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# 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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Complete List of Authors:	Haahr, Thor; Aarhus Universitet, Department of Clinical Medicine Freiesleben, Nina; Copenhagen University Hospital, Hvidovre Hospital, Department of Obstetrics and Gynaecology, The Fertility Clinic Pinborg, Anja; Rigshospitalet, University of Copenhagen, Fertility Clinic Nielsen, Henriette; Copenhagen University Hospital, Fertility Clinic Hartvig, Vibeke; Stork Fertility Clinic Mikkelsen, Anne-Lis; Sjællands Universitetshospital Køge, The Fertility Clinic Parks, Thomas; Osel inc Uldbjerg, Niels; Aarhus Universitetshospital, Department of Obstetrics and Gynecology Jensen, Jørgen; Statens Serum Institut, Mycoplasma Laboratory Humaidan, Peter; The Fertility Clinic, Skive Regional Hospital
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4 1 **Title:** A protocol for a double-blind, placebo-controlled multicenter trial on the effect of clindamycin  
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6 2 and a live biotherapeutic on the reproductive outcomes of IVF patients with abnormal vaginal  
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8 3 microbiota  
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11  
12 4 **Authors:** Thor Haahr<sup>1\*</sup>, Nina la Cour Freiesleben<sup>2</sup>, Anja Pinborg<sup>3</sup>, Henriette Svarre Nielsen<sup>3</sup>, Vibeke  
13  
14 5 Hartvig<sup>4</sup>, Anne-Lis Mikkelsen<sup>5</sup>, Thomas Parks<sup>6</sup>, Niels Ulbjerg<sup>7</sup>, Jørgen Skov Jensen<sup>8</sup>, Peter  
15  
16 6 Humaidan<sup>1</sup>.  
17

18 7  
19 8 <sup>1</sup>Department of Clinical Medicine, Aarhus University, Denmark and the Fertility Clinic Skive, Skive  
20  
21 9 Regional Hospital, Denmark  
22

23 10 <sup>2</sup>The Fertility Clinic, Department of Obstetrics and Gynecology, Hvidovre Hospital, Copenhagen  
24  
25 11 University Hospital, Denmark  
26

27 12 <sup>3</sup>Fertility Clinic, Rigshospitalet 4071, Copenhagen University Hospital, Copenhagen, Denmark  
28

29 13 <sup>4</sup>Stork Fertility Clinic, Copenhagen, Denmark  
30

31 14 <sup>5</sup>Fertility Clinic, Zealand University Hospital, Denmark.  
32

33 15 <sup>6</sup>Osel, Inc., Mountain View, CA, United States  
34

35 16 <sup>7</sup>Department of Obstetrics and Gynecology, Aarhus University Hospital, Skejby, Denmark  
36

37 17 <sup>8</sup>Statens Serum Institute, Research Unit for Reproductive Microbiology, Copenhagen, Denmark  
38

39 18  
40 19 Trial sponsor:

41 20 Professor Peter Humaidan  
42

43 21 Address: The Fertility Clinic, Skive Regional Hospital, Resenvej 25, 7800 Skive, Denmark  
44

45 22 Phone: +45 78445760  
46

47 23 \*Corresponding author: [thohaa@rm.dk](mailto:thohaa@rm.dk)  
48

49 24 Address: The Fertility Clinic, Skive Regional Hospital, Resenvej 25, 7800 Skive, Denmark  
50

51 25 Phone: +45 78445760  
52

53 26 <https://orcid.org/0000-0001-9304-5299>  
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4 **28 Abstract:**

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7 **29 Introduction**

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9 **30** Recent studies in IVF patients have associated abnormal vaginal microbiota with poor clinical  
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11 **31** pregnancy rates of 6-9% per embryo transfer. The biological plausibility for this finding is  
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13 **32** hypothesized to be ascending infection to the endometrium which in turn hampers embryo  
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15 **33** implantation. The prevalence ranges from 9-28%. The important question is whether screening and  
16  
17 **34** treatment of abnormal vaginal microbiota would improve reproductive outcomes in IVF patients.  
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19 **35** Herein, we describe a protocol for an ongoing double-blind, placebo-controlled multicenter trial of  
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21 **36** IVF patients with abnormal vaginal microbiota randomized in three parallel groups 1:1:1.  
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25 **37**  
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27 **38 Methods and analysis**

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29 **39** This is a drug intervention study where IVF patients will be screened for abnormal vaginal  
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31 **40** microbiota, using a qPCR assay targeting *Atopobium vaginae* and *Gardnerella vaginalis*. If positive,  
32  
33 **41** patients will be randomized to one of the three study arms. The first arm consists of clindamycin  
34  
35 **42** 300mg x2 daily for 7 days followed by topical *Lactobacillus crispatus* CTV-05 until clinical  
36  
37 **43** pregnancy scan week 7-9. The second arm consists of clindamycin and placebo *Lactobacillus*  
38  
39 **44** *crispatus* CTV-05, whereas patients in the third arm will be treated with placebo/placebo. We used a  
40  
41 **45** superiority design to estimate that active treatment in both arms will increase the primary outcome,  
42  
43 **46** clinical pregnancy rate per transfer, from 20% to 40%. A potential difference between the two active  
44  
45 **47** arms was considered exploratory. With a power of 80% and an alpha at 5%, the sample size is  
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47 **48** estimated to be 333 patients randomized. A pre-planned interim analysis is scheduled at 167 patients  
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49 **49** randomized.  
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53 **50**  
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55 **51 Ethics and dissemination**  
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4 52 All patients have to give informed consent. Dissemination of results is ensured in clinical trial  
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6 53 agreements whether they be positive or not.  
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11 55 Registration

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13 56 This trial is registered in all relevant national agencies as well as in EudraCT with registration number  
14  
15 57 2016-002385-31. Moreover, the trial is ICH-GCP monitored.  
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20 59 **Keywords: IVF, Microbiota, Bacterial vaginosis, Clindamycin, Gardnerella, RCT**  
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4 60 **Article summary: ‘Strengths and limitations of this study’**  
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- 8 61 • Diagnosis of abnormal vaginal microbiota was validated in pilot studies  
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10 62 • The first RCT in IVF patients with abnormal vaginal microbiota investigating treatment effect  
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12 63 on reproductive outcome of clindamycin and live lactobacillus treatment  
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15 64 • The *Lactobacillus crispatus* CTV-05 treatment is an investigational live biotherapeutic  
16  
17 65 product regulated by the US FDA  
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19 66 • Inclusion criteria are relatively broad  
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## 67 Introduction

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



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4 91 interpret clinically and iii) the establishment of quantitative thresholds using key vaginal bacteria to  
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6 92 detect IVF patients at risk of a poor reproductive outcome. Hence, a new terminology termed  
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9 93 abnormal vaginal microbiota (AVM) was proposed for IVF patients (5). AVM was significantly  
10  
11 94 associated with poor clinical pregnancy rates as compared to normal vaginal microbiota patients, 9%  
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13 95 (2/22) versus 44% (27/62) (5). Later, these findings were corroborated by Koedooder et al. (6) who  
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16 96 found clinical pregnancy rates of 6% (2/34) versus 42% (65/154) in patients with unfavorable and  
17  
18 97 favorable vaginal microbiota, respectively.

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20 98 In the field of reproductive medicine, there have been two different approaches to investigate the  
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23 99 potential influence of the genital tract microbiota on IVF outcomes: either i) to directly investigate  
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25 100 the endometrial microbiota by transcervical swabs/suctions (13–15) or ii) to investigate the vaginal  
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27 101 microbiota as a proxy for the endometrial microbiota (5,6,16). The bacterial load in the uterus as  
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30 102 compared to the vagina is very low (17), and for this reason the studies on endometrial microbiota  
31  
32 103 have been criticized for reporting contamination from the transcervical sampling approach - and not  
33  
34 104 a genuine endometrial microbiota. Nevertheless, endometrial samples from women undergoing  
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36 105 hysterectomy provide evidence for a genuine endometrial microbiota (17,18) that seems to be highly  
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39 106 influenced by the vaginal microbiota (18,19). Several groups are developing or further optimizing  
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41 107 molecular based approaches to diagnose IVF women at risk of poor reproductive outcomes caused  
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43 108 by genital tract dysbiosis. However, only one study validated a molecular diagnostic approach in IVF  
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46 109 women against the gold standard for vaginal dysbiosis – Nugent score of Gram stained vaginal smears  
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48 110 (5). Two other studies applied arbitrary cut-offs for *Lactobacillus* dominance in the vaginal  
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50 111 microbiota (6,16). Subsequently, these studies were criticized for insufficient methods (20,21),  
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53 112 including the application of arbitrary thresholds based on relative abundances which does not  
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55 113 sufficiently take into account differences in the total abundance bacteria (20–22).

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4 114 The recommended first-line treatments for BV are antibiotic therapy with either metronidazole or  
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7 115 clindamycin as reported by the 2015 CDC (Center for Disease Prevention and Control) Sexually  
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9 116 Transmitted Disease Guideline and the 2018 European IUSTI/WHO (International Union against  
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11 117 Sexually Transmitted Infections) guideline. Clindamycin was reported to effectively eradicate BV-  
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14 118 related bacteria in the endometrium of patients with endometritis (23), while it was also proven to  
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16 119 enter the endometrial tissue in high concentrations if administered orally (24). In contrast,  
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18 120 metronidazole was less effective against *Gardnerella vaginalis* both *in vivo* (25) and *in vitro* (23).  
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21 121 Finally, a recent systematic review and meta-analysis reported that the use of additional probiotic  
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23 122 treatment alongside standard treatment of bacterial vaginosis could improve BV cure rates,  
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25 123 RR = 1.28, 95% CI (1.05, 1.56) (26). However, currently there is no consensus on which vaginal  
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27 124 *Lactobacillus* product should be recommended.

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30 125 The pioneering work by Ravel and colleagues (2011) established that the vaginal microbiota is  
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32 126 heterogeneous and four *Lactobacillus* dominated community state types (CSTs) can be identified  
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34 127 using taxonomic stratification at the species level, with each CST dominated by a different  
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36 128 *Lactobacillus* species or being a diversity CST not dominated by *Lactobacillus* (2). Although such  
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39 129 stratification was based on hierarchical clustering and relative abundance – in contrast to absolute  
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41 130 abundance, these CSTs have been adopted by the majority of researchers in the vaginal microbiome  
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43 131 field. Consistently, publications have reported the *L. crispatus* CST to be associated with optimal  
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45  
46 132 genital health and reproductive outcomes (16,27–30). Abundant *in vitro* evidence point towards a  
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48 133 beneficial production of lactic acid isomers that seem to be unique for *L. crispatus* as compared to  
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50 134 other common vaginal lactobacilli (31,32). At the time of planning the present study, only one *L.*  
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52 135 *crispatus* product, LACTIN-V, existed as an investigational live biotherapeutic product regulated by  
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55 136 FDA – at that time in Phase 2 development (11).

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4 138 Taking into consideration the abovementioned evidence, the research question of the present study  
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6 139 is: does antibiotic alone or in combination with live biotherapeutic treatment of an abnormal vaginal  
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9 140 microbiota improve the reproductive outcomes of IVF patients? The intervention is clindamycin  
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11 141 either alone or in combination with LACTIN-V, a live biotherapeutic product containing *L.crispatus*  
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13 142 CTV-05 (11). The study is designed as a double-blind, placebo-controlled multicenter trial of three  
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16 143 parallel groups randomized 1:1:1. Randomization is by computer generated code and allocation  
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18 144 concealment is performed by the pharmacy who will send out medication to the participating clinics  
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20 145 with identical appearance and randomization numbers. The randomization code is with the pharmacy  
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23 146 and can only be opened in case of emergency by the principal investigators or as planned by the  
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25 147 sponsor-investigator. The benefit of the intervention would potentially lead to increased pregnancy  
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27 148 rates and, for those suffering from symptomatic BV, also relief of BV symptoms. In contrast, the  
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30 149 expected adverse reactions of concern are especially gastrointestinal symptoms caused by  
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32 150 clindamycin, whereas LACTIN-V might cause increased vaginal discharge but is otherwise not  
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34 151 expected to cause adverse reactions as based on prior studies (11,33).

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39 153 **Methods and analysis**

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41 154 *Setting and eligibility criteria*

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43 155 The present trial is conducted at four University affiliated clinics and one private fertility clinic in

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46 156 Denmark. The list of study sites is available with EudraCT clinical trial identifier: 2016-002385-31,

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48 157 first registration day 2016-07-11. The current version of the protocol is 7, 2019-10-10. Patients are

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50 158 enrolled in a cohort study (Clinicaltrials.gov NCT03420859) from which we will recruit patients for

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53 159 the randomized trial (EudraCT: 2016-002385-31). Eligibility criteria are described in Table 1.

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55 160 In brief, IVF patients attending their first, second or third IVF stimulation cycle or embryo transfer

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57 161 will be approached for informed consent by the study nurse or treating physician. Patients are told

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4 162 about the project in a private room with the right to have an assessor, allowing time to reflect whether  
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6 163 they will participate. They are handed out written information material with a link to the study website  
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9 164 with full information about the project – [www.reproflor.dk](http://www.reproflor.dk). The vaginal swab can be taken by the  
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11 165 treating physician or the patient herself after careful instruction. In this case, patients are instructed  
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13 166 to place the swab at least 8 cm into the vaginal cavity for 5 seconds and rotate clockwise. This is to  
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16 167 ensure that the vaginal bacteria in the fornix or in its close proximity will be caught by the flocked  
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18 168 swab. Subsequently, the vaginal swab will be sent to a central laboratory at Statens Serum Institut,  
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20 169 Copenhagen to be analyzed for AVM within 7 days as determined in a previous study (5). If AVM  
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22  
23 170 positive, patients are asked to provide informed consent that they are willing to participate in the  
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25 171 randomized controlled trial. Patients should ideally be randomized on the first day of ovarian  
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27 172 stimulation with exogenous gonadotropins, allowing a minimum of 12 days of study medication to  
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30 173 be acceptable for inclusion in the study. If elective frozen embryo transfer (FET) is planned, patients  
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32 174 should be randomized during the first days of the FET cycle allowing for at least 12 days of study  
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34 175 medication.

### 36 176 *Interventions*

39 177 Active Treatment 1: **Oral Clindamycin** 300 mg 2 times per day for 7 days followed by **LACTIN-V**  
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42 178 (Osel, Inc.) until completion of the clinical pregnancy scan at week 7-9. LACTIN-V (2 x 10<sup>9</sup>  
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44 179 CFU/dose, 200 mg, delivered with pre-filled, single use vaginal applicator) regimen is once daily  
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46 180 from the clindamycin stop for 7 consecutive days.

49 181 Active Treatment 2: **Oral Clindamycin** 300 mg 2 times per day for 7 days followed by **LACTIN-V**  
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51 182 **placebo** (Osel, Inc.) until completion of the clinical pregnancy scan at week 7-9. The LACTIN-V  
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54 183 placebo regimen is once daily from the clindamycin stop for 7 consecutive days.

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184 Inactive treatment (placebo): Matching **clindamycin placebo** 2 times per day for 7 days followed by

185 **LACTIN-V placebo** (Osel Inc.) until completion of the clinical pregnancy scan at week 7-9.

186 LACTIN-V placebo regimen is once daily from clindamycin stop and the following 7 days.

187 If there are embryos to transfer (90% of patients), then LACTIN-V/placebo treatment is continued

188 twice weekly until clinical pregnancy scan, however with a maximum of 21 applicators per patient.

189 If the patient has no embryos to transfer or is confirmed not pregnant (negative hCG test), then

190 LACTIN-V treatment can be stopped, albeit at least 7 days of LACTIN-V administration need to be

191 administered. An overview of the study medication and allocation can be seen in Table 2. Patients

192 are not allowed to take other antibiotics (unless medically indicated), probiotics, neuromuscular

193 blocking drugs, immunosuppressive medication or investigational drug preparations other than the

194 study product.

### 195 *Labelling and packaging*

196 Labelling and packaging of the medication are performed by Glostrup Pharmacy, Denmark in

197 accordance with ICH-GCP guideline and EU GMP Annex 13. Patients are informed that it is

198 important not to have penile-vaginal intercourse within 12 hours after LACTIN V application. Patient

199 compliance will be measured by tablet counting of the medication packs (clindamycin). Any unused

200 LACTIN-V applicators should be delivered to the clinics, otherwise they are considered used.

201 Patients can withdraw their informed consent at any given time and without any reason according to

202 Danish law. The reason for discontinuation has to be stated in the electronic Case Report Form

203 (eCRF). Moreover, in case of protocol deviations, this also has to be stated in the eCRF and the

204 principal investigator should decide whether trial medication can continue or not. Furthermore, trial

205 medication is stopped, should the patient develop hypersensitivity, allergy or severe diarrhea that a

206 primary investigator suspects may be trial medication related. Vaginal swabs will be taken alongside

207 treatment to monitor the vaginal microbiota and its response to treatment, see Table 3 and appendix

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4 208 1. Specifically, vaginal swabs will be taken on the day of randomization immediately before study  
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6 209 medication, after clindamycin treatment, on the day of embryo transfer and again on the day of  
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9 210 clinical pregnancy scan. A total of 20 patients will be asked to deliver vaginal samples for each day  
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11 211 they take medication and the swab should be taken immediately before the medication on that specific  
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14 212 day.

### 15 16 213 *Outcomes*

17  
18 214 The primary outcome is the clinical pregnancy rate per first embryo transfer defined as ultrasound  
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20 215 proven fetal heartbeat in gestational week 7-9. The secondary outcome are the live birth rate per  
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22 216 embryo transfer, biochemical pregnancy rate (hCG positive at 9-11 days after embryo transfer  
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24 217 according to local laboratory standards), implantation rate, early miscarriage, late miscarriage,  
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26 218 preterm birth rates, birth weight, and adverse effects of the medication through a safety analysis. As  
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28 219 part of the cohort study (Clinicaltrials.gov, NCT03420859), the effect of treatment on the vaginal  
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30 220 microbiota of the mother throughout pregnancy will be determined using qPCR and next generation  
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32 221 sequencing methods. Later, we plan to investigate cumulative live birth results of subsequent transfer  
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34 222 of spare frozen thawed embryos of patients attending the study in a fresh cycle.  
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### 40 223 *Sample size*

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42 224 In 2014, the average clinical pregnancy rate per embryo transfer in our fertility clinic was  
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44 225 approximately 40% for an IVF cycle. In our pilot study (5), the unadjusted risk difference between  
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46 226 the AVM group and the normal group was 34% (95%CI 17-52%). This was a relatively large  
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48 227 confidence interval which was not biologically meaningful. Taken together, we estimated a  
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50 228 superiority design were women in each AVM arm treated with active medication will have at least a  
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52 229 40% chance for clinical pregnancy per embryo transfer as compared to the placebo arm which was  
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54 230 estimated to have a maximum of 20% chance of clinical pregnancy/transfer. By two samples  
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56 231 proportion test with a power of 80% and an alpha at 5%, the aim was to randomize 92 patients in each  
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4 232 group. A potential difference between the two active arms was considered exploratory and  
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6 233 consequently this was not part of the power calculation, but we decided to include the same number  
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9 234 of patients in the active/active arm to investigate a potential added benefit of live biotherapeutic  
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11 235 treatment.

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15 236 An interim analysis will be performed, and to adjust for this, we add 10% to the 92 randomized  
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17 237 patients as suggested in Wittes et al (34). Approximately 10% of couples will have no embryos for  
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19 238 transfer; we adjusted for this by adding another 10% to each randomized group, i.e.  $19 + 92 = 111$   
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21 239 (see Figure 1). Considering an estimated 20% AVM rate, a total of 1850 IVF patients will be screened  
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24 240 to randomize 333 patients (three arms). It was estimated that inclusion will be distributed according  
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26 241 to the size of the centers. Furthermore, we make the following assumptions: i) very limited loss to  
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28 242 follow-up, ii) near full compliance to study medication and iii) homogeneity in the treatment effect.

### 31 32 243 *Allocation*

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34 244 Randomization is performed by Glostrup pharmacy by a computer-generated code. The medication  
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36 245 packs labelled with the randomization number are received at the IVF centers from the pharmacy in  
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39 246 blocks of 15, five of each of the three treatments, to secure equal distribution of treatment arms at the  
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41 247 centers. The medication have identical appearance and only the randomization number differ, hence  
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43 248 both patients and study personnel are blinded for the intervention. A block of 15 medication packs  
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45  
46 249 will be sent from the pharmacy from start of study and new blocks can be requested when 5  
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48 250 medication packs are left. The 15 medication packs are mixed and appear identical to both personnel  
49  
50 251 and patients. The randomization number is continuous and unique for each patient, starting from 1 to  
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52  
53 252 333 and the number is prelabelled from the pharmacy before distribution to the clinics.

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56 253 The randomization list is secured by the pharmacy throughout the trial, and only the sponsor has the  
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58 254 authority to unblind the trial. However, in case of medical emergency the principal investigator (PI)

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4 255 can call the pharmacy to unblind. Each participant's medication package is labeled with a  
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6 256 randomization number that is linked to their study ID number in the eCRF. Although both patients  
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8  
9 257 and clinicians will be blinded to allocation, they may suspect active medication in case of side-effects.  
10  
11 258 This small risk of bias seems to be unavoidable. However, to investigate such an effect, patients are  
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14 259 asked if they believed that they received active or inactive medication.  
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### 17 260 *Data collection methods*

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19 261 Study data are collected and managed using REDCap (Research Electronic Data Capture) electronic  
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21  
22 262 data capture tools hosted at Aarhus University (35,36). REDCap is a secure, web-based software  
23  
24 263 platform designed to support data capture for research studies, providing 1) an intuitive interface for  
25  
26 264 validated data capture; 2) audit trails for tracking data manipulation and export procedures; 3)  
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29 265 automated export procedures for seamless data downloads to common statistical packages; and 4)  
30  
31 266 procedures for data integration and interoperability with external sources. All data collectors of the  
32  
33 267 study have to be trained in Good Clinical Practice (GCP) procedures and as minimum to have passed  
34  
35 268 the course provided by the Danish GCP institution. All inclusion and exclusion criteria as well as  
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38 269 outcome data will be monitored by external GCP monitors to ensure optimal data quality. Data  
39  
40 270 collection forms and other data entry related information can be requested from the corresponding  
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42 271 author.  
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46 272 Protocol deviations have to be stated in the eCRF. Loss to follow-up is unlikely for patients in IVF  
47  
48 273 treatment who will be highly motivated to come to the clinic. However, patients who are not pregnant  
49  
50 274 may opt to go to other clinics for further treatment and, thus will be lost to follow-up. Patients who  
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52  
53 275 are not pregnant (negative HCG-test) and continue LACTIN-V treatment are informed to contact the  
54  
55 276 respective clinics in case of adverse events and these will be captured in the eCRF. If patients decide  
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57 277 to end study product treatment, they are informed to contact the clinics and to deliver the unused  
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4 278 LACTIN-V to the clinic at which point they would be asked about any adverse events. The eCRF  
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6 279 instruments have range checks and other data rules that have to be passed to ensure optimal data  
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8  
9 280 input.

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12 281 *Statistical methods*

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14 282 The total significance level of the study was set to be 5%. Based on the O'Brien-Fleming method, the  
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16  
17 283 total significance was split into 0.1% for the interim and 4.9% for the final analysis (37). Therefore,  
18  
19 284 a p-value with 99.9% confidence interval is calculated in the interim analysis to test the possible  
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21  
22 285 effect of one or both active treatment arms (combined or separately) on clinical pregnancy rate per  
23  
24 286 embryo transfer (primary outcome) compared to placebo. An analysis between the two active  
25  
26 287 treatment arms will also be conducted. All four analyses: 1) active/active vs active/placebo, 2)  
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28 288 active/placebo vs placebo/placebo, 3) active/active vs placebo/placebo, 4) active/active AND  
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31 289 active/placebo vs placebo/placebo will be done as first a crude estimate and then secondly adjustment  
32  
33 290 with confounders for double embryo transfer, quality of the embryo (Cleavage/blastocyst), female  
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35 291 age (continuous variable) and center effect (public/private). If the trial is discontinued according to the  
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37  
38 292 criteria stated under the paragraph "interim analysis", a full statistical analysis will be made as  
39  
40 293 described below. The final statistical analysis includes crude odds ratios (ORs) and 94.9% confidence  
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42 294 intervals (CI's), that are calculated from Chi-square test for possible effect of active treatment on  
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44  
45 295 study outcomes (e.g. clinical pregnancy rate [primary outcome]) compared with placebo. Analyses  
46  
47 296 will be conducted at intention-to-treat level and for those completing the treatment protocol without  
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49 297 violation. Specific analyses will also include regression analyses, e.g. logistic and linear regressions,  
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52 298 taking confounding factors into account.

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56 300 *Interim analysis*

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4 301 An interim analysis of the four analyses mentioned above will be performed to evaluate the clinical  
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6 302 pregnancy rate per embryo transfer when 167 patients have been randomized. If study medication is  
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9 303 affecting the clinical pregnancy rate statistically significant in either of the groups, the trial will  
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11 304 discontinue. Furthermore, the drop-out rate will be evaluated considering both the number of positive  
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13 305 AVM declining to participate and the number of patients who drop-out after randomization. A drop-  
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16 306 out rate above 20% will lead to discontinuation. External statisticians from Aarhus University,  
17  
18 307 Denmark will conduct the interim analysis. Only a small study board, including sponsor and principal  
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20 308 investigators will know the result of the interim analysis. Sponsor-investigator make the decision to  
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23 309 continue or discontinue the trial. The study will continue in case there is no statistical difference in  
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25 310 either of the 4 tests, drop-out rate is acceptable, and the logistical requirements to finish the study can  
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27 311 be met within reasonable time considering e.g. expiry of study medication and time to recruit all  
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30 312 patients. The time to undertake the interim analysis and the decision to continue or discontinue is  
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32 313 approximately 3 weeks. The trial is on hold during these 3 weeks.  
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#### 36 315 *Data monitoring*

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39 316 Investigator(s)/institution(s) will be permitted direct access to source data/documents for trial-related  
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41 317 monitoring, audits, IRB/IEC review, and regulatory inspection(s). Primary investigators only have  
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43 318 access to patients from their own center. This study will be monitored by the Danish GCP units,  
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45  
46 319 primarily the GCP-unit at Aarhus University and GCP-unit Copenhagen University Hospital.  
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48 320 Furthermore, this trial is open for audit and quality assurance by the Danish Medicines Agency as  
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50 321 specified by Danish law.  
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#### 55 323 *Adverse events and reactions*

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4 324 Adverse events and adverse reactions will be registered in a questionnaire handed out by study  
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6 325 personnel to the patient on the day of embryo transfer and on the day of the clinical pregnancy scan.  
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9 326 In case there is no embryos for transfer patients will be approached to answer the questionnaire either  
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11 327 by email or at oocyte retrieval day. Patients who enter luteal phase stimulation (Duostim) or  
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13 328 segmentation (freeze-all) will use the same questionnaire on the oocyte retrieval day of the cycle  
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16 329 where they have study medication, corresponding to approximately 14 days of study medication. In  
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18 330 the questionnaire, patients will also be asked to answer questions regarding gastrointestinal symptoms  
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20 331 that might be related to the treatment with antibiotic clindamycin. There will be at least 8 weeks of  
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23 332 follow-up to register any late-occurring adverse events/reactions. Patients will be asked the same  
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25 333 questionnaire concerning potential late occurring LACTIN-V related side-effects on the day of the  
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27 334 clinical pregnancy scan. Moreover, patients are asked if they have symptoms at all study visits and  
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30 335 these symptoms are recorded in the eCRF, including an adverse reaction judgement from the treating  
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32 336 physician.  
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36 338 *Serious adverse events (SAE)*  
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39 339 At each center, primary investigators will report serious adverse events (SAE) to sponsor within 24  
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41 340 hours by email or phone. Sponsor ensures that all Suspected Unexpected Serious Adverse Reactions  
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43 341 (SUSARs) that are fatal or life-threatening are recorded and reported to the Danish Medicines Agency  
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46 342 and the scientific Ethics Committee as soon as possible and no later than 7 days after the sponsor  
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48 343 became aware of such possible side effect. Within 8 days after a SUSAR has been reported, the  
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50 344 sponsor must notify the Danish Medicines Agency and the Ethics Committee with all relevant  
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53 345 information on the follow-up of any SUSAR that may occur. All other unexpected serious or  
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55 346 suspected serious adverse reactions will be reported to the Danish Medicines Agency and the  
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57 347 scientific Ethics Committee within 15 days after the sponsor become aware of these. An annual safety  
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4 348 report regarding the trial participants will be performed, consisting of serious adverse event suspected  
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6 349 to be related to the investigational drug will be submitted to Danish Medicines Agency and the Ethics  
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9 350 Committee. At end of study, all AEs and SAEs will be reported according to regulations in Denmark.  
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13 352 *Ethics*

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16 353 Approvals from the Regional Scientific Ethical Committee (M-2017-157-17), the Danish Data  
17  
18 354 Protection Agency (1-16-02-790-17) and Danish Medicines Agency (2016-002385-31) were  
19  
20 355 obtained prior to trial initiation December 7<sup>th</sup>, 2017. Danish law will be complied with regarding the  
21  
22 356 handling of personal information. Protocol amendments will be provided to the relevant parties,  
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24  
25 357 including the Regional Scientific Ethical Committees and Danish Medicines Agency. All protocol  
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27 358 amendments have to be approved by the Danish Medicines Agency and the scientific ethical  
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30 359 committee before taken into use. Logging of trial amendments is secured at both these institutions,  
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32 360 the sponsor-investigator as well as updated at EudraCT. Patient confidentiality is ensured by data  
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34 361 capture in REDCap<sup>TM</sup>. All patients are covered by a public insurance in Denmark.  
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39 363 *Access to data*

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41 364 Only the sponsor-investigator has full access to the dataset. The interim analysis will be performed  
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43 365 by external statisticians at the local university according to the pre-set plan explained above. Primary  
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45 366 investigators and statisticians may have access to data at the discretion of the sponsor-investigator.  
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48 367 External parties can only gain access to trial data following establishment of a data handling  
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50 368 agreement.  
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55 370 *Dissemination*  
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4 371 Positive, negative as well as inconclusive results will be published, aiming for high impact journals  
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6 372 with full data transparency. Dissemination of results is ensured in clinical trial agreements between  
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9 373 the participating institutions and Sponsors institution, Aarhus University, Denmark. The Vancouver  
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11 374 guidelines for authorship will be followed.

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16 376 *Trial status*

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18 377 The first patient was screened December 7<sup>th</sup>, 2017. By September 7<sup>th</sup>, 2019 we had screened 533  
19  
20 378 patients and randomized 119 patients. Interim analysis is expected by March 2020. End of trial is  
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23 379 expected to be summer 2021.

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27 381 *Author Contributions*

28  
29 382 PH, JSJ, NU and TH were the primary writers and inventors of this protocol. PH is the sponsor-  
30  
31  
32 383 investigator. TP provided information on LACTIN-V and contributed to the study design and protocol  
33  
34 384 development. TH, NICF, AP, VH, ALM and HSN are primary investigators at the involved clinics  
35  
36 385 and contributed to the protocol and amendments during the initiation phase of the study.

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41  
42  
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44  
45  
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47  
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49  
50 390 Stork. Moreover, we wish to thank Aparna Udipi from Aarhus University, Denmark for statistical  
51  
52  
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54  
55 392 Denmark for their contribution concerning medication allocation to the clinics.

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58 393 *Funding statement*

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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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26 529 **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)
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**Table 2: Study medication scheme**

	Clindamycin “Alternova”	LACTIN-V™
Dose	300mg	200mg/2x10 <sup>9</sup> 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				
<b>TIMEPOINT</b>	<b>Max 3 months prior to allocation day</b>	<b>Minimum 12 days prior to embryo transfer</b>	<b>7 days later</b>	<b>Embryo transfer</b>	<b>Pregnancy scan</b>	<b>Gestional week 22, 37, after birth</b>
<b>ENROLMENT:</b>						

1						
2						
3						
4	<b>Eligibility screen</b>	X				
5						
6	<b>Informed consent</b>	X				
7						
8	<b>Vaginal swab</b>	X				
9						
10	<b>Allocation</b>		X			
11						
12						
13	<b>INTERVENTIONS:</b>					
14						
15						
16	<b>Clindamycin</b>	X	←→			
17						
18	<b>Lactin-V</b>			X	←→	
19						
20	<b>IVF treatment</b>	X	←→			
21						
22						
23						
24						
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26						
27						
28	<b>ASSESSMENTS:</b>					
29						
30	<b>Vaginal swab</b>	X	X	X	X	X
31						
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33	536					
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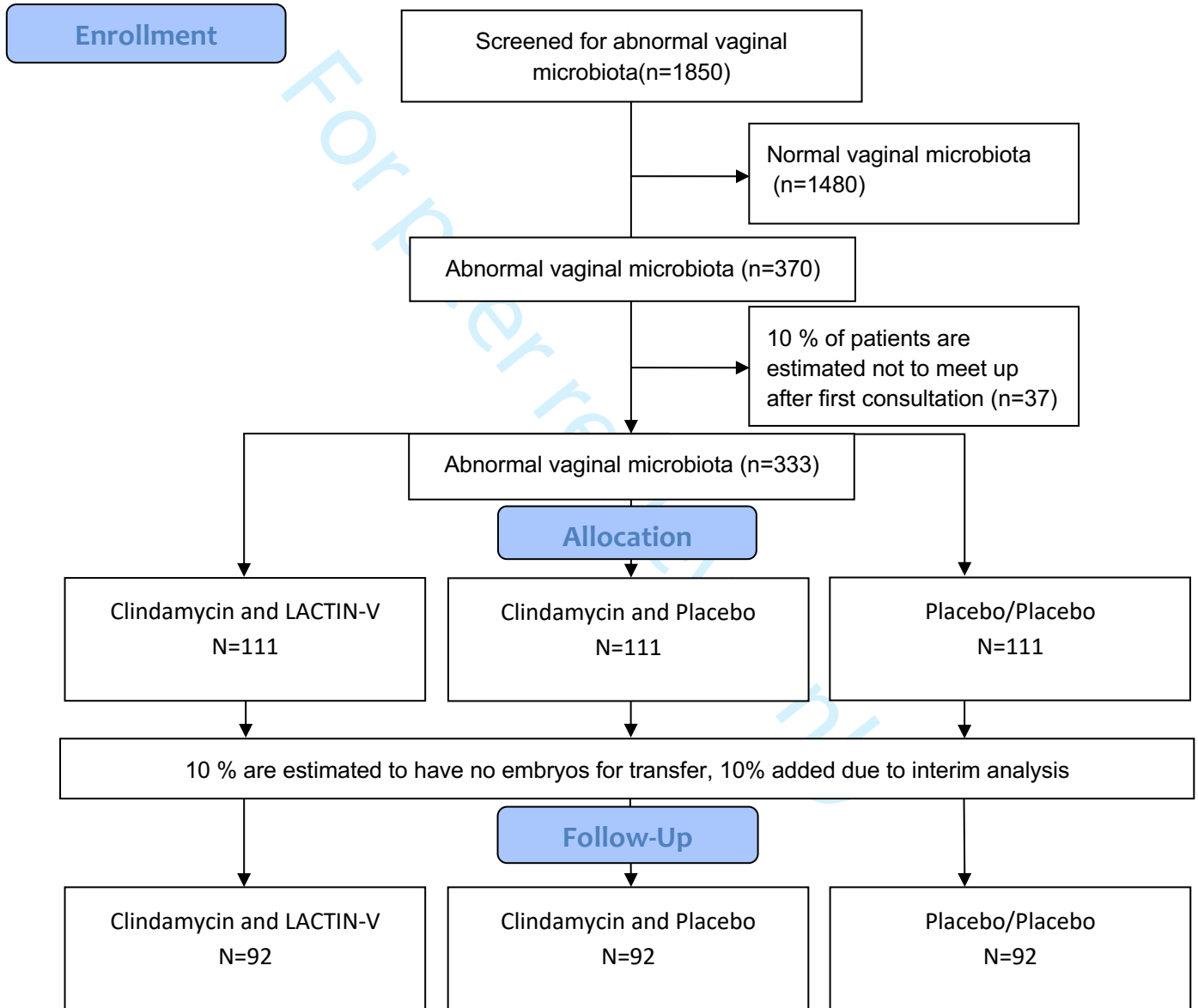
538 **Figure 1: Study Flowchart**

539 See figure attached.

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

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CONSORT 2010 Flow Diagram



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4 1 Appendix 1  
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6 2 Samples will be collected from the vagina as outlined in table 3 and in the protocol. Moreover, we  
7  
8  
9 3 collect seminal samples from the partner at oocyte retrieval day and a fecal specimen from the  
10  
11 4 newborn diaper within 3 weeks from birth. Vaginal swabs that are taken at the clinic are taken with  
12  
13 5 a flocked swab and placed in Eswab (Copan™) except for the seminal sample, which is kept in a  
14  
15 6 1.8 mL cryotube, Nunc™, cryotube™, Thermo Scientific. The vaginal swabs taken at home in  
16  
17 7 pregnancy after birth and the fecal swab are taken by the patient herself using flocked swabs and  
18  
19 8 placed in an eNAT tube (Copan™). All the vaginal samples are taken after these instructions: the  
20  
21 9 vaginal swab has to be placed at least 8 cm into the vaginal cavity for 5 seconds and rotated  
22  
23 10 clockwise. The fecal swab has to be a deep sample and has to be rotated in the feces for 5 seconds.  
24  
25 11 The semen sample is collected after homogenization and then 200 microL are transferred to the  
26  
27 12 cryotube using a pipette.  
28  
29 13 Only the vaginal swab at screening visit is sent immediately to the central laboratory at Statens  
30  
31 14 Serum Institute for analysis within 7 days. All vaginal swabs taken during study visits and the  
32  
33 15 seminal sample are immediately frozen at minus 80 degrees at the respective clinics. All samples  
34  
35 16 are stored at the clinics until they are to be transferred by dry ice shipment to the central laboratory.  
36  
37 17 Finally, the home-samples (vaginal samples and the fecal sample) are sent with return envelopes to  
38  
39 18 the patients when they are supposed to take the sample and samples are received back at the  
40  
41 19 Fertility Clinic in Skive, Denmark where they are stored at -80 degrees. For the substudy explained  
42  
43 20 in the protocol concerning the 20 patients taking daily vaginal home-samples (Copan ESwab™),  
44  
45 21 these are kept in the patient's own freezer (-20 degrees) until embryo transfer day where the first  
46  
47 22 12-16 samples will be delivered to the clinic and stored at minus 80 degrees. The remaining 5-9  
48  
49 23 samples are kept in the patient's own freezer (-20) until her next visit which may be either hCG test  
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51 24 day, clinical pregnancy scan or a visit during the subsequent cycle due to no pregnancy.  
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STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

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

Section/item	Item No	Description	Addressed on page number
<b>Administrative information</b>			
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 ___



1	<b>Introduction</b>			
2				
3	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
4				
5				
6		6b	Explanation for choice of comparators	7
7				
8	Objectives	7	Specific objectives or hypotheses	8
9				
10	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
11				
12				
13				
14	<b>Methods: Participants, interventions, and outcomes</b>			
15				
16	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
17				
18				
19	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
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24	Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	9
25				
26				
27				
28		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
29				
30				
31		11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	10,15
32				
33				
34		11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	10
35				
36	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
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1	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
2				
3				
4	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 _____
5				
6				
7	Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	_____ 8 _____
8				
9				

### Methods: Assignment of interventions (for controlled trials)

#### Allocation:

13				
14	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 _____
15				
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19	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 _____
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23	Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	_____ 8, 12 _____
24				
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27	Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	_____ 8,12 _____
28				
29				
30		17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	_____ 12,13 _____
31				
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### Methods: Data collection, management, and analysis

34				
35				
36	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 _____
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1		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 _____
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4	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 _____
5				
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7				
8	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 _____
9				
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11		20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	_____ 15 _____
12				
13		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 _____
14				
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17	<b>Methods: Monitoring</b>			
18				
19	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 _____
20				
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25		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 _____
26				
27				
28	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 _____
29				
30				
31	Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	_____ 15 _____
32				
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35	<b>Ethics and dissemination</b>			
36				
37	Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	_____ 17 _____
38				
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1	Protocol	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_____
2	amendments			
3				
4				
5	Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	_____8_____
6				
7				
8		26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	_____N/A_____
9				
10				
11	Confidentiality	27	How personal information about potential and enrolled participants will be collected, stored, and maintained in order to protect confidentiality before, during, and after the trial	_____17_____
12				
13				
14	Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	_____19_____
15				
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18	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_____
19				
20				
21	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_____
22				
23				
24	Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, health care professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	_____17_____
25				
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28				
29		31b	Authorship eligibility guidelines and any intended use of professional writers	_____17_____
30				
31		31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	_____17_____
32				
33	<b>Appendices</b>			
34				
35	Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	www.reproflor.dk
36				
37				
38	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_____
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1 \*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items.  
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# 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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<b>Primary Subject Heading</b>:	Reproductive medicine
Secondary Subject Heading:	Sexual health, Infectious diseases
Keywords:	REPRODUCTIVE MEDICINE, BACTERIOLOGY, MICROBIOLOGY, GYNAECOLOGY

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4 1 **Title:** A protocol for a double-blind, placebo-controlled multicenter trial on the effect of clindamycin  
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6 2 and a live biotherapeutic on the reproductive outcomes of IVF patients with abnormal vaginal  
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8 3 microbiota  
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12 4 **Authors:** Thor Haahr<sup>1\*</sup>, Nina la Cour Freiesleben<sup>2</sup>, Anja Pinborg<sup>3</sup>, Henriette Svarre Nielsen<sup>3</sup>, Vibeke  
13  
14 5 Hartvig<sup>4</sup>, Anne-Lis Mikkelsen<sup>5</sup>, Thomas Parks<sup>6</sup>, Niels Ulbjerg<sup>7</sup>, Jørgen Skov Jensen<sup>8</sup>, Peter  
15  
16 6 Humaidan<sup>1</sup>.  
17

18 7  
19 8 <sup>1</sup>Department of Clinical Medicine, Aarhus University, Denmark and the Fertility Clinic Skive, Skive  
20  
21 9 Regional Hospital, Denmark  
22

23 10 <sup>2</sup>The Fertility Clinic, Department of Obstetrics and Gynecology, Hvidovre Hospital, Copenhagen  
24  
25 11 University Hospital, Denmark  
26

27 12 <sup>3</sup>Fertility Clinic, Rigshospitalet 4071, Copenhagen University Hospital, Copenhagen, Denmark  
28

29 13 <sup>4</sup>Stork Fertility Clinic, Copenhagen, Denmark  
30

31 14 <sup>5</sup>Fertility Clinic, Zealand University Hospital, Denmark.  
32

33 15 <sup>6</sup>Osel, Inc., Mountain View, CA, United States  
34

35 16 <sup>7</sup>Department of Obstetrics and Gynecology, Aarhus University Hospital, Skejby, Denmark  
36

37 17 <sup>8</sup>Statens Serum Institute, Research Unit for Reproductive Microbiology, Copenhagen, Denmark  
38

39 18  
40 19 Trial sponsor:

41 20 Professor Peter Humaidan  
42

43 21 Address: The Fertility Clinic, Skive Regional Hospital, Resenvej 25, 7800 Skive, Denmark  
44

45 22 Phone: +45 78445760  
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47 23 \*Corresponding author: [thohaa@rm.dk](mailto:thohaa@rm.dk)  
48

49 24 Address: The Fertility Clinic, Skive Regional Hospital, Resenvej 25, 7800 Skive, Denmark  
50

51 25 Phone: +45 78445760  
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53 26 <https://orcid.org/0000-0001-9304-5299>  
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55 27 **Word count:** 4,809 excluding references and tables.  
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4 **28 Abstract:**

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7 **29 Introduction**

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9 **30** Recent studies in *in vitro* fertilization (IVF) patients have associated abnormal vaginal microbiota  
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11 **31** (AVM) with poor clinical pregnancy rates of 6-9% per embryo transfer. The biological plausibility  
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13 **32** for this finding is hypothesized to be ascending infection to the endometrium which in turn hampers  
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15 **33** embryo implantation. The prevalence of AVM ranges from 4-38%. New molecular diagnosis may  
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17 **34** offer advantages compared to microscopical diagnosis; however, the important question is whether  
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19 **35** screening and treatment of AVM would improve reproductive outcomes in IVF patients. Herein, we  
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21 **36** describe a protocol for an ongoing double-blind, placebo-controlled multicenter trial of IVF patients  
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23 **37** with molecular defined AVM randomized in three parallel groups 1:1:1.  
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30 **39 Methods and analysis**

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32 **40** This is a drug intervention study where IVF patients will be screened for AVM, using a qPCR assay  
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34 **41** targeting *Atopobium vaginae* and *Gardnerella vaginalis*. If positive, patients will be randomized to  
35  
36 **42** one of the three study arms. The first arm consists of clindamycin 300mg x2 daily for 7 days followed  
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38 **43** by topical *Lactobacillus crispatus* CTV-05 until clinical pregnancy scan week 7-9. The second arm  
39  
40 **44** consists of clindamycin and placebo *Lactobacillus crispatus* CTV-05, whereas patients in the third  
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42 **45** arm will be treated with placebo/placebo. We used a superiority design to estimate that active  
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44 **46** treatment in both arms will increase the primary outcome, clinical pregnancy rate per embryo transfer,  
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46 **47** from 20% to 40%. A potential difference between the two active arms was considered exploratory.  
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48 **48** With a power of 80% and an alpha at 5%, the sample size is estimated to be 333 patients randomized.  
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50 **49** A pre-planned interim analysis is scheduled at 167 patients randomized.  
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57 **51 Ethics and dissemination**  
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4 52 All patients have to give informed consent. Dissemination of results is ensured in clinical trial  
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6 53 agreements whether they be positive or not.  
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11 55 Registration

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13 56 This ICH-GCP monitored trial is registered in relevant national agencies. EudraCT number 2016-  
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15 57 002385-31.  
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18 58

19  
20 59 **Keywords: IVF, Microbiota, Bacterial vaginosis, Clindamycin, Gardnerella, RCT,**

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23 60 **Lactobacillus**  
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4 **61 Article summary: ‘Strengths and limitations of this study’**  
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- 8 **62** • Molecular based diagnosis of abnormal vaginal microbiota was validated in pilot studies  
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10 **63** • The first RCT in IVF patients with abnormal vaginal microbiota investigating treatment effect  
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12 **64** on reproductive outcome of clindamycin and live lactobacillus treatment  
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15 **65** • The *Lactobacillus crispatus* CTV-05 treatment is an investigational live biotherapeutic  
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17 **66** product regulated by the US FDA  
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20 **67** • Inclusion criteria are relatively broad  
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## 68 Introduction

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

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

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

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

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116 relative abundances which does not sufficiently take into account differences in the total abundance  
117 (21–23).

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

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

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

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4 140 that time in Phase 2 development(11). Recently, adjuvant LACTIN-V after vaginal metronidazole  
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7 141 was reported to lower BV recurrence rates in a phase 2b trial, RR 0.66 (95%CI 0.44-0.87)(35).

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11 143 Taking into consideration the abovementioned evidence, the research question of the present study  
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14 144 is: does antibiotic alone or in combination with live biotherapeutic treatment of an abnormal vaginal  
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16 145 microbiota improve the reproductive outcomes of IVF patients? The intervention is clindamycin  
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18 146 either alone or in combination with LACTIN-V, a live biotherapeutic product containing *L.crispatus*  
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20 147 CTV-05 (11). The study is designed as a double-blind, placebo-controlled multicenter trial of three  
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23 148 parallel groups randomized 1:1:1. Randomization is by computer generated code and allocation  
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25 149 concealment is performed by the pharmacy who will send out medication to the participating clinics  
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27 150 with identical appearance and randomization numbers. The randomization code is with the pharmacy  
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30 151 and can only be opened in case of emergency by the principal investigators or as planned by the  
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32 152 sponsor-investigator. The benefit of the intervention would potentially lead to increased pregnancy  
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34 153 rates and, for those suffering from symptomatic BV, also relief of BV symptoms. In contrast, the  
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36 154 expected adverse reactions of concern are especially gastrointestinal symptoms caused by  
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39 155 clindamycin, whereas LACTIN-V might cause increased vaginal discharge but is otherwise not  
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41 156 expected to cause adverse reactions as based on prior studies(11,36).

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## 44 45 158 **Methods and analysis**

### 46 47 48 159 *Setting and eligibility criteria*

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50 160 The present trial is conducted at four University affiliated clinics and one private fertility clinic in  
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53 161 Denmark. The list of study sites is available with EudraCT clinical trial identifier: 2016-002385-31,  
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55 162 first registration day 2016-07-11. The current version of the protocol is 9, 2020-02-07. Patients are  
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4 163 enrolled in a cohort study (Clinicaltrials.gov NCT03420859) from which we will recruit patients for  
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7 164 the randomized trial (EudraCT: 2016-002385-31). Eligibility criteria are described in Table 1.

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9 165 In brief, IVF patients attending their first, second or third IVF stimulation cycle or embryo transfer  
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11 166 therefrom will be approached for informed consent by the study nurse or treating physician. Patients  
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14 167 are told about the project in a private room with the right to have an assessor, allowing time to reflect  
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16 168 whether they will participate. They are handed out written information material with a link to the  
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18 169 study website with full information about the project – [www.reproflor.dk](http://www.reproflor.dk). The vaginal swab can be  
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21 170 taken by the treating physician or the patient herself after careful instruction. In this case, patients are  
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23 171 instructed to place the swab at least 8 cm into the vaginal cavity for 5 seconds and rotate clockwise.  
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25 172 This is to ensure that the vaginal bacteria in the fornix or in its close proximity will be caught by the  
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27 173 flocked swab. Subsequently, the vaginal swab will be sent to a central laboratory at Statens Serum  
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30 174 Institut, Copenhagen to be analyzed for AVM within 7 days as determined in a previous study(5). If  
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32 175 AVM positive, patients are asked to provide informed consent that they are willing to participate in  
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34 176 the randomized controlled trial. Patients should ideally be randomized on the first day of ovarian  
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37 177 stimulation with exogenous gonadotropins, allowing a minimum of 12 days of study medication to  
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39 178 be acceptable for inclusion in the study. If elective frozen embryo transfer (FET) is planned, patients  
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41 179 should be randomized during the first days of the FET cycle allowing for at least 12 days of study  
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44 180 medication. If patients enter the trial and have less than 12 days of study medication despite the  
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46 181 abovementioned inclusion criteria (e.g. when hormonal stimulation is shortened due to an unexpected  
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48 182 ovarian response), it is considered a protocol violation and they will be excluded from the per protocol  
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50 183 analysis, i.e. not from ITT-analysis.

#### 51 52 184 *Interventions*

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55 185 Active Treatment 1: **Oral Clindamycin** 300 mg 2 times per day for 7 days followed by **LACTIN-V**  
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58 186 (Osel, Inc.) until completion of the clinical pregnancy scan at week 7-9. LACTIN-V containing *L.*  
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187 *crispatus* CTV-05 ( $2 \times 10^9$  CFU/dose, 200 mg, delivered with pre-filled, single use vaginal applicators) regimen is once daily from the clindamycin stop for 7 consecutive days.

189 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.

192 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.

195 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.

197 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.

#### 205 *Labelling and packaging*

206 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.

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

### 223 *Outcomes*

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

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#### 235 *Sample size*

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

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

#### 254 *Allocation*

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

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4 259 and only the randomization number differ, hence both patients and study personnel are blinded for  
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6 260 the intervention. A block of 15 medication packs will be sent from the pharmacy from start of study  
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9 261 and new blocks can be requested when 5 medication packs are left. The 15 medication packs are  
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11 262 mixed and appear identical to both personnel and patients. The randomization number is continuous  
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13 263 and unique for each patient, starting from 1 to 333 and the number is prelabelled from the pharmacy  
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16 264 before distribution to the clinics. The last 3 medication packs from 330-333 is also one block.  
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19 265 The randomization list is secured by the pharmacy throughout the trial, and only the sponsor has the  
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21 266 authority to unblind the trial. However, in case of medical emergency the principal investigator (PI)  
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24 267 can call the pharmacy to unblind. Each participant's medication package is labeled with a  
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26 268 randomization number that is linked to their study ID number in the eCRF. Although both patients  
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28 269 and clinicians will be blinded to allocation, they may suspect active medication in case of side-effects.  
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31 270 This small risk of bias seems to be unavoidable. However, to investigate such an effect, patients are  
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33 271 asked if they believed that they received active or inactive medication.  
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### 36 272 *Data collection methods*

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39 273 Study data are collected and managed using REDCap (Research Electronic Data Capture) electronic  
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41 274 data capture tools hosted at Aarhus University, Denmark(38,39). REDCap is a secure, web-based  
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43 275 software platform designed to support data capture for research studies, providing 1) an intuitive  
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46 276 interface for validated data capture; 2) audit trails for tracking data manipulation and export  
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48 277 procedures; 3) automated export procedures for seamless data downloads to common statistical  
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50 278 packages; and 4) procedures for data integration and interoperability with external sources. All data  
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53 279 collectors of the study have to be trained in Good Clinical Practice (GCP) procedures and as minimum  
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55 280 to have passed the course provided by the Danish GCP institution. All inclusion and exclusion criteria  
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57 281 as well as outcome data will be monitored by external GCP monitors to ensure optimal data quality.  
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4 282 Data collection forms and other data entry related information can be requested from the  
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7 283 corresponding author.  
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10 284 Protocol deviations have to be stated in the eCRF. Loss to follow-up is unlikely for patients in IVF  
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12 285 treatment who will be highly motivated to come to the clinic. However, patients who are not pregnant  
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15 286 may opt to go to other clinics for further treatment and, thus will be lost to follow-up. Patients who  
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17 287 are not pregnant (negative HCG-test) and continue LACTIN-V treatment are informed to contact the  
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19 288 respective clinics in case of adverse events and these will be captured in the eCRF. If patients decide  
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22 289 to end study product treatment, they are informed to contact the clinics and to deliver the unused  
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24 290 LACTIN-V to the clinic at which point they would be asked about any adverse events. The eCRF  
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26 291 instruments have range checks and other data rules that have to be passed to ensure optimal data  
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29 292 input. In case of missing outcome data we plan to use the framework proposed by White et al  
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31 293 (2011)(40).  
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#### 34 294 *Statistical methods*

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36 295 The total significance level of the study was set to be 5%. Based on the O'Brien-Fleming method, the  
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39 296 total significance was split into 0.1% for the interim and 4.9% for the final analysis (37). Therefore,  
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41 297 a p-value with 99.9% confidence interval is calculated in the interim analysis to test the possible  
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43 298 effect of one or both active treatment arms (combined or separately) on clinical pregnancy rate per  
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46 299 embryo transfer (primary outcome) compared to placebo. A Walds Chi-square test for possible effect  
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48 300 will be conducted comparing all 3 arms. Moreover, four analyses: 1) active/active vs active/placebo,  
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50 301 2) active/placebo vs placebo/placebo, 3) active/active vs placebo/placebo, 4) average effect of  
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53 302 active/active AND active/placebo vs placebo/placebo will be done as first a crude estimate and then  
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55 303 secondly adjustment with confounders for double embryo transfer, quality of the embryo  
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57 304 (Cleavage/blastocyst), female age (continuous variable) and center effect (public/private). If the trial  
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4 305 is discontinued according to the criteria stated under the paragraph “interim analysis”, a full statistical  
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6 306 analysis will be made as described below. First, a Walds Chi-square test for possible effect of active  
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9 307 treatment on clinical pregnancy rate [primary outcome] will be made across all 3 groups. Moreover,  
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11 308 pairwise comparisons for the abovementioned 4 tests will be made with odds ratios (OR) and risk  
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13 309 ratios (RR) and 94.9% confidence intervals (CI’s) calculated from logistic and linear regressions  
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16 310 models, taking abovementioned confounding factors into account. Analyses will be conducted at  
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18 311 intention-to-treat level (all randomized patients) and for those completing the treatment protocol  
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20 312 without violation. Intention-to-treat is considered the primary analysis.  
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#### 24 25 314 *Interim analysis*

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28 315 An interim analysis of the four analyses mentioned above will be performed to evaluate the clinical  
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30 316 pregnancy rate per embryo transfer when 167 patients have been randomized and completed the study  
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32 317 for primary outcome evaluation. If study medication is affecting the clinical pregnancy rate  
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34 318 statistically significant in either of the analyses, the trial will discontinue. Furthermore, the drop-out  
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37 319 rate will be evaluated considering both the number of positive AVM declining to participate and the  
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39 320 number of patients who drop-out after randomization. A drop-out rate above 20% will lead to  
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42 321 discontinuation. External statisticians from Aarhus University, Denmark will conduct the interim  
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44 322 analysis. Only a small study board, including sponsor and principal investigators will know the result  
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46 323 of the interim analysis. Sponsor-investigator make the decision to continue or discontinue the trial.  
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49 324 The study will continue in case there is no statistical difference in either of the tests, drop-out rate is  
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51 325 acceptable, and the logistical requirements to finish the study can be met within reasonable time  
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53 326 considering e.g. expiry of study medication and time to recruit all patients. The time to undertake the  
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55 327 interim analysis and the decision to continue or discontinue is approximately 3 weeks.  
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329 *Data monitoring*

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

336

337 *Adverse events and reactions*

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

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352 *Serious adverse events (SAE)*



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

### 366 *Ethics*

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

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4 377 *Access to data*

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6 378 Only the sponsor-investigator has full access to the dataset. The interim analysis will be performed  
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9 379 by external statisticians at the local university according to the pre-set plan explained above. Primary  
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11 380 investigators and statisticians may have access to data at the discretion of the sponsor-investigator.  
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13 381 External parties can only gain access to trial data following establishment of a data handling  
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16 382 agreement.

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20 384 *Dissemination*

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23 385 Positive, negative as well as inconclusive results will be published, aiming for high impact journals  
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25 386 with full data transparency. Dissemination of results is ensured in clinical trial agreements between  
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27 387 the participating institutions and Sponsors institution, Aarhus University, Denmark. The Vancouver  
28  
29  
30 388 guidelines for authorship will be followed.

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32 389  
33  
34 390 *Trial status*

35  
36 391 The first patient was screened December 7<sup>th</sup>, 2017. By September 7<sup>th</sup>, 2019 we had screened 533  
37  
38  
39 392 patients and randomized 119 patients. Interim analysis is expected by March 2020. End of trial is  
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41 393 expected to be summer 2021.

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43 394  
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45 395 *Author Contributions*

46  
47  
48 396 PH, JSJ, NU and TH were the primary writers and inventors of this protocol. PH is the sponsor-  
49  
50 397 investigator. TP provided information on LACTIN-V and contributed to the study design and protocol  
51  
52  
53 398 development. TH, NICF, AP, VH, ALM and HSN are primary investigators at the involved clinics  
54  
55 399 and contributed to the protocol and amendments during the initiation phase of the study.

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58 400 *Acknowledgements*

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403 Statens Serum Institut, Copenhagen and the Fertility Clinics in Skive, Hvidovre, Rigshospitalet and  
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405 advice concerning this trial. Finally, we wish to thank Kristian Nielsen and Glostrup Pharmacy,  
406 Denmark for their contribution concerning medication allocation to the clinics.

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414 TH.

#### 415 *Competing interests statement*

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

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

#### 426 *Patients and public involvement*

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

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53 551 **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 <sup>9</sup> 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.

	<p>medication. Investigational drug preparations other than the study product.</p>	
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\* Patients not pregnant are informed to contact the department in case of any LACTIN-V related side-effect.

**Table 3:** Study timeline

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



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<b>ASSESSMENTS:</b>						
<i>Vaginal swab</i>	X	X	X	X	X	X

For peer review only

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560 **Figure 1: Study Flowchart**

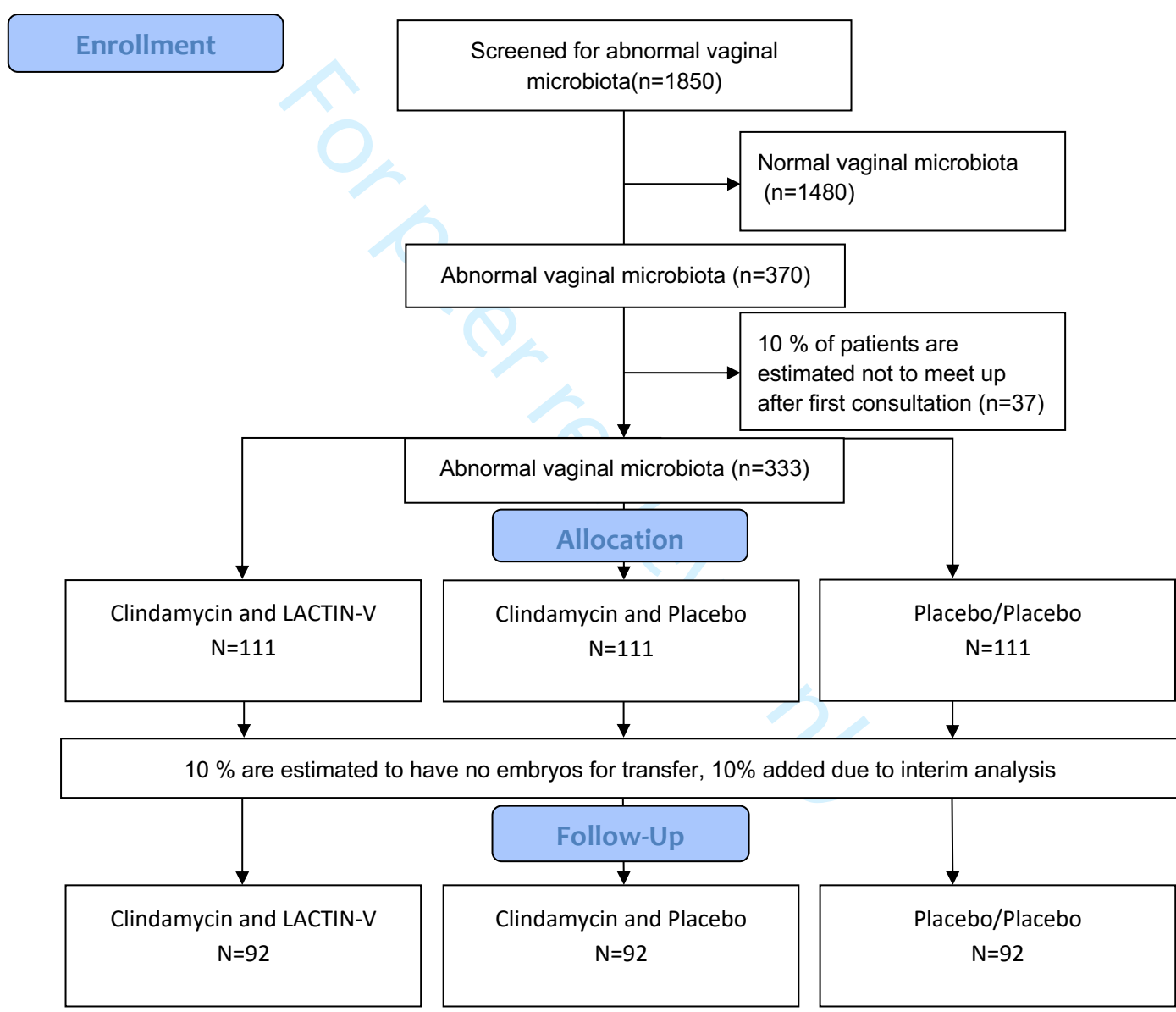
561 See figure attached.

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

For peer review only

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### CONSORT 2010 Flow Diagram



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6 2 Samples will be collected from the vagina as outlined in table 3 and in the protocol. Moreover, we  
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9 3 collect seminal samples from the partner at oocyte retrieval day and a fecal specimen from the  
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11 4 newborn diaper within 3 weeks from birth. Vaginal swabs that are taken at the clinic are taken with  
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13 5 a flocked swab and placed in Eswab (Copan™) except for the seminal sample, which is kept in a  
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15 6 1.8 mL cryotube, Nunc™, cryotube™, Thermo Scientific. The vaginal swabs taken at home in  
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17 7 pregnancy after birth and the fecal swab are taken by the patient herself using flocked swabs and  
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19 8 placed in an eNAT tube (Copan™). All the vaginal samples are taken after these instructions: the  
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21 9 vaginal swab has to be placed at least 8 cm into the vaginal cavity for 5 seconds and rotated  
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23 10 clockwise. The fecal swab has to be a deep sample and has to be rotated in the feces for 5 seconds.  
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25 11 The semen sample is collected after homogenization and then 200 microL are transferred to the  
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27 12 cryotube using a pipette.  
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29 13 Only the vaginal swab at screening visit is sent immediately to the central laboratory at Statens  
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31 14 Serum Institute for analysis within 7 days. All vaginal swabs taken during study visits and the  
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33 15 seminal sample are immediately frozen at minus 80 degrees at the respective clinics. All samples  
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35 16 are stored at the clinics until they are to be transferred by dry ice shipment to the central laboratory.  
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37 17 Finally, the home-samples (vaginal samples and the fecal sample) are sent with return envelopes to  
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39 18 the patients when they are supposed to take the sample and samples are received back at the  
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41 19 Fertility Clinic in Skive, Denmark where they are stored at -80 degrees. For the sub-study explained  
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43 20 in the protocol concerning the 20 patients taking daily vaginal home-samples (Copan ESwab™),  
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45 21 these are kept in the patient's own freezer (-20 degrees) until embryo transfer day where the first  
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47 22 12-16 samples will be delivered to the clinic and stored at minus 80 degrees. The remaining 5-9  
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49 23 samples are kept in the patient's own freezer (-20) until her next visit which may be either hCG test  
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51 24 day, clinical pregnancy scan or a visit during the subsequent cycle due to no pregnancy.  
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STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

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

Section/item	Item No	Description	Addressed on page number
<b>Administrative information</b>			
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 ___

## 1 Introduction

2			
3	Background and	6a	Description of research question and justification for undertaking the trial, including summary of relevant
4	rationale		studies (published and unpublished) examining benefits and harms for each intervention
5			
6		6b	Explanation for choice of comparators
7			
8	Objectives	7	Specific objectives or hypotheses
9			
10	Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group),
11			allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)
12			
13			
14	<b>Methods: Participants, interventions, and outcomes</b>		
15			
16	Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will
17			be collected. Reference to where list of study sites can be obtained
18			
19	Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and
20			individuals who will perform the interventions (eg, surgeons, psychotherapists)
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23			
24	Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be
25			administered
26			
27		11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose
28			change in response to harms, participant request, or improving/worsening disease)
29			
30		11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence
31			(eg, drug tablet return, laboratory tests)
32			
33		11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial
34			
35	Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood
36			pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg,
37			median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen
38			efficacy and harm outcomes is strongly recommended
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1	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
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3				
4	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 _____
5				
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7	Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	_____ 8 _____
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10	<b>Methods: Assignment of interventions (for controlled trials)</b>			
11				
12	Allocation:			
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14	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 _____
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19	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 _____
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24	Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	_____ 8, 12 _____
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27	Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	_____ 8,12 _____
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30		17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	_____ 12,13 _____
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34	<b>Methods: Data collection, management, and analysis</b>			
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36	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 _____
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1		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 _____
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4	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 _____
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8	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 _____
9				
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11		20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	_____ 15 _____
12				
13		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 _____
14				
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17	<b>Methods: Monitoring</b>			
18				
19	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 _____
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25		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 _____
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28	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 _____
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31	Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	_____ 15 _____
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35	<b>Ethics and dissemination</b>			
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37	Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	_____ 17 _____
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1	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_____
2				
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4				
5	Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	_____8_____
6				
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8		26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	_____N/A_____
9				
10				
11	Confidentiality	27	How personal information about potential and enrolled participants will be collected, stored, and maintained in order to protect confidentiality before, during, and after the trial	_____17_____
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14	Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	_____19_____
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18	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_____
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21	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_____
22				
23				
24	Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, health care professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	_____17_____
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28		31b	Authorship eligibility guidelines and any intended use of professional writers	_____17_____
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30		31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	_____17_____
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33	<b>Appendices</b>			
34				
35	Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	www.reproflor.dk
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38	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_____
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1 \*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items.  
2 Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons  
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# 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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4 1 **Title:** A protocol for a double-blind, placebo-controlled multicenter trial on the effect of clindamycin  
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6 2 and a live biotherapeutic on the reproductive outcomes of IVF patients with abnormal vaginal  
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8 3 microbiota  
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12 4 **Authors:** Thor Haahr<sup>1\*</sup>, Nina la Cour Freiesleben<sup>2</sup>, Anja Pinborg<sup>3</sup>, Henriette Svarre Nielsen<sup>3</sup>, Vibeke  
13  
14 5 Hartvig<sup>4</sup>, Anne-Lis Mikkelsen<sup>5</sup>, Thomas Parks<sup>6</sup>, Niels Ulbjerg<sup>7</sup>, Jørgen Skov Jensen<sup>8</sup>, Peter  
15  
16 6 Humaidan<sup>1</sup>.  
17

18 7  
19 8 <sup>1</sup>Department of Clinical Medicine, Aarhus University, Denmark and the Fertility Clinic Skive, Skive  
20  
21 9 Regional Hospital, Denmark  
22

23 10 <sup>2</sup>The Fertility Clinic, Department of Obstetrics and Gynecology, Hvidovre Hospital, Copenhagen  
24  
25 11 University Hospital, Denmark  
26

27 12 <sup>3</sup>Fertility Clinic, Rigshospitalet 4071, Copenhagen University Hospital, Copenhagen, Denmark  
28

29 13 <sup>4</sup>Stork Fertility Clinic, Copenhagen, Denmark  
30

31 14 <sup>5</sup>Fertility Clinic, Zealand University Hospital, Denmark.  
32

33 15 <sup>6</sup>Osel, Inc., Mountain View, CA, United States  
34

35 16 <sup>7</sup>Department of Obstetrics and Gynecology, Aarhus University Hospital, Skejby, Denmark  
36

37 17 <sup>8</sup>Statens Serum Institute, Research Unit for Reproductive Microbiology, Copenhagen, Denmark  
38

39 18  
40 19 Trial sponsor:

41 20 Professor Peter Humaidan  
42

43 21 Address: The Fertility Clinic, Skive Regional Hospital, Resenvej 25, 7800 Skive, Denmark  
44

45 22 Phone: +45 78445760  
46

47 23 \*Corresponding author: [thohaa@rm.dk](mailto:thohaa@rm.dk)  
48

49 24 Address: The Fertility Clinic, Skive Regional Hospital, Resenvej 25, 7800 Skive, Denmark  
50

51 25 Phone: +45 78445760  
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53 26 <https://orcid.org/0000-0001-9304-5299>  
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55 27 **Word count:** 4,809 excluding references and tables.  
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4 **28 Abstract:**

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7 **29 Introduction**

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9 **30** Recent studies in *in vitro* fertilization (IVF) patients have associated abnormal vaginal microbiota  
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11 **31** (AVM) with poor clinical pregnancy rates of 6-9% per embryo transfer. The biological plausibility  
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13 **32** for this finding is hypothesized to be ascending infection to the endometrium which in turn hampers  
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15 **33** embryo implantation. The prevalence of AVM ranges from 4-38%. New molecular diagnosis may  
16  
17 **34** offer advantages compared to microscopical diagnosis; however, the important question is whether  
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19 **35** screening and treatment of AVM would improve reproductive outcomes in IVF patients. Herein, we  
20  
21 **36** describe a protocol for an ongoing double-blind, placebo-controlled multicenter trial of IVF patients  
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23 **37** with molecular defined AVM randomized in three parallel groups 1:1:1.  
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30 **39 Methods and analysis**

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32 **40** This is a drug intervention study where IVF patients will be screened for AVM, using a qPCR assay  
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34 **41** targeting *Atopobium vaginae* and *Gardnerella vaginalis*. If positive, patients will be randomized to  
35  
36 **42** one of the three study arms. The first arm consists of clindamycin 300mg x2 daily for 7 days followed  
37  
38 **43** by topical *Lactobacillus crispatus* CTV-05 until clinical pregnancy scan week 7-9. The second arm  
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40 **44** consists of clindamycin and placebo *Lactobacillus crispatus* CTV-05, whereas patients in the third  
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42 **45** arm will be treated with placebo/placebo. We used a superiority design to estimate that active  
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44 **46** treatment in both arms will increase the primary outcome, clinical pregnancy rate per embryo transfer,  
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46 **47** from 20% to 40%. A potential difference between the two active arms was considered exploratory.  
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48 **48** With a power of 80% and an alpha at 5%, the sample size is estimated to be 333 patients randomized.  
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50 **49** A pre-planned interim analysis is scheduled at 167 patients randomized.  
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57 **51 Ethics and dissemination**  
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4 52 All patients have to give informed consent. Dissemination of results is ensured in clinical trial  
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6 53 agreements whether they be positive or not. Ethics committee, Central Denmark Region approved  
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9 54 this protocol.  
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13 56 Registration

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15 57 ICH-GCP monitored trial, EudraCT: 2016-002385-31.  
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20 59 **Keywords: IVF, Microbiota, Bacterial vaginosis, Clindamycin, Gardnerella, RCT,**  
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23 60 **Lactobacillus**  
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4 **61 Article summary: ‘Strengths and limitations of this study’**  
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- 8 **62** • Molecular based diagnosis of abnormal vaginal microbiota was validated in pilot studies  
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10 **63** • The first RCT in IVF patients with abnormal vaginal microbiota investigating treatment effect  
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12 **64** on reproductive outcome of clindamycin and live lactobacillus treatment  
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15 **65** • The *Lactobacillus crispatus* CTV-05 treatment is an investigational live biotherapeutic  
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17 **66** product regulated by the US FDA  
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19 **67** • ICH-GCP monitored trial  
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22 **68** • Inclusion criteria are relatively broad  
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## 69 Introduction

70 Bacterial Vaginosis (BV) is a common vaginal dysbiosis in reproductive age women with a  
71 prevalence of 29% (95% CI 27-31%) as reported in a US population based survey, N=3739 (1). It is  
72 well-known that there is a higher BV rate among African-Americans compared to Caucasian women  
73 (1). However, this finding could be affected by the fact that asymptomatic African-Americans seem  
74 to have a more diverse physiological vaginal microbiota as compared to Caucasians(2,3). Other risk  
75 factors include frequent vaginal douching and number of lifetime sex partners(1). In the *in vitro*  
76 fertilization (IVF) population, a recent meta-analysis (N=2980) reported that the prevalence of BV  
77 exhibited huge interstudy heterogeneity ranging from 4-38% (4). In this study, BV was clearly  
78 associated with tubal factor infertility, but not endometriosis. The most recent studies using a  
79 molecular based analysis to determine an abnormal vaginal microbiota observed a prevalence of 17%  
80 and 28%, respectively (5,6). It is known that despite diagnosed with BV by the gold standard Nugent  
81 method (7), more than 80% of BV positives remain asymptomatic(1). Hence, the important question  
82 is whether the many asymptomatic BV cases should be screened and treated. Clinical guidelines  
83 recommend screening and treatment for BV in patients undergoing gynecological surgery or invasive  
84 diagnostic procedures through the vagina to minimize infection(8). However, most clinical guidelines  
85 do not support screening and treatment for asymptomatic BV to optimize reproductive outcome – a  
86 topic which has been thoroughly investigated in obstetric populations for preterm birth prevention  
87 (9,10). Today, a new frontier is emerging with optimized molecular based diagnosis of bacterial  
88 dysbiosis and new treatment possibilities including well-studied and well-characterized probiotics –  
89 that have been designated “live biotherapeutic products” by FDA(11,12).

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

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

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

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117 relative abundances which does not sufficiently take into account differences in the total abundance  
118 (21–23).

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

126 Finally, a recent systematic review and meta-analysis reported that the use of additional probiotic  
127 treatment alongside standard treatment of BV could improve BV cure rates, RR = 1.28, 95% CI (1.05,  
128 1.56) (27). However, due to primarily poor study quality(28), there is currently no consensus on  
129 which vaginal *Lactobacillus* product, if any, should be recommended (29).

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

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4 141 that time in Phase 2 development(11). Recently, adjuvant LACTIN-V after vaginal metronidazole  
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7 142 was reported to lower BV recurrence rates in a phase 2b trial, RR 0.66 (95%CI 0.44-0.87)(35).  
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9 143  
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11 144 Taking into consideration the abovementioned evidence, the research question of the present study  
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14 145 is: does antibiotic alone or in combination with live biotherapeutic treatment of an abnormal vaginal  
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16 146 microbiota improve the reproductive outcomes of IVF patients? The intervention is clindamycin  
17  
18 147 either alone or in combination with LACTIN-V, a live biotherapeutic product containing *L.crispatus*  
19  
20 148 CTV-05 (11). The study is designed as a double-blind, placebo-controlled multicenter trial of three  
21  
22  
23 149 parallel groups randomized 1:1:1. Randomization is by computer generated code and allocation  
24  
25 150 concealment is performed by the pharmacy who will send out medication to the participating clinics  
26  
27 151 with identical appearance and randomization numbers. The randomization code is with the pharmacy  
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29  
30 152 and can only be opened in case of emergency by the principal investigators or as planned by the  
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32 153 sponsor-investigator. The benefit of the intervention would potentially lead to increased pregnancy  
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34 154 rates and, for those suffering from symptomatic BV, also relief of BV symptoms. In contrast, the  
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36 155 expected adverse reactions of concern are especially gastrointestinal symptoms caused by  
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39 156 clindamycin, whereas LACTIN-V might cause increased vaginal discharge but is otherwise not  
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41 157 expected to cause adverse reactions as based on prior studies(11,36).  
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## 46 159 **Methods and analysis**

### 48 160 *Setting and eligibility criteria*

50 161 The present trial is conducted at four University affiliated clinics and one private fertility clinic in  
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53 162 Denmark. The list of study sites is available with EudraCT clinical trial identifier: 2016-002385-31,  
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55 163 first registration day 2016-07-11. The current version of the protocol is 9, 2020-02-07. Patients are  
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4 164 enrolled in a cohort study (Clinicaltrials.gov NCT03420859) from which we will recruit patients for  
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6  
7 165 the randomized trial (EudraCT: 2016-002385-31). Eligibility criteria are described in Table 1.

8  
9 166 In brief, IVF patients attending their first, second or third IVF stimulation cycle or embryo transfer  
10  
11 167 therefrom will be approached for informed consent by the study nurse or treating physician. Patients  
12  
13  
14 168 are told about the project in a private room with the right to have an assessor, allowing time to reflect  
15  
16 169 whether they will participate. They are handed out written information material with a link to the  
17  
18 170 study website with full information about the project – [www.reproflor.dk](http://www.reproflor.dk). The vaginal swab can be  
19  
20  
21 171 taken by the treating physician or the patient herself after careful instruction. In this case, patients are  
22  
23 172 instructed to place the swab at least 8 cm into the vaginal cavity for 10 seconds and rotate. This is to  
24  
25 173 ensure that the vaginal bacteria in the fornix or in its close proximity will be caught by the flocked  
26  
27 174 swab. Subsequently, the vaginal swab will be sent to a central laboratory at Statens Serum Institut,  
28  
29  
30 175 Copenhagen to be analyzed for AVM within 7 days as previously reported (5). If AVM positive,  
31  
32 176 patients are asked to provide informed consent that they are willing to participate in the randomized  
33  
34 177 controlled trial. Patients should ideally be randomized on the first day of ovarian stimulation with  
35  
36 178 exogenous gonadotropins, allowing a minimum of 12 days of study medication to be acceptable for  
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38  
39 179 inclusion in the study. If elective frozen embryo transfer (FET) is planned, patients should be  
40  
41 180 randomized during the first days of the FET cycle allowing for at least 12 days of study medication.  
42  
43 181 If patients enter the trial and have less than 12 days of study medication despite the abovementioned  
44  
45 182 inclusion criteria (e.g. when hormonal stimulation is shortened due to an unexpected ovarian  
46  
47  
48 183 response), it is considered a protocol violation and they will be excluded from the per protocol  
49  
50 184 analysis, i.e. not from ITT-analysis.

### 51 52 53 185 *Interventions*

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55 186 Active Treatment 1: **Oral Clindamycin** 300 mg 2 times per day for 7 days followed by **LACTIN-V**  
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58 187 (Osel, Inc.) until completion of the clinical pregnancy scan at week 7-9. LACTIN-V containing *L.*  
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188 *crispatus* CTV-05 (2 x 10<sup>9</sup> CFU/dose, 200 mg, delivered with pre-filled, single use vaginal applicators) regimen is once daily from the clindamycin stop for 7 consecutive days.

190 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.

193 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.

196 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 Mannitolium. The placebo LACTIN-V formulation contains the same inactive ingredients as LACTIN-V, without *Lactobacillus crispatus* CTV-05.

#### 206 *Labelling and packaging*

207 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

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

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

### 228 *Outcomes*

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



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4 236 sequencing methods. The colonization of the *L. crispatus* CTV-05 strain will also be investigated  
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6 237 using qPCR. It is pre-planned that reproductive outcome analysis will lead to a first publication by  
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9 238 itself, whereas the more laborious sequencing results will arrive in a later publication. Later, we plan  
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11 239 to investigate cumulative live birth results of subsequent transfer of spare frozen thawed embryos of  
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13 240 patients attending the study in a fresh cycle.

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17 241 *Sample size*

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19 242 In 2014, the average clinical pregnancy rate per embryo transfer in our fertility clinic was  
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21 243 approximately 40% for an IVF cycle. In our pilot study(5), the adjusted odds ratio between the AVM  
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23 244 group and the normal group was 0.06, 95% CI (0.01–0.47) for clinical pregnancy per embryo transfer.  
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26 245 Taken together, we estimated a superiority design where women in each AVM arm and treated with  
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28 246 active medication will have at least a 40% chance for clinical pregnancy per embryo transfer as  
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31 247 compared to the placebo arm which was estimated to have a maximum of 20% chance of clinical  
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33 248 pregnancy/transfer. By two samples proportion test with a power of 80% and an alpha at 5%, the aim  
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35 249 was to randomize 92 patients in each group. A potential difference between the two active arms was  
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38 250 considered exploratory and consequently this was not part of the power calculation, but we decided  
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40 251 to include the same number of patients in the active/active arm to investigate a potential added benefit  
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42 252 of live biotherapeutic treatment.

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45 253 An interim analysis will be performed, and to adjust for this, we add 10% to the 92 randomized  
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47 254 patients as suggested in Wittes et al (37). Approximately 10% of couples will have no embryos for  
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49 255 transfer; we adjusted for this by adding another 10% to each randomized group, i.e.  $19 + 92 = 111$   
50  
51 256 (see Figure 1). Considering an estimated 20% AVM rate, a total of 1850 IVF patients will be screened  
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53  
54 257 to randomize 333 patients (three arms). It was estimated that inclusion will be distributed according  
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56 258 to the size of the centers. Furthermore, we make the following assumptions: i) very limited loss to  
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58 259 follow-up, ii) near full compliance to study medication and iii) homogeneity in the treatment effect.  
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#### 260 *Allocation*

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

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

#### 278 *Data collection methods*

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

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4 283 procedures; 3) automated export procedures for seamless data downloads to common statistical  
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6 284 packages; and 4) procedures for data integration and interoperability with external sources. All data  
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9 285 collectors of the study have to be trained in Good Clinical Practice (GCP) procedures and as minimum  
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11 286 to have passed the course provided by the Danish GCP institution. All inclusion and exclusion criteria  
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13 287 as well as outcome data will be monitored by external GCP monitors to ensure optimal data quality.  
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16 288 Data collection forms and other data entry related information can be requested from the  
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18 289 corresponding author.  
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22 290 Protocol deviations have to be stated in the eCRF. Loss to follow-up is unlikely for patients in IVF  
23  
24 291 treatment who will be highly motivated to come to the clinic. However, patients who are not pregnant  
25  
26 292 may opt to go to other clinics for further treatment and, thus will be lost to follow-up. The eCRF  
27  
28 293 instruments have range checks and other data rules that have to be passed to ensure optimal data  
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30  
31 294 input. In case of missing outcome data we plan to use the framework proposed by White et al  
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33 295 (2011)(40).  
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### 36 296 *Statistical methods*

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39 297 The total significance level of the study was set to be 5%. Based on the O'Brien-Fleming method, the  
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41 298 total significance was split into 0.1% for the interim and 4.9% for the final analysis (37). Therefore,  
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43 299 a p-value with 99.9% confidence interval is calculated in the interim analysis to test the possible  
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46 300 effect of one or both active treatment arms (combined or separately) on clinical pregnancy rate per  
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48 301 embryo transfer (primary outcome) compared to placebo. A Walds Chi-square test for possible effect  
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50 302 will be conducted comparing all 3 arms. Moreover, four analyses: 1) active/active vs active/placebo,  
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53 303 2) active/placebo vs placebo/placebo, 3) active/active vs placebo/placebo, 4) average effect of  
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55 304 active/active AND active/placebo vs placebo/placebo will be done as first a crude estimate and then  
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57 305 secondly adjustment with confounders for double embryo transfer, quality of the embryo  
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4 306 (Cleavage/blastocyst), female age (continuous variable) and center effect (public/private). If the trial  
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7 307 is discontinued according to the criteria stated under the paragraph “interim analysis”, a full statistical  
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9 308 analysis will be made as described below. First, a Walds Chi-square test for possible effect of active  
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11 309 treatment on clinical pregnancy rate [primary outcome] will be made across all 3 groups. Moreover,  
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13 310 pairwise comparisons for the abovementioned 4 tests will be made with odds ratios (OR) and risk  
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16 311 ratios (RR) and 94.9% confidence intervals (CI’s) calculated from logistic and linear regressions  
17  
18 312 models, taking abovementioned confounding factors into account. Analyses will be conducted at  
19  
20 313 intention-to-treat (ITT) level defined as all randomized patients who have an embryo transfer  
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23 314 following study treatment cycle, including also deferred/frozen embryo transfers due to e.g. OHSS  
24  
25 315 risk. Patients are excluded from ITT analysis if they do not have embryos for transfer or in case  
26  
27 316 embryo transfer is deferred to a later stage than actual study treatment which is approximately 9weeks  
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29  
30 317 total. Per protocol analysis will also be considered i.e. an analysis for patients having an embryo  
31  
32 318 transfer as described above and not violating the protocol as described herein. Intention-to-treat is  
33  
34 319 considered the primary analysis.  
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#### 36 320 37 38 39 321 *Interim analysis*

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42 322 An interim analysis as described above will be performed to evaluate the clinical pregnancy rate per  
43  
44 323 embryo transfer when 167 patients have been randomized and completed the study for primary  
45  
46 324 outcome evaluation. If study medication is affecting the clinical pregnancy rate statistically  
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48 325 significant in either of the analyses, the trial will discontinue. Furthermore, the drop-out rate will be  
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51 326 evaluated considering both the number of positive AVM declining to participate and the number of  
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53 327 patients who drop-out after randomization. A drop-out rate above 20% will lead to discontinuation.  
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55 328 External statisticians from Aarhus University, Denmark will conduct the interim analysis. Only a  
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58 329 small study board, including sponsor and principal investigators will know the result of the interim  
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330 analysis. Sponsor-investigator make the decision to continue or discontinue the trial. The study will  
331 continue in case there is no statistical difference in either of the tests, drop-out rate is acceptable, and  
332 the logistical requirements to finish the study can be met within reasonable time considering e.g.  
333 expiry of study medication and time to recruit all patients. The time to undertake the interim analysis  
334 and the decision to continue or discontinue is approximately 3 weeks.

#### 336 *Data monitoring*

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

#### 344 *Adverse events and reactions*

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

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4 354 effects on the day of the clinical pregnancy scan. Moreover, patients are asked if they have symptoms  
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6 355 at all study visits and if these symptoms are considered adverse reactions they are recorded in the  
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9 356 eCRF, including an adverse reaction judgement from the treating physician.  
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#### 11 357 12 13 358 *Serious adverse events (SAE)*

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16 359 At each center, primary investigators will report serious adverse events (SAE) to sponsor within 24  
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18 360 hours by email or phone. Sponsor ensures that all Suspected Unexpected Serious Adverse Reactions  
19  
20 361 (SUSARs) that are fatal or life-threatening are recorded and reported to the Danish Medicines Agency  
21  
22 362 and the scientific Ethics Committee as soon as possible and no later than 7 days after the sponsor  
23  
24 363 became aware of such possible side effect. Within 8 days after a SUSAR has been reported, the  
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26 364 sponsor must notify the Danish Medicines Agency and the Ethics Committee with all relevant  
27  
28 365 information on the follow-up of any SUSAR that may occur. All other unexpected serious or  
29  
30 366 suspected serious adverse reactions will be reported to the Danish Medicines Agency and the  
31  
32 367 scientific Ethics Committee within 15 days after the sponsor become aware of these. An annual safety  
33  
34 368 report regarding the trial participants will be performed, consisting of serious adverse event suspected  
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36 369 to be related to the investigational drug will be submitted to Danish Medicines Agency and the Ethics  
37  
38 370 Committee. At end of study, all adverse events and serious adverse events will be reported according  
39  
40 371 to regulations in Denmark.  
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#### 46 372 47 48 373 *Ethics*

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50 374 Approvals from the Regional Scientific Ethical Committee, Central Denmark Region (M-2017-157-  
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52 375 17), the Danish Data Protection Agency (1-16-02-790-17) and Danish Medicines Agency (2016-  
53  
54 376 002385-31) were obtained prior to trial initiation December 7<sup>th</sup>, 2017. Danish law will be complied  
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56 377 with regarding the handling of personal information. Protocol amendments will be provided to the  
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378 relevant parties, including the Regional Scientific Ethical Committees and Danish Medicines Agency.

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

#### 384 *Access to data*

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

#### 391 *Dissemination*

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

#### 397 *Trial status*

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

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4 402 *Patients and public involvement*  
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8 403 Neither patients nor the public was directly involved in the planning of this trial.  
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13 405 *Author Contributions*  
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15  
16 406 PH, JSJ, NU and TH were the primary writers and inventors of this protocol. PH is the sponsor-  
17  
18 407 investigator. TP provided information on LACTIN-V and contributed to the study design and protocol  
19  
20 408 development. TH, NICF, AP, VH, ALM and HSN are primary investigators at the involved clinics  
21  
22  
23 409 and contributed to the protocol and amendments during the initiation phase of the study.  
24  
25

26 410 *Acknowledgements*  
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29  
30 411 The authors wish to thank all contributing patients, clinicians, nurses, lab technicians etc. involved  
31  
32 412 with this study. Specifically, we thank the personnel at the reproductive microbiology laboratory at  
33  
34 413 Statens Serum Institut, Copenhagen and the Fertility Clinics in Skive, Hvidovre, Rigshospitalet and  
35  
36 414 Stork. Moreover, we wish to thank Aparna Udipi from Aarhus University, Denmark for statistical  
37  
38  
39 415 advice concerning this trial. Finally, we wish to thank Kristian Nielsen and Glostrup Pharmacy,  
40  
41 416 Denmark for their contribution concerning medication allocation to the clinics.  
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45 417 *Funding statement*  
46

47 418 PH, TH and JSJ received - through their institutions - an unrestricted research grant from Osel, Inc.  
48  
49 419 which produces LACTIN-V, the live biotherapeutic product containing *Lactobacillus crispatus* CTV-  
50  
51 420 05. A clinical trial agreement was made ensuring full data ownership and publication rights to PH.  
52  
53  
54 421 Other grants were Axel Muusfeldts foundation Grant number 2018-1311, A.P. Møller Foundation for  
55  
56 422 Medical research Grant number 18-L-0173, Central Denmark Region Hospital MIDT foundation  
57  
58 423 Grant number 421506 and a PhD scholarship from Aarhus University, Denmark to TH.  
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424 *Competing interests statement*

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

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11 558 **Table 1: In- and exclusion criteria**

<b>Inclusion criteria:</b>	<b>Exclusion criteria:</b>
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)

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56 560 **Table 2: Study medication scheme**  
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	Clindamycin “Alternova”	LACTIN-V™
Dose	300mg	200mg/2x10 <sup>9</sup> 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.
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\* Patients not pregnant are informed to contact the department in case of any LACTIN-V related side-effect.

**Table 3:** Study timeline

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

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<b>Clindamycin</b>	X	←	→			
<b>Lactin-V</b>			X	←	→	
<b>IVF treatment</b>	X	←	→			
<b>ASSESSMENTS:</b>						
<b>Vaginal swab</b>	X		X	X	X	X

For peer review only

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567 **Figure 1: Study Flowchart**

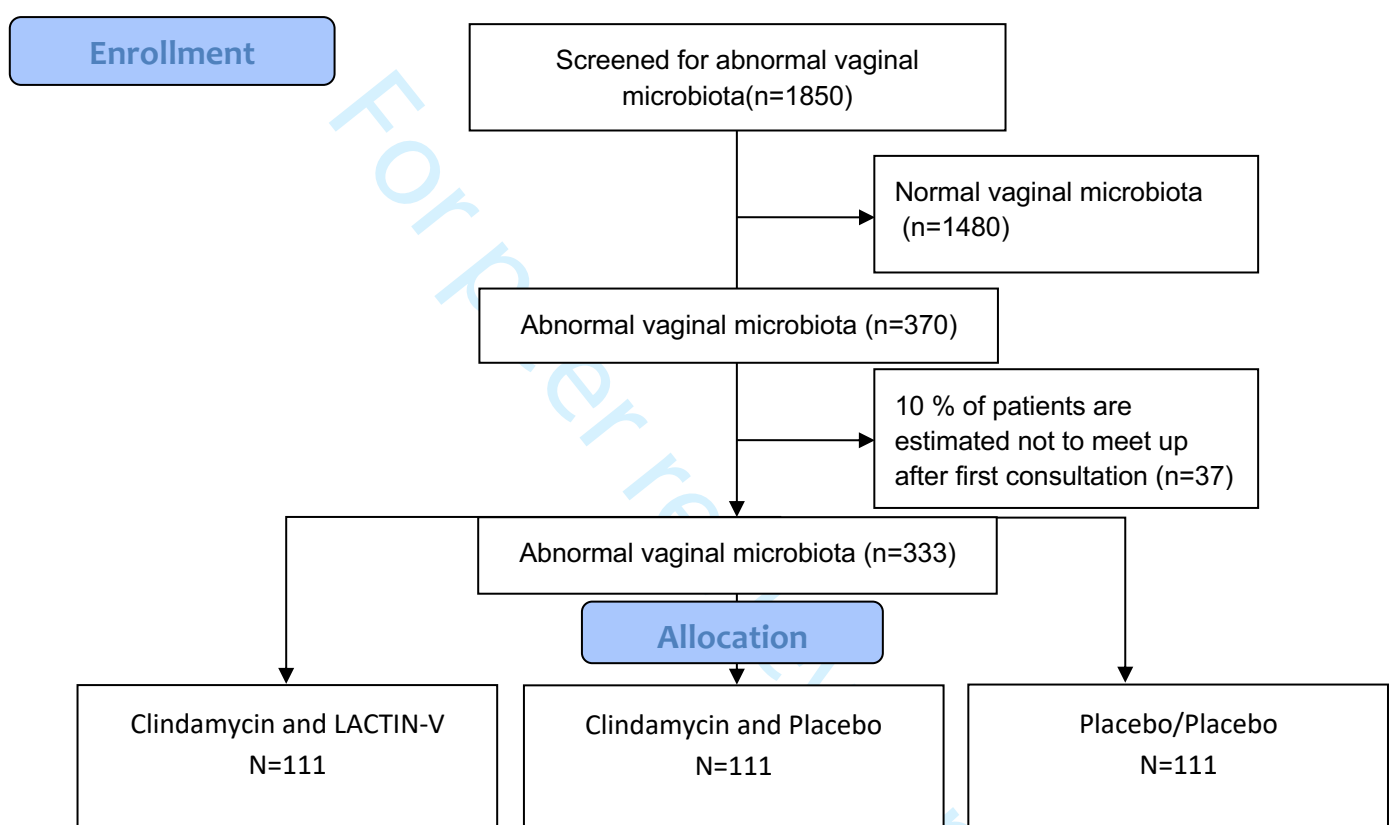
568 See figure attached.

569 Figure legend: We add 20% more patients to the powered sample size of 92 randomized patients to  
570 adjust for couples who have no embryos for transfer and to adjust for the interim analysis, i.e.  $19 +$   
571  $92 = 111$ . Considering an estimated 20% AVM rate, a total of 1850 IVF patients will be screened to  
572 randomize 333 patients (three arms).

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### CONSORT 2010 Flow Diagram





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4 1 Appendix 1  
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6 2 Samples will be collected from the vagina as outlined in table 3 and in the protocol. Moreover, we  
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9 3 collect seminal samples from the partner at oocyte retrieval day and a fecal specimen from the  
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11 4 newborn diaper within 3 weeks from birth. Vaginal swabs that are taken at the clinic are taken with  
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13 5 a flocked swab and placed in Eswab (Copan™) except for the seminal sample, which is kept in a  
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15 6 1.8 mL cryotube, Nunc™, cryotube™, Thermo Scientific. The vaginal swabs taken at home in  
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17 7 pregnancy after birth and the fecal swab are taken by the patient herself using flocked swabs and  
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19 8 placed in an eNAT tube (Copan™). All the vaginal samples are taken after these instructions: the  
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21 9 vaginal swab has to be placed at least 8 cm into the vaginal cavity for 5 seconds and rotated  
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23 10 clockwise. The fecal swab has to be a deep sample and has to be rotated in the feces for 5 seconds.  
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25 11 The semen sample is collected after homogenization and then 200 microL are transferred to the  
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27 12 cryotube using a pipette.  
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29 13 Only the vaginal swab at screening visit is sent immediately to the central laboratory at Statens  
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31 14 Serum Institute for analysis within 7 days. All vaginal swabs taken during study visits and the  
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33 15 seminal sample are immediately frozen at minus 80 degrees at the respective clinics. All samples  
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35 16 are stored at the clinics until they are to be transferred by dry ice shipment to the central laboratory.  
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37 17 Finally, the home-samples (vaginal samples and the fecal sample) are sent with return envelopes to  
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39 18 the patients when they are supposed to take the sample and samples are received back at the  
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41 19 Fertility Clinic in Skive, Denmark where they are stored at -80 degrees. For the sub-study explained  
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43 20 in the protocol concerning the 20 patients taking daily vaginal home-samples (Copan ESwab™),  
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45 21 these are kept in the patient's own freezer (-20 degrees) until embryo transfer day where the first  
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47 22 12-16 samples will be delivered to the clinic and stored at minus 80 degrees. The remaining 5-9  
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49 23 samples are kept in the patient's own freezer (-20) until her next visit which may be either hCG test  
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51 24 day, clinical pregnancy scan or a visit during the subsequent cycle due to no pregnancy.  
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STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

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

Section/item	Item No	Description	Addressed on page number
<b>Administrative information</b>			
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 ___

## 1 Introduction

2			
3	Background and	6a	Description of research question and justification for undertaking the trial, including summary of relevant
4	rationale		studies (published and unpublished) examining benefits and harms for each intervention
5			_____ 5-8 _____
6		6b	Explanation for choice of comparators
7			_____ 7 _____
8	Objectives	7	Specific objectives or hypotheses
9			_____ 8 _____
10	Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group),
11			allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)
12			_____ 8 _____
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14	<b>Methods: Participants, interventions, and outcomes</b>		
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16	Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will
17			be collected. Reference to where list of study sites can be obtained
18			_____ 8 _____
19	Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and
20			individuals who will perform the interventions (eg, surgeons, psychotherapists)
21			_____ 8-9 _____
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24	Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be
25			administered
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27		11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose
28			change in response to harms, participant request, or improving/worsening disease)
29			_____ 14 _____
30		11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence
31			(eg, drug tablet return, laboratory tests)
32			_____ 10,15 _____
33		11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial
34			_____ 10 _____
35	Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood
36			pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg,
37			median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen
38			efficacy and harm outcomes is strongly recommended
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1	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
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4	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 _____
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7	Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	_____ 8 _____
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10	<b>Methods: Assignment of interventions (for controlled trials)</b>			
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12	Allocation:			
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14	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 _____
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19	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 _____
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23	Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	_____ 8, 12 _____
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27	Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	_____ 8,12 _____
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30		17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	_____ 12,13 _____
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34	<b>Methods: Data collection, management, and analysis</b>			
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36	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 _____
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1		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 _____
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4	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 _____
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8	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 _____
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11		20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	_____ 15 _____
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13		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 _____
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17	<b>Methods: Monitoring</b>			
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19	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 _____
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25		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 _____
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28	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 _____
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31	Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	_____ 15 _____
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35	<b>Ethics and dissemination</b>			
36				
37	Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	_____ 17 _____
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1	Protocol	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_____
2	amendments			
3				
4				
5	Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	_____8_____
6				
7				
8		26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	_____N/A_____
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11	Confidentiality	27	How personal information about potential and enrolled participants will be collected, stored, and maintained in order to protect confidentiality before, during, and after the trial	_____17_____
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14	Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	_____19_____
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18	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_____
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21	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_____
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23				
24	Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, health care professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	_____17_____
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29		31b	Authorship eligibility guidelines and any intended use of professional writers	_____17_____
30				
31		31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	_____17_____
32				
33	<b>Appendices</b>			
34				
35	Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	www.reproflor.dk
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38	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_____
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1 \*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items.  
2 Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons  
3 "[Attribution-NonCommercial-NoDerivs 3.0 Unported](https://creativecommons.org/licenses/by-nc-nd/3.0/)" license.  
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