibited huge interstudy heterogeneity ranging from 4-38% (4). In this study, BV was clearly associated with tubal factor infertility, but not endometriosis. The most recent studies using a molecular based analysis to determine an abnormal vaginal microbiota observed a prevalence of 17% and 28%, respectively (5,6). It is known that despite diagnosed with BV by the gold standard Nugent method (7), more than 80% of BV positives remain asymptomatic(1). Hence, the important question is whether the many asymptomatic BV cases should be screened and treated. Clinical guidelines recommend screening and treatment for BV in patients undergoing gynecological surgery or invasive diagnostic procedures through the vagina to minimize infection(8). However, most clinical guidelines do not support screening and treatment for asymptomatic BV to optimize reproductive outcome – a topic which has been thoroughly investigated in obstetric populations for preterm birth prevention (9,10). Today, a new frontier is emerging with optimized molecular based diagnosis of bacterial dysbiosis and new treatment possibilities including well-studied and well-characterized probiotics – that have been designated “live biotherapeutic products” by FDA(11,12).

Haahr et al. (2016) reported the advantages of a molecular based diagnosis of vaginal dysbiosis in IVF patients(5). The main advantages were, i) a more objective diagnosis as microscopists had significant interrater variability with the prior gold standard, Nugent score, ii) dichotomization of the

Nugent intermediate group which was difficult to interpret clinically and, iii) the establishment of quantitative thresholds using key vaginal bacteria to detect IVF patients at risk of a poor reproductive outcome. Hence, a new terminology termed abnormal vaginal microbiota (AVM) was proposed for IVF patients (5,13). AVM was significantly associated with poor clinical pregnancy rates as compared to normal vaginal microbiota patients, 9% (2/22) versus 44% (27/62)(5). Later, these findings were corroborated by Koedooder et al. (6) who found clinical pregnancy rates of 6% (2/34) versus 42% (65/154) in patients with unfavorable and favorable vaginal microbiota, respectively.

In the field of reproductive medicine, there have been two different approaches to investigate the potential influence of the genital tract microbiota on IVF outcomes: either i) to directly investigate the endometrial microbiota by transcervical swabs/suctions (14–16) or ii) to investigate the vaginal microbiota as a proxy for the endometrial microbiota (5,6,17). The bacterial load in the uterus as compared to the vagina is very low (18), and for this reason the studies on endometrial microbiota have been criticized for reporting contamination from the transcervical sampling approach - and not a genuine endometrial microbiota. Nevertheless, endometrial samples from women undergoing hysterectomy provide evidence for a genuine endometrial microbiota (18,19) that seems to be highly influenced by the vaginal microbiota (19), especially in the case of BV where the odds of having endometrial colonization, including *Gardnerella vaginalis* biofilm infection, was significant as compared to normal vaginal microbiota patients: OR 5.7 (95% CI, 1.8–18.3, P = 0.002) (20). Several groups are developing or further optimizing molecular based approaches to diagnose IVF women at risk of poor reproductive outcomes caused by genital tract dysbiosis. However, only one study validated a molecular diagnostic approach in IVF women against the gold standard for vaginal dysbiosis – Nugent score of Gram stained vaginal smears(5). Two other studies applied arbitrary cut-offs for *Lactobacillus* dominance in the vaginal microbiota (6,17). Subsequently, these studies were criticized for insufficient methods (21,22), including the application of arbitrary thresholds based on

relative abundances which does not sufficiently take into account differences in the total abundance (21–23).

The recommended first-line treatments for BV are antibiotic therapy with either metronidazole or clindamycin as reported by the 2015 CDC (Center for Disease Prevention and Control) Sexually Transmitted Disease Guideline and the 2018 European IUSTI/WHO (International Union against Sexually Transmitted Infections) guideline. Clindamycin was reported to effectively eradicate BV-related bacteria in the endometrium of patients with endometritis (24), while it was also proven to enter the endometrial tissue in high concentrations if administered orally (25). In contrast, metronidazole was less effective against *Gardnerella vaginalis* both *in vivo* (26) and *in vitro* (24). Finally, a recent systematic review and meta-analysis reported that the use of additional probiotic treatment alongside standard treatment of BV could improve BV cure rates, RR = 1.28, 95% CI (1.05, 1.56) (27). However, due to primarily poor study quality(28), there is currently no consensus on which vaginal *Lactobacillus* product, if any, should be recommended (29).

The pioneering work by Ravel and colleagues (2011) established that the vaginal microbiota identified four *Lactobacillus* dominated community state types (CSTs) using taxonomic stratification at the species level, with each CST dominated by a different vaginal *Lactobacillus* species, or a diverse CST not dominated by *Lactobacillus (2)*. Although such stratification was based on hierarchical clustering and relative abundance – in contrast to absolute abundance, these CSTs have been adopted by the majority of researchers in the vaginal microbiome field. Consistently, publications have reported the *L. crispatus* CST to be associated with optimal genital health and reproductive outcomes (13,17,30–32). Moreover, abundant *in vitro* evidence point towards a beneficial production of both D and L lactic acid isomers by *L. crispatus* that not all other common vaginal lactobacilli produce (33,34). At the time of planning the present study, only one *L. crispatus* product, LACTIN-V, existed as an investigational live biotherapeutic product regulated by FDA – at

that time in Phase 2 development(11). Recently, adjuvant LACTIN-V after vaginal metronidazole was reported to lower BV recurrence rates in a phase 2b trial, RR 0.66 (95%CI 0.44-0.87)(35).

Taking into consideration the abovementioned evidence, the research question of the present study is: does antibiotic alone or in combination with live biotherapeutic treatment of an abnormal vaginal microbiota improve the reproductive outcomes of IVF patients? The intervention is clindamycin either alone or in combination with LACTIN-V, a live biotherapeutic product containing *L.crispatus* CTV-05 (11). The study is designed as a double-blind, placebo-controlled multicenter trial of three parallel groups randomized 1:1:1. Randomization is by computer generated code and allocation concealment is performed by the pharmacy who will send out medication to the participating clinics with identical appearance and randomization numbers. The randomization code is with the pharmacy and can only be opened in case of emergency by the principal investigators or as planned by the sponsor-investigator. The benefit of the intervention would potentially lead to increased pregnancy rates and, for those suffering from symptomatic BV, also relief of BV symptoms. In contrast, the expected adverse reactions of concern are especially gastrointestinal symptoms caused by clindamycin, whereas LACTIN-V might cause increased vaginal discharge but is otherwise not expected to cause adverse reactions as based on prior studies(11,36).

Methods and analysis

Setting and eligibility criteria

The present trial is conducted at four University affiliated clinics and one private fertility clinic in Denmark. The list of study sites is available with EudraCT clinical trial identifier: 2016-002385-31, first registration day 2016-07-11. The current version of the protocol is 9, 2020-02-07. Patients are

enrolled in a cohort study (Clinicaltrials.gov NCT03420859) from which we will recruit patients for the randomized trial (EudraCT: 2016-002385-31). Eligibility criteria are described in Table 1.

In brief, IVF patients attending their first, second or third IVF stimulation cycle or embryo transfer therefrom will be approached for informed consent by the study nurse or treating physician. Patients are told about the project in a private room with the right to have an assessor, allowing time to reflect whether they will participate. They are handed out written information material with a link to the study website with full information about the project – www.reproflor.dk. The vaginal swab can be taken by the treating physician or the patient herself after careful instruction. In this case, patients are instructed to place the swab at least 8 cm into the vaginal cavity for 10 seconds and rotate. This is to ensure that the vaginal bacteria in the fornix or in its close proximity will be caught by the flocked swab. Subsequently, the vaginal swab will be sent to a central laboratory at Statens Serum Institut, Copenhagen to be analyzed for AVM within 7 days as previously reported (5). If AVM positive, patients are asked to provide informed consent that they are willing to participate in the randomized controlled trial. Patients should ideally be randomized on the first day of ovarian stimulation with exogenous gonadotropins, allowing a minimum of 12 days of study medication to be acceptable for inclusion in the study. If elective frozen embryo transfer (FET) is planned, patients should be randomized during the first days of the FET cycle allowing for at least 12 days of study medication. If patients enter the trial and have less than 12 days of study medication despite the abovementioned inclusion criteria (e.g. when hormonal stimulation is shortened due to an unexpected ovarian response), it is considered a protocol violation and they will be excluded from the per protocol analysis, i.e. not from ITT-analysis.

Interventions

Active Treatment 1: **Oral Clindamycin** 300 mg 2 times per day for 7 days followed by **LACTIN-V** (Osel, Inc.) until completion of the clinical pregnancy scan at week 7-9. LACTIN-V containing *L.*

crispatus CTV-05 (2 x 10^9 CFU/dose, 200 mg, delivered with pre-filled, single use vaginal applicators) regimen is once daily from the clindamycin stop for 7 consecutive days.

Active Treatment 2: **Oral Clindamycin** 300 mg 2 times per day for 7 days followed by **LACTIN-V placebo** (Osel, Inc.) until completion of the clinical pregnancy scan at week 7-9. The LACTIN-V placebo regimen is once daily from the clindamycin stop for 7 consecutive days.

Inactive treatment (placebo): Matching **Clindamycin placebo** 2 times per day for 7 days followed by **LACTIN-V placebo** (Osel Inc.) until completion of the clinical pregnancy scan at week 7-9. LACTIN-V placebo regimen is once daily from clindamycin stop and the following 7 days.

If there are embryos to transfer (approx. 90% of patients), then LACTIN-V/placebo treatment is continued twice weekly until clinical pregnancy scan, however with a maximum of 21 applicators per patient. If the patient has no embryos to transfer or is confirmed not pregnant (negative hCG test), then LACTIN-V treatment can be stopped by the patient, albeit at least 7 days of LACTIN-V administration need to be administered. An overview of the study medication and allocation can be seen in Table 2. Patients are not allowed to take other antibiotics (unless medically indicated), probiotics, neuromuscular blocking drugs, immunosuppressive medication or investigational drug preparations other than the study product. Placebo clindamycin consists of encapsulated Mannitolum. The placebo LACTIN-V formulation contains the same inactive ingredients as LACTIN-V, without *Lactobacillus crispatus* CTV-05.

Labelling and packaging

Labelling and packaging of the medication are performed by Glostrup Pharmacy, Denmark in accordance with ICH-GCP guideline and EU GMP Annex 13. Patients are informed that it is important not to have penile-vaginal intercourse within 12 hours after LACTIN V application. Patient compliance will be measured by tablet counting of the medication packs (clindamycin). Patients who are not pregnant (negative HCG-test) and who decided to continue LACTIN-V treatment are

informed to contact the respective clinics in case of adverse events and these will be captured in the eCRF. If patients decide to end study product treatment, they are informed to contact the clinics and to deliver the unused LACTIN-V to the clinic at which point they would be asked about any adverse events. Study personnel will verify in the electronic Case Report Form (eCRF) what patients decided to do with remaining LACTIN-V applicators after a negative hCG test or no embryos for transfer.

Patients can withdraw their informed consent at any given time and without any reason according to Danish law. If available, the reason for discontinuation has to be stated in the eCRF. Moreover, in case of protocol deviations, this also has to be stated in the eCRF and the principal investigator should decide whether trial medication can continue or not. Furthermore, trial medication is stopped, should the patient develop hypersensitivity, allergy or severe diarrhea that a primary investigator suspects may be trial medication related. Vaginal swabs will be taken alongside treatment to monitor the vaginal microbiota and its response to treatment, see Table 3 and appendix 1. Specifically, vaginal swabs will be taken on the day of randomization immediately before study medication, after clindamycin treatment, on the day of embryo transfer and again on the day of clinical pregnancy scan. In a sub-study, a total of 20 patients will be asked to deliver vaginal samples for each day they take medication and the swab should be taken immediately before the medication on that specific day.

Outcomes

The primary outcome is the clinical pregnancy rate per first embryo transfer defined as ultrasound proven fetal heartbeat in gestational week 7-9. The secondary outcomes are the live birth rate per embryo transfer, biochemical pregnancy rate (hCG positive at 9-11 days after embryo transfer according to local laboratory standards), implantation rate, early miscarriage, late miscarriage, preterm birth rates, birth weight, and adverse effects of the medication through a safety analysis. The effect of treatment on the vaginal microbiota of the mother throughout study participation and potential pregnancy will be determined using quantitative PCR (qPCR) and next generation

sequencing methods. The colonization of the *L. crispatus* CTV-05 strain will also be investigated using qPCR. It is pre-planned that reproductive outcome analysis will lead to a first publication by itself, whereas the more laborious sequencing results will arrive in a later publication. Later, we plan to investigate cumulative live birth results of subsequent transfer of spare frozen thawed embryos of patients attending the study in a fresh cycle.

Sample size

In 2014, the average clinical pregnancy rate per embryo transfer in our fertility clinic was approximately 40% for an IVF cycle. In our pilot study(5), the adjusted odds ratio between the AVM group and the normal group was 0.06, 95% CI (0.01–0.47) for clinical pregnancy per embryo transfer. Taken together, we estimated a superiority design where women in each AVM arm and treated with active medication will have at least a 40% chance for clinical pregnancy per embryo transfer as compared to the placebo arm which was estimated to have a maximum of 20% chance of clinical pregnancy/transfer. By two samples proportion test with a power of 80% and an alpha at 5%, the aim was to randomize 92 patients in each group. A potential difference between the two active arms was considered exploratory and consequently this was not part of the power calculation, but we decided to include the same number of patients in the active/active arm to investigate a potential added benefit of live biotherapeutic treatment.

An interim analysis will be performed, and to adjust for this, we add 10% to the 92 randomized patients as suggested in Wittes et al (37). Approximately 10% of couples will have no embryos for transfer; we adjusted for this by adding another 10% to each randomized group, i.e. 19 + 92 =111 (see Figure 1). Considering an estimated 20% AVM rate, a total of 1850 IVF patients will be screened to randomize 333 patients (three arms). It was estimated that inclusion will be distributed according to the size of the centers. Furthermore, we make the following assumptions: i) very limited loss to follow-up, ii) near full compliance to study medication and iii) homogeneity in the treatment effect.

Allocation

Randomization is performed by Glostrup pharmacy by a computer-generated code (www.randomization.com). The medication packs labelled with the randomization number are received at the IVF centers from the pharmacy in blocks of 15, five of each of the three treatments, to secure equal distribution of treatment arms at the centers. The medication has identical appearance and only the randomization number differ, hence both patients and study personnel are blinded for the intervention. A block of 15 medication packs will be sent from the pharmacy from start of study and new blocks can be requested when 5 medication packs are left. The 15 medication packs are mixed and appear identical to both personnel and patients. The randomization number is continuous and unique for each patient, starting from 1 to 333 and the number is prelabelled from the pharmacy before distribution to the clinics. The last 3 medication packs from 331-333 is also one block.

The randomization list is secured by the pharmacy throughout the trial, and only the sponsor has the authority to unblind the trial. However, in case of medical emergency the principal investigator (PI) can call the pharmacy to unblind. Each participant's medication package is labeled with a randomization number that is linked to their study ID number in the eCRF. Although both patients and clinicians will be blinded to allocation, they may suspect active medication in case of side-effects. This small risk of bias seems to be unavoidable. However, to investigate such an effect, patients are asked if they believed that they received active or inactive medication.

Data collection methods

Study data are collected and managed using REDCap (Research Electronic Data Capture) electronic data capture tools hosted at Aarhus University, Denmark(38,39). REDCap is a secure, web-based software platform designed to support data capture for research studies, providing 1) an intuitive interface for validated data capture; 2) audit trails for tracking data manipulation and export

procedures; 3) automated export procedures for seamless data downloads to common statistical packages; and 4) procedures for data integration and interoperability with external sources. All data collectors of the study have to be trained in Good Clinical Practice (GCP) procedures and as minimum to have passed the course provided by the Danish GCP institution. All inclusion and exclusion criteria as well as outcome data will be monitored by external GCP monitors to ensure optimal data quality. Data collection forms and other data entry related information can be requested from the corresponding author.

Protocol deviations have to be stated in the eCRF. Loss to follow-up is unlikely for patients in IVF treatment who will be highly motivated to come to the clinic. However, patients who are not pregnant may opt to go to other clinics for further treatment and, thus will be lost to follow-up. The eCRF instruments have range checks and other data rules that have to be passed to ensure optimal data input. In case of missing outcome data we plan to use the framework proposed by White et al (2011)(40).

Statistical methods

The total significance level of the study was set to be 5%. Based on the O'Brien-Fleming method, the total significance was split into 0.1% for the interim and 4.9% for the final analysis (37). Therefore, a p-value with 99.9% confidence interval is calculated in the interim analysis to test the possible effect of one or both active treatment arms (combined or separately) on clinical pregnancy rate per embryo transfer (primary outcome) compared to placebo. A Walds Chi-square test for possible effect will be conducted comparing all 3 arms. Moreover, four analyses: 1) active/active vs active/placebo, 2) active/placebo vs placebo/placebo, 3) active/active vs placebo/placebo, 4) average effect of active/active AND active/placebo vs placebo/placebo will be done as first a crude estimate and then secondly adjustment with confounders for double embryo transfer, quality of the embryo

(Cleavage/blastocyst), female age (continuous variable) and center effect (public/private). If the trial is discontinued according to the criteria stated under the paragraph "interim analysis", a full statistical analysis will be made as described below. First, a Walds Chi-square test for possible effect of active treatment on clinical pregnancy rate [primary outcome] will be made across all 3 groups. Moreover, pairwise comparisons for the abovementioned 4 tests will be made with odds ratios (OR) and risk ratios (RR) and 94.9% confidence intervals (CI's) calculated from logistic and linear regressions models, taking abovementioned confounding factors into account. Analyses will be conducted at intention-to-treat (ITT) level defined as all randomized patients who have an embryo transfer following study treatment cycle, including also deferred/frozen embryo transfers due to e.g. OHSS risk. Patients are excluded from ITT analysis if they do not have embryos for transfer or in case embryo transfer is deferred to a later stage than actual study treatment which is approximately 9weeks total. Per protocol analysis will also be considered i.e. an analysis for patients having an embryo transfer as described above and not violating the protocol as described herein. Intention-to-treat is considered the primary analysis.

Interim analysis

An interim analysis as described above will be performed to evaluate the clinical pregnancy rate per embryo transfer when 167 patients have been randomized and completed the study for primary outcome evaluation. If study medication is affecting the clinical pregnancy rate statistically significant in either of the analyses, the trial will discontinue. Furthermore, the drop-out rate will be evaluated considering both the number of positive AVM declining to participate and the number of patients who drop-out after randomization. A drop-out rate above 20% will lead to discontinuation. External statisticians from Aarhus University, Denmark will conduct the interim analysis. Only a small study board, including sponsor and principal investigators will know the result of the interim

analysis. Sponsor-investigator make the decision to continue or discontinue the trial. The study will continue in case there is no statistical difference in either of the tests, drop-out rate is acceptable, and the logistical requirements to finish the study can be met within reasonable time considering e.g. expiry of study medication and time to recruit all patients. The time to undertake the interim analysis and the decision to continue or discontinue is approximately 3 weeks.

Data monitoring

Investigator(s)/institution(s) will be permitted direct access to source data/documents for trial-related monitoring, audits, IRB/IEC review, and regulatory inspection(s). Primary investigators only have access to patients from their own center in the eCRF. This study will be monitored by the Danish GCP units, primarily the GCP-unit at Aarhus University and GCP-unit Copenhagen University Hospital. Furthermore, this trial is open for audit and quality assurance by the Danish Medicines Agency as specified by Danish law.

Adverse events and reactions

Adverse events and adverse reactions will be registered in a questionnaire handed out by study personnel to the patient on the day of embryo transfer and on the day of the clinical pregnancy scan. In case there is no embryos for transfer, patients will be approached to answer the questionnaire either by email or at oocyte retrieval day. Patients who enter luteal phase stimulation (Duostim) or segmentation ("freeze-all") will use the same questionnaire on the oocyte retrieval day of the cycle where they have started study medication, corresponding to approximately 14 days of study medication. In the questionnaire, patients will also be asked to answer questions regarding gastrointestinal symptoms that might be related to the treatment with antibiotic clindamycin. Patients will be asked the same questionnaire concerning potential late occurring LACTIN-V related side-

effects on the day of the clinical pregnancy scan. Moreover, patients are asked if they have symptoms at all study visits and if these symptoms are considered adverse reactions they are recorded in the eCRF, including an adverse reaction judgement from the treating physician.

Serious adverse events (SAE)

At each center, primary investigators will report serious adverse events (SAE) to sponsor within 24 hours by email or phone. Sponsor ensures that all Suspected Unexpected Serious Adverse Reactions (SUSARs) that are fatal or life-threatening are recorded and reported to the Danish Medicines Agency and the scientific Ethics Committee as soon as possible and no later than 7 days after the sponsor became aware of such possible side effect. Within 8 days after a SUSAR has been reported, the sponsor must notify the Danish Medicines Agency and the Ethics Committee with all relevant information on the follow-up of any SUSAR that may occur. All other unexpected serious or suspected serious adverse reactions will be reported to the Danish Medicines Agency and the scientific Ethics Committee within 15 days after the sponsor become aware of these. An annual safety report regarding the trial participants will be performed, consisting of serious adverse event suspected to be related to the investigational drug will be submitted to Danish Medicines Agency and the Ethics Committee. At end of study, all adverse events and serious adverse events will be reported according to regulations in Denmark.

Ethics

Approvals from the Regional Scientific Ethical Committee, Central Denmark Region (M-2017-157-17), the Danish Data Protection Agency (1-16-02-790-17) and Danish Medicines Agency (2016-002385-31) were obtained prior to trial initiation December 7th, 2017. Danish law will be complied with regarding the handling of personal information. Protocol amendments will be provided to the

relevant parties, including the Regional Scientific Ethical Committees and Danish Medicines Agency. All protocol amendments have to be approved by the Danish Medicines Agency and the scientific ethical committee before taken into use. Logging of trial amendments is secured at both these institutions, the sponsor-investigator as well as updated at EudraCT. Patient confidentiality is ensured by data capture in REDCap™. All patients are covered by a public insurance in Denmark.

Access to data

Only the sponsor-investigator has full access to the dataset. The interim analysis will be performed by external statisticians at the local university according to the pre-set plan explained above. Primary investigators and statisticians may have access to data at the discretion of the sponsor-investigator. External parties can only gain access to trial data following establishment of a data handling agreement.

Dissemination

Positive, negative as well as inconclusive results will be published, aiming for high impact journals with full data transparency. Dissemination of results is ensured in clinical trial agreements between the participating institutions and Sponsors institution, Aarhus University, Denmark. The Vancouver guidelines for authorship will be followed.

Trial status

The first patient was screened December 7th, 2017. By September 7th, 2019 we had screened 533 patients and randomized 119 patients. Interim analysis is expected by March 2020. End of trial is expected to be summer 2021.

Patients and public involvement

Neither patients nor the public was directly involved in the planning of this trial.

Author Contributions

PH, JSJ, NU and TH were the primary writers and inventors of this protocol. PH is the sponsor-investigator. TP provided information on LACTIN-V and contributed to the study design and protocol development. TH, NlCF, AP, VH, ALM and HSN are primary investigators at the involved clinics and contributed to the protocol and amendments during the initiation phase of the study.

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Competing interests statement

PH, JSJ, NU, TP and TH are listed as inventors in an international patent application (PCT/US2018/040882), involving the therapeutic use of vaginal lactobacilli to improve IVF outcomes. TP is an employee of Osel, Inc. Not related to this trial, TH received honoraria for lectures from Ferring, IBSA, Besins and Merck. PH received unrestricted research grants from MSD, Merck, and Ferring as well as honoraria for lectures from MSD, Merck, Gedeon-Richter, Theramex, and IBSA. JSJ received speaker's fee from Hologic, BD, SpeeDx, and Cepheid and serves scientific advisory board of Roche Molecular Systems, Abbott Molecular, and Cepheid. NF received unrestricted research grant from Gedeon Richter and honoraria for lectures from Merck. HSN received unrestricted research grant from Ferring and honoraria for lectures from Merck, IBSA and Ferring.

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Table 1: In- and exclusion criteria

Inclusion criteria:	**Exclusion criteria:**
Abnormal vaginal microbiota as described above. The screening swab should be repeated if more than 3 months old at randomization day	HIV, Hepatitis B or C positivity.
First, second or third IVF stimulation cycle or embryo transfer therefrom.	HPV CIN 2 or higher.
BMI<35	Known or suspected hypersensitivity to clindamycin.
Informed consent.	Former or current inflammatory bowel disease
18-42 years old	Severe concomitant disease, including diabetes.
A maximum of 2 embryos to be transferred	Artificial heart valve
	Intrauterine malformations with operation indication as determined by treating physician (Polyps, Septum, fibroma)

Table 2: Study medication scheme

	Clindamycin "Alternova"	LACTIN-V™
Dose	300mg	200mg/2x10^9 CFU/applicator
Dose schedule	Two times per day minimum 6 hours interval. Max. 14 tablets	Before sleeping Max. 21 applicators
Allocation	Patients start medication at least 12 days prior to embryo transfer in a fresh or a frozen cycle	Patients start medication at least 12 days prior to embryo transfer in a fresh or a frozen cycle
Route of administration	Oral	Vaginal/topical
Treatment period	7 days	Once per day in 7 days followed by administration twice weekly until clinical pregnancy scan or confirmed not pregnant. In the event of negative hCG test (not pregnant), patients are, however, allowed to continue LACTIN-V treatment until all applicators have been used*.
Follow-up period in the present RCT	Clinical pregnancy scan 7-9 weeks later	Clinical pregnancy scan 7-9 weeks later
Medication permitted	All other than the below mentioned	All other than the below mentioned

Medication not permitted	Other antibiotics (unless medically indicated), probiotics, neuromuscular blocking drugs, immunosuppressive medication. Investigational drug preparations other than the study product.	Antibiotics (unless medically indicated), other probiotics and investigational drug preparations other than the study product.

* Patients not pregnant are informed to contact the department in case of any LACTIN-V related side-effect.

Table 3: Study timeline

	Enrolment	**Allocation**				
TIMEPOINT	**Max 3 months prior to allocation day**	**Minimum 12 days prior to embryo transfer**	***7 days later***	***Embryo transfer***	***Pregnancy scan***	***Gestional week 22, 37, after birth***
ENROLMENT:						
Eligibility screen	X					
Informed consent	X					
Vaginal swab	X					
Allocation		X				
INTERVENTIONS:						

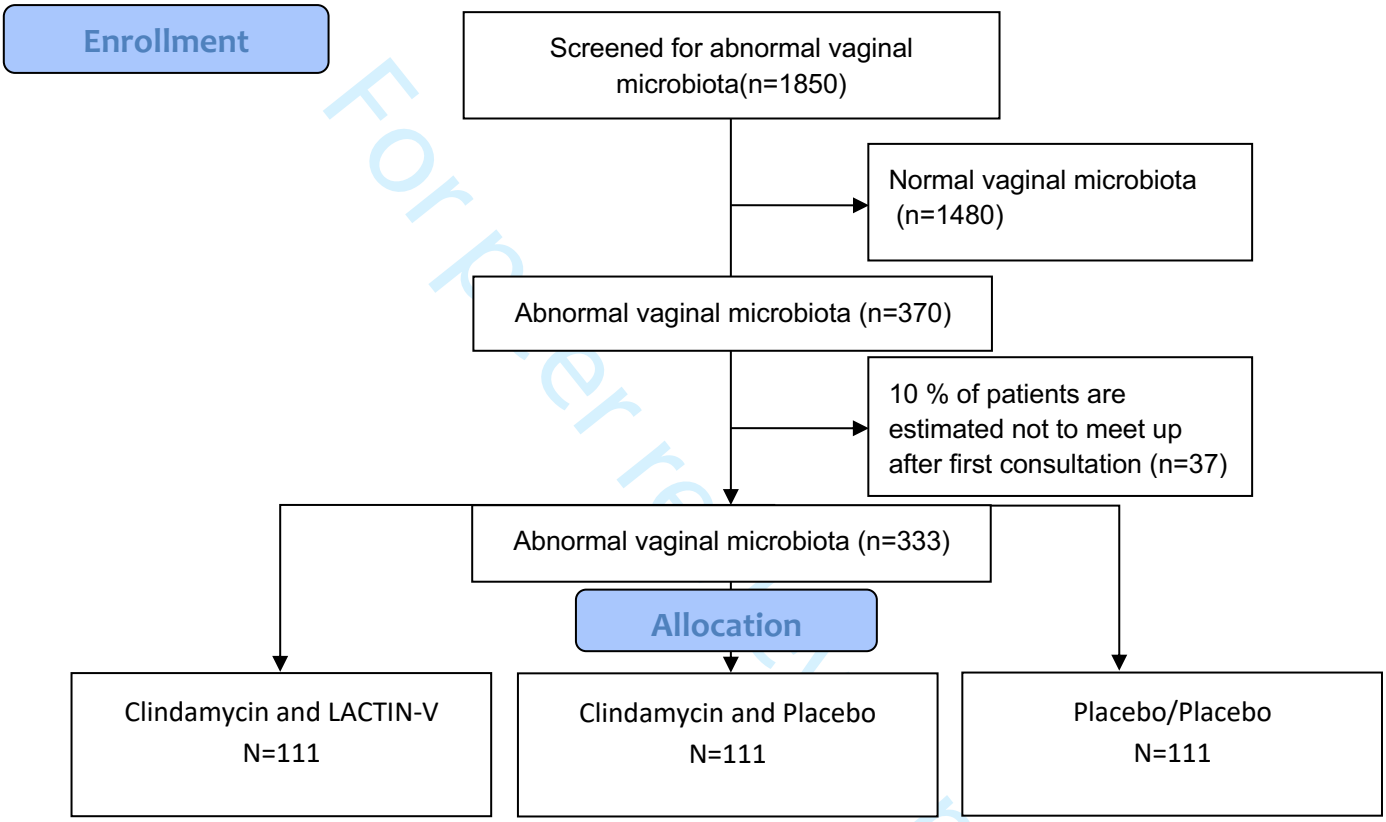
Clindamycin		X				
Lactin-V			X			
IVF treatment		X				
ASSESSMENTS:						
Vaginal swab		X	X	X	X	X

Figure 1: Study Flowchart

See figure attached.

Figure legend: We add 20% more patients to the powered sample size of 92 randomized patients to adjust for couples who have no embryos for transfer and to adjust for the interim analysis, i.e. 19 + 92 =111. Considering an estimated 20% AVM rate, a total of 1850 IVF patients will be screened to randomize 333 patients (three arms).

CONSORT 2010 Flow Diagram



Appendix 1

Samples will be collected from the vagina as outlined in table 3 and in the protocol. Moreover, we collect seminal samples from the partner at oocyte retrieval day and a fecal specimen from the newborn diaper within 3 weeks from birth. Vaginal swabs that are taken at the clinic are taken with a flocked swab and placed in Eswab (Copan™) except for the seminal sample, which is kept in a 1.8 mL cryotube, Nunc™, cryotube™, Thermo Scientific. The vaginal swabs taken at home in pregnancy after birth and the fecal swab are taken by the patient herself using flocked swabs and placed in an eNAT tube (Copan™). All the vaginal samples are taken after these instructions: the vaginal swab has to be the placed at least 8 cm into the vaginal cavity for 5 seconds and rotated clockwise. The fecal swab has to be a deep sample and has to be rotated in the feces for 5 seconds. The semen sample is collected after homogenization and then 200 microL are transferred to the cryotube using a pipette.

Only the vaginal swab at screening visit is sent immediately to the central laboratory at Statens Serum Institute for analysis within 7 days. All vaginal swabs taken during study visits and the seminal sample are immediately frozen at minus 80 degrees at the respective clinics. All samples are stored at the clinics until they are to be transferred by dry ice shipment to the central laboratory. Finally, the home-samples (vaginal samples and the fecal sample) are sent with return envelopes to the patients when they are supposed to take the sample and samples are received back at the Fertility Clinic in Skive, Denmark where they are stored at -80 degrees. For the sub-study explained in the protocol concerning the 20 patients taking daily vaginal home-samples (Copan ESwab™), these are kept in the patient's own freezer (-20 degrees) until embryo transfer day where the first 12-16 samples will be delivered to the clinic and stored at minus 80 degrees. The remaining 5-9 samples are kept in the patient's own freezer (-20) until her next visit which may be either hCG test day, clinical pregnancy scan or a visit during the subsequent cycle due to no pregnancy.

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

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	3
	2b	All items from the World Health Organization Trial Registration Data Set	2,8-15
Protocol version	3	Date and version identifier	8
Funding	4	Sources and types of financial, material, and other support	18
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	1,18
	5b	Name and contact information for the trial sponsor	1
	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	3,17,18
	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)	15

Introduction

Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	5-8
	6b	Explanation for choice of comparators	7
Objectives	7	Specific objectives or hypotheses	8
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)	8

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	8
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)	8-9
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	9
	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)	14
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	10,15
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	10
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	11

Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	table 3 p.24
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	11
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	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	8,12
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	8,12
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	8, 12
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	8,12
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	12,13
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	13,15

	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	13
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	13
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	15
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	15
	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)	15
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	15
	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	14,15
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	15,16
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	15
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	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)	17
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	8
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	N/A
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	17
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	19
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	17
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	17
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	17
	31b	Authorship eligibility guidelines and any intended use of professional writers	17
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	17
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	www.reproflor.dk
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	See appendix 1

*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.