PhD protocol 2

Modulating the Vaginal Microbiome after Implantation failure

A randomized placebo controlled study of vaginal lactobacilli supplements

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I. Setting

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II. Introduction and Background

Introduction, background and purpose

Infertility affects around 1 in 6 couples and is recognized as a public health issue by the World Health Organization. In 33% of couples no cause is found, but it is becoming clear that undiagnosed endometrial dysfunction may be a significant factor(Malchau *et al.*, 2017). IVF offers an effective treatment for many, but only 50% will achieve a pregnancy and 8-10% will suffer recurrent implantation failure (RIF) despite the transfer of high quality embryos (Koot *et al.*, 2012; Koot and Macklon, 2013). However, therapies aimed at assisting implantation or treating endometrial infertility remains largely empirical and ineffective (Hviid and Macklon, 2017). This reflects in part the lack of validated means of assessing and diagnosing endometrial functional defects. It is clear that several etiologies may underlie implantation failure and there is an urgent need to develop means of diagnosing specific endometrial functional abnormalities (Robertson *et al.*, 2016). Only then can targeted therapies be offered.

In recent years, a number of novel insights into factors governing endometrial function have emerged and provide potential new avenues for intervention. One putative marker of endometrial receptivity that has recently attracted interest is the profile of bacteria (termed the 'microbiome') present in the vagina or endometrium. Disruption of the vaginal microbiome by overgrowth of specific commensal strains has long been associated with symptoms of itching and discharge. The diagnosis of bacterial vaginosis continues to be based on the recognition of histological markers in vaginal swabs. (Mastromarino *et al.*, 2013). However in recent years, more sensitive and specific tests based on RNA identification of individual bacteria have enabled a more precise and quantitative assessment of the bacteria present. These technologies are shedding new light on the microbiome within different organs of the body and their impact on health (Lamont *et al.*, 2011). In the field of reproductive medicine, a close correlation has recently been reported between the vaginal and endometrial microbiome and the chance of conceiving through IVF (Hyman *et al.*, 2012; Moreno *et al.*, 2017) and our group is currently testing the hypothesis that disruption of the vaginal and endometrial microbiome may be associated with recurrent implantation failure after IVF treatment.

Recent studies have indicated that a vaginal microbiome consisting of < 90% lactobacilli is associated with failure of IVF treatment (Data submitted for publication, ART PRED, Amsterdam) However, it remains unclear whether interventions designed to alter the microbiome are effective in changing the suboptimal microbiome diagnosed with these new tests, or how stable any induced change in microbiome might be. These questions are of particular importance to women planning IVF treatment, as an effective treatment may alter their prognosis in subsequent cycles. A number of approaches have been proposed, such as treatment with antibiotics and the use of probiotics. Vivag Plus (Bifodan, Hundested, marketed by Orkla, Ishøj) is a vaginal probiotic capsule containing lactobacilli species effective in relieving symptoms of bacterial vaginosis (Lægemiddelstyrelsen, 2017). It is hypothesized that this probiotic may also be effective in restoring lactobacilli dominance in the vaginal microbiome in women shown to have a profile associated with implantation failure. To test this hypothesis, the following research questions will be addressed:

Research questions

1. Compared with a placebo vaginal capsule, does the application of a lactobacilli loaded capsule for 10 days change a lactobacillus poor or by predictive vaginal test otherwise unfavorable vaginal microbiome, to a lactobacillus rich or by predictive vaginal test otherwise favorable microbiome in the same menstrual cycle as intervention?
2. Is the change in the microbiome still present one month after the intervention?

From this, our primary hypothesis is that compared with placebo, use of lactobacillus loaded vaginal capsule is associated with a significant increase in the proportion of women who demonstrate a shift from unfavorable to favorable vaginal microbiome.

III. Methods

Study design and population

74 women diagnosed with suboptimal microbiome from either vaginal swabs or endometrial fluid sampling will be invited to participate in our double-blinded, randomized, controlled pilot study offering lactobacillus treatment to an intervention group of approximately 37 participants. The control group will receive placebo treatment with a placebo vaginal capsule. Information on medical and fertility history will be collected from patient journal after signed written consent from participants. Information on sexual life will be collected when participating in the study. Timing of last intercourse has shown to have an effect on the vaginal microbiome.

The Implantation Clinic

Since April 2017 Zealand University Hospital has been offering additional diagnostic work-up to women experiencing recurrent implantation failure (RIF= more than three consecutive transfers of high quality embryos). Patients demonstrating a suboptimal microbiome, will be invited to participate in the study. The results from the vaginal- and/or endometrial microbiome, are both part of the RIF-study and not part of diagnostic work-up. Information on microbiome from the RIF-study will be given to investigators in the present microbiome intervention study.

Additional subjects will be recruited from women attending the Fertility who have revealed a suboptimal vaginal microbiome following testing by their clinician and who meet the criteria for inclusion in the study.

The following flowchart gives an overview over the complete study.

Study flowchart

Figure 1: Overall study flow chart

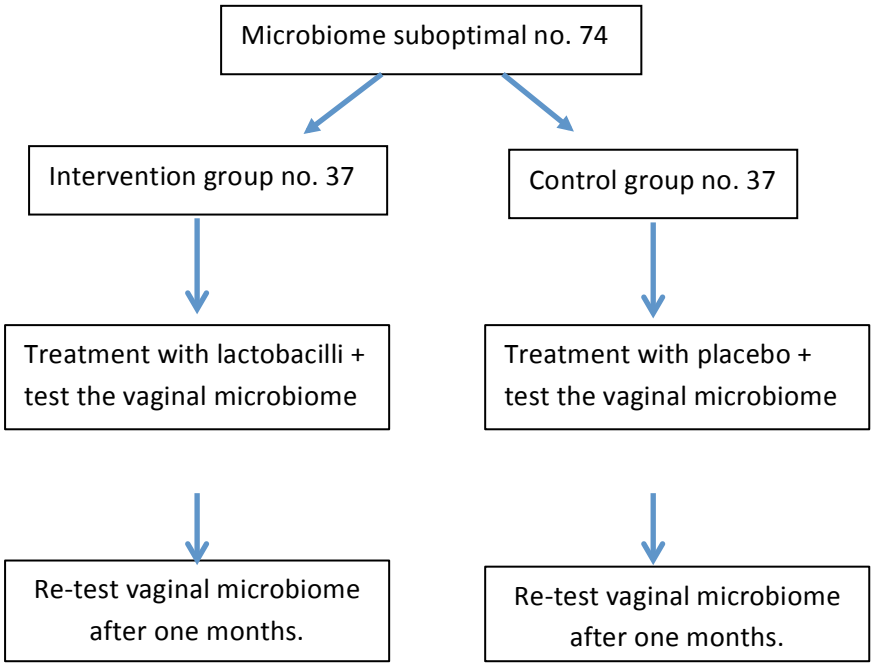
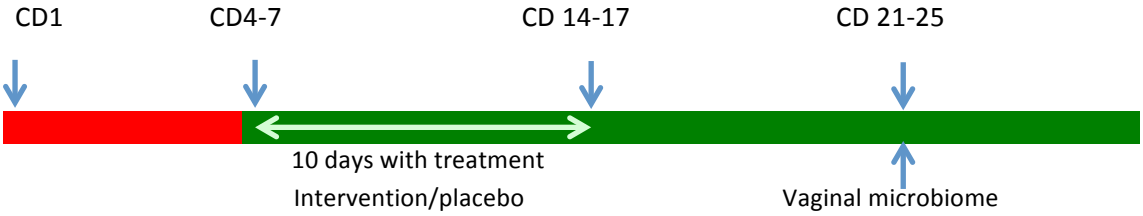


Figure 2

Cycle with intervention (CD=cycle day)



Cycle after intervention:

Re-test vaginal microbiome

Participant inclusion- and exclusion criteria

Patients diagnosed with suboptimal microbiome

- Age < 43 years
- Non-smoker
- Vaginal and/or endometrial microbiome suboptimal (<90% dominated lactobacilli)
- Attending Implantation Clinic, patient or control, and agreed to be contacted in case of other research study
- Patients attending fertility treatment in the Fertility Clinic in Køge and who have revealed a suboptimal microbiome following testing by their clinician, and who meet the criteria for inclusion in the study.

Exclusion criteria

- Age < 18 years
- Use of oral antibiotics between diagnose and inclusion
- Use of oral or vaginal probiotics with lactobacilli between diagnosis and inclusion
- Pregnant at enrollment, since inclusion will happen after menstruation, pregnancy is not possible at the time of inclusion
- Allergy towards any of the content in the tablets

Laboratory tests and examinations

1. Gynecological examination: At all visits, the participants will undergo gynecological examination.
2. Vaginal microbiome: During the examination, a swab with vaginal secretion will be taken. In collaboration with Artpred (Amsterdam, The Netherlands) the vaginal microbiome will be identified by deep sequencing of the IS region of the 16SRNA gene using validated protocols. Samples will be collected using special swabs in fornix posterior in the vagina. After sampling the material will be inserted in a specialized buffer and saved for deep sequencing and qPCR. IS diagnostics will perform analyses on behalf of ARTPred.

Side effects, risks, and disadvantages

	Side effects	Risks	Disadvantage
Gynecological examination	No	No	Requires presence
Vaginal swab	No	No	Requires presence
Placebo supplements	No	No	Must be applied vaginally, increased vaginal discharge may occur
Vivag Plus supplement	No side effects registered	No	Must be applied vaginally, increased vaginal discharge may occur

Security measures include, sampling only collected by clinicians trained in the procedure. Participants will be informed about the procedure by phone before coming to the clinic.

Tests will be taken before treatment, after treatment, and one months after intervention or placebo at cycle day 21-25.

Randomization

Study participants will be randomized after inclusion and written consent forms have been signed. The study will be double-blinded. The manufacturer will distribute the packages unlabeled to Region Hovedstadens Apotek. The randomization and labeling will be orchestrated by the pharmacy using a validated computer based randomization program. The clinician and the participant will be blinded to the content of the packages. All packages will contain a total of 10 vaginal capsules and duration of treatment will be ten days. The duration of treatment is based on two previous studies, describing prolonged cure rate after treatment for ten days(Stray-pedersen *et al.*, 2008; Pendharkar *et al.*, 2015).

Intervention: Lactobacillus treatment

The lactobacillus treatment will consist of Vivag Plus vaginal supplements. The vaginal capsules consist of cultures of *Lactobacillus gasseri* and *Lactobacillus rhamnosus* distributed and produced by Bifodan, approved for vaginal use and treatment of suboptimal vaginal bacteria. The vaginal capsule will be administered in the following dose; one tablet every night before sleep after menstruation, repeated for ten days. Each participant will receive a total of 10 vaginal capsules at inclusion. See figure above for details.

Placebo

Participants randomized to receive placebo, will receive supplements similar to the Vivag Plus, without the active substance; *Lactobacillus gasseri* and *Lactobacillus rhamnosus*. Placebo treatment is provided and developed by Bifodan. Placebo will be taken in the exact same dose as the intervention drug, Vivag Plus. Each participant will receive a total of 10 vaginal capsules at inclusion. See figure above for details.

Participants treated with oral antibiotics during the trial period will be excluded from the study. If pregnancy occurs during the study period, the participant will be followed through the rest of the study. The study drug or placebo is safe and harmless to use during pregnancy.

Active study drug

Content

Hard vaginal gelatine capsule, with more than 10^8 CFU of *Lactobacillus gasseri* EB01 DSM14869 and more than 10^8 CFU of *Lactobacillus rhamnosus* PB01 DSM14870.

Manufacturer

The manufacturer will produce, in anonymous packages according to good manufacturing practice (GMP), both active study drug and placebo.

Packaging, labeling, and handling of study drug

Bifodan A/S will produce the active study drug and placebo and distribute the drugs to the pharmacy. The Capital Region pharmacy will make packages, randomization, randomization envelopes, and distribute them to the study coordinator. The randomization envelopes will be used in case of the need to "de-code" an individual included participant. The study co-investigator will perform immediately de-coding for each participant when necessary, and without interference from the primary investigator. The text on the study drug will be made according to GMP. Distribution, inventory and return of excess medicine will be registered throughout the study. Participants will be asked to return the empty packages as well as excess medicine after treatment. They will be asked to bring the packages at the first visit after treatment. All handling of study drug will be done according to GMP. Please see labeling example in amendment.

Adverse reactions of the active intervention, Vivag Plus.

There are no known side effects or adverse reactions known to the study drug. See The Danish Summary of Product Characteristics, "produktresume".

Registration of adverse events and/or reactions

There will be continuous monitoring and registration of compliance to the intervention, throughout the study. Participants will be asked to bring any excess study drugs at their first visit to the clinic, after the intervention. All excess study drugs will be registered in the CRF. Excess study drugs, handed back to the clinic will be destroyed. If participants forget to bring the excess study drug, registration of compliance will be done according to the participant's memory. Rescue medicine is not applicable. Effect of treatment will be monitored throughout the primary and secondary end points. Throughout the study participants will be questioned about adverse events (AE=an unexpected medical event) and adverse reactions (AR= an unexpected/unintended response to a medical product) at every visit to the clinic. AE and AR will be registered in the trial clinical case reports and reported to the Sponsor within 24 hours.. All serious AE and serious AR (results in death, is life threatening, causes hospitalisation, or extension of hospital stay, results in disability or reduced incapacity) will be registered and investigator will report this to sponsor/principal investigator immediately and to the Danish Medicines Agency and the Ethical Committee, according to Lægemiddelloven §89. All serious AE will be assessed for causality before classified as a serious AR. Sponsor/principal investigator must also report suspected serious adverse reactions (SUSARs) immediately to The Danish Medicines Agency and ethics committee. All information regarding any SUSAR that is deadly or life threatening will be reported immediately and at the latest within 7 days after sponsor/principal investigator has been informed of the SUSAR. The sponsor/principal investigator reports all relevant information on sponsors and investigators follow up of the report within 8 days after the report. The sponsor must report all other SUSARs within 15 days of being informed of it. Every report should include comments on any implications and consequences for the trial.

The Danish Summary of Product Characteristics (Produktresume) is the reference document for evaluating whether a SAR is expected or suspected unexpected (SUSAR).

The ethics committee and the Danish Medicines Agency will receive yearly reports on security, and lists of serious AR. Reports will be send by the sponsor/principal investigator.

Statistical considerations, primary endpoint, study participants, and size

Within the clinical context in which IVF treatment is carried out, it is important to ascertain whether the modulation in microbiome caused by an intervention remains long enough to impact on the outcome of a subsequent treatment. In this regard, the key question becomes whether or not the impact of the intervention is still detectable one months after the intervention. The primary endpoint will therefore be the proportion of participants who demonstrate lactobacillus rich microbiome at one month follow up after randomization.
The planned size and duration of this study are based on the following considerations.

1. It is assumed that 80% of women randomized to the intervention will demonstrate a lactobacillus dominant vaginal microbiome on retesting later in the cycle, whereas 20% of those randomized to the placebo will do so, reflecting the impact of other modulating factors independent of the intervention.
2. Its assumed that subsequent spontaneous conversions between lactobacillus dominant and non-dominant profiles will occur in both groups at a similar rate, and it is estimated that this will occur in 20% of women.
3. Given these figures, after the intervention/placebo, it is estimated that 64% of those randomized to the intervention will show a lactobacillus dominant microbiome, one months following the intervention compared to 36% of those receiving the control intervention. In order to show this difference in proportions with 80% power with p value less than 0.05, 37 patients in in each arm will be required.
4. Previous studies have reported that around 40% of women embarking on IVF treatment have a lactobacillus poor microbiome and that this can be associated with a poor outcome. This percentage with this microbiome profile is therefore likely to be higher in the study population consisting largely of women with a history of repeated IVF failures. We therefore anticipate that with the current referral rate to the Implantation Clinic and from the patients undergoing treatment in our Fertility Clinic diagnosed with a sub-optimal microbome, 37 women and 37 controls can be recruited within a period of one year.

Differences between the intervention and control group in the primary endpoint will be tested for significance using the chi^2 test. The significance level will be set at a p-value less then 0.05.

Participants dropping out before the primary endpoint has been reached, will be replaced by additional recruits. However, it is anticipated that the drop-out will be <5%. Participants dropping out of the study at any point will be followed until the end of the study period for the individual participant. Participants failing to complete intervention with less then 50% of the study trial medicine, will be replaced.
The end of the study is defined as inclusion and test of the last participants one months after the first vaginal examination after intervention. See Figure 2. End of Trial for each participants will be the last visit to the clinic (third visit).

Study duration

Participant recruitment: September 3rd 2018 – December 31st 2019
Data Analysis: January 1st 2020 – February 1st 2020
Publication of results: March 1st 2020 – May 1st 2020

Biological material from participants

Microbiome material from the vagina

Size: One swab imbedded with bacteria from the top of the vagina.
Purpose: deep sequencing 16SRNA and qPCR from the bacteria to verify any suboptimal microbiome.
Analyzing: The vaginal swab will be send to be analysed abroad by ARTPred, in Holland. The samples will be stored and sent in a frozen state (-80 degrees or on dry ice respectively) in small batches of 10-30 samples. Storage of samples will take place in our fertility lab in Køge, where also sample collection will take place. Samples will be kept pseudo- anonymized and will be fully anonymized after the study is complete.
Analysis: ARTPred, The Netherlands will perform analyses. All samples will be sent pseudo-anonymized and in line with data protection legislation and the law of data protection.

Information from patient journals and research biobank

Information on medical and reproductive history will be collected from patient journal. All medical and fertility information from journals will be used within the study and in our Implantation Clinic. No personal information will be sent abroad. Samples sent will be identified with a participant number and date of sample collection. The participants will admit to use of journal medical- and reproductive history via consent forms. The Danish law on data protection will be followed.
A research biobank will be made to store the samples, between collection and analyses. Permission from the Data Protection Agency will be made simultaneously. All samples will be destroyed after analyses.

Data collection

Case report forms (CRF) are used to collect data and participant information regarding history of fertility treatment, menstrual cycle, age, BMI, last menstruation, sexual history, and results from tests. All in line with Good Clinical Practice (GCP). At every participant visit a CRF will be filled in electronically using "Easytrial", with the required information to meet the endpoints. An access to the above will be provided for inspection from the GCP-monitor, The Danish Medicines Agency or the Danish Data Protection Agency. Data collection will be performed in accordance with the guidelines of CONSORT, in order to achieve transparent reporting of trials. All collected CRF will be kept in Easytrial and will be deleted 5 years and one months after inclusion of the last participant. After this period of time, data will be anonymized.

IV. Quality Assurance

Helsinki Declaration
The study will be carried out in accordance with the Helsinki Declaration, EU Directive on GCP and ICH-GCP guidelines after approval by the Regional Ethics Committee, Danish Health and Medicines Agency and the Danish Data Protection Agency. The law of personal data treatment will be complied at all times.
All elements in the trial are planned and conducted in accordance with current Danish legislation and the ICH GCP Guidelines.

EudraCT
The study will be registered at EudraCT and Clinicaltrials.gov.

GCP monitor
The Danish Medicine Agency and a GCP monitor from The GCP Unit at Copenhagen University will monitor the project and make sure the study comply with applicable legislation as stated above.
The Sponsor/Principal Investigator will also monitor the study to do quality control.
In order to complete the above, the relevant authorities will be given access to the study participant's patient charts regarding fertility, and all other study material. The study participants will be well-informed about this before joining the study.

V. Ethical Considerations

Ethical considerations
The patients in this study will already have undergone the initial work-up leading to the diagnosis suboptimal microbiome. At present no treatment has been shown to effectively treat or change the unfeasible microbiome present in the vagina and/or endometrium. Studies show that a *Lactobacillus* loaded environment in the female reproductive tract is important in order to have successful artificial reproductive treatment. The tests performed in this study are non-invasive and can be performed during fertility treatment, however our participants will be advised not to undergo fertility treatment during the study period, because of the hormonal effect from fertility treatment on the bacteria in the vagina. Clouding the results collected.

Recruiting participants and informed consent
Participants undergoing endometrial profiling in our Implantation Clinic and diagnosed with suboptimal vaginal and/or endometrial microbiome will be invited to participate, when they are informed about their work-up in the clinic. Investigation of the vaginal microbiome will be offered to patients undergoing fertility treatment in our Fertility Clinic. The vaginal swab will be performed at a routine visit in the clinic. This will provide the patients and the clinician with a prediction of the outcome of fertility treatment. Patients with a sub-optimal microbiome will hereafter be invited to participate in the present randomized controlled study. This can be done by phone or at a planned consultation. Before enrollment the participants will receive study information and "Participants rights when participating in a research project" by email or handed out during consultation. Hereafter, a consultation will be performed where the oral information will take place and a printed study information

and “Participants rights when participating in a research project” will be given. The oral information will be given according to guidelines in the field. See attached material. The oral information will be given by PhD-student Malene Meisner Hviid or health personal who have received the proper training and education. The consultation will take place in one of the clinics assigned rooms and behind closed doors. Before the participants consultation in the clinic they are informed about their right to an assessor. The informed consent will be collected after a suitable time of consideration.

Submission of results

All results, positive, negative and inconclusive will be registered and available from clinicaltrialsregister.eu or clinicaltrials.gov.

The principal investigator will submit the results in EudraCT (European Clinical Trials Database) after the study is complete (within one year).

Scientific ethical statement

It is our believe that results collected after completion of this study will exceed the possible side effects and risks connected to participation in the study. In addition to this, obtaining new knowledge in the field will help and guide women in the future suffering from suboptimal vaginal and/or endometrial microbiome. Without the possibility to conduct research trials of this character, no progress in the research field on microbiome in the female reproductive tract will be made.

Risk assessment and alternative approaches/plans

The primary risks of this pilot randomized controlled study reside in achieving the numbers of participants desired in order to complete the study.

VI. Financial Aspects

Economics

Professor Nick Macklon initiated the project with funds from “Future Research Fonds” receiving Ph.D salary for three years, 1,500,000.00 mil DKK and Savværksejer Jeppe Juhls og Hustrus Ovita Juhls legat, 250.000 DKK.

ARTPred in the Netherlands provide the analysis of bacterial swaps, 100 euro pr. test. Orkla Care will provide the vaginal supplements Vivag Plus and the Placebo supplements, produced by Bifodan. Professor Nick Macklon and PhD-student Malene Meisner Hviid have no financial interest in the companies supporting the project with analysis and treatment.

Compensation to participants

No financial support will be given to participants in this double-blinded randomized controlled study.

Relevant clauses in the contract

There are no clauses made between scientists and companies involved in this protocol.

Insurance

All participants in the trial are covered under “Patient insurance.”

VII. References

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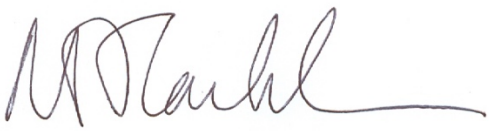
VII. Signature

Sponsor/ Principal investigator

07/08/2018



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