Title: Does intervention with clindamycin and a live biotherapeutic drug containing *Lactobacillus crispatus* impact the reproductive outcome of IVF patients with abnormal vaginal microbiota: a randomised double-blind, placebo-controlled multicentre trial

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Abstract (300 words, max 300)

Background

Genital tract microbiota is associated with reproductive outcomes in IVF patients. Thus, the aim of the present trial was to investigate whether intervention with antibiotics and live lactobacilli would improve clinical pregnancy rates in IVF patients with abnormal vaginal microbiota (AVM).

Methods

Randomised, double-blind, placebo-controlled drug intervention trial at four fertility clinics in Denmark. IVF patients were diagnosed with AVM defined by high quantitative PCR loads of *Fannyhessea vaginae* and *Gardnerella spp*. were randomised into three parallel groups 1:1:1. Group one (CLLA) received clindamycin 300 mg ×2 daily for 7 days followed by vaginal *Lactobacillus crispatus* until the day of pregnancy scan, using the investigational drug LACTIN-V. Group two (CLPL) received clindamycin and placebo LACTIN-V, and finally, group three (PLPL) received an identical placebo of both drugs. The primary outcome was ultrasound proven foetal heartbeat in gestational week 7–9. Primary analysis was modified intention to treat (mITT) defined as all patients with embryo transfer less than 63 days from the active treatment start until day 1 in the embryo transfer cycle.

EU Clinical trials register: 2016-002385-31; Completed.

Findings

Between December 7th 2017 and September 21 2022, a total of 1535 patients were screened, and 338 patients were randomised. In the mITT analysis, the clinical pregnancy rate per embryo transfer was 42% (95%CI 32-52%), 46% (95%CI 36-56%) and 45% (95%CI 35-56%) in the CLLA, CLPL, PLPL groups respectively. The average effect of the two active groups compared to placebo was close to

unity, adjusted risk ratio 0.98 (95%CI 0.74-1.29). Patients in the active treatment arms significantly more often reported abdominal pain and diarrhoea compared to patients in the PLPL group.

Interpretation

The present RCT does not support a policy of screening and treating IVF patients for BV-type vaginal dysbiosis prior to embryo transfer in order to improve reproductive outcomes.

Funding

Through their institutions, PH, TH and JSJ received an unrestricted research grant from Osel, Inc. which produces LACTIN-V, the live biotherapeutic product containing *Lactobacillus crispatus* CTV-05. A clinical trial agreement was made ensuring full data ownership and publication rights to PH. Other grants were Axel Muusfeldts Foundation grant number 2018-1311, A.P. Møller Foundation for Medical Research grant number 18-L-0173, Central Denmark Region Hospital MIDT Foundation grant number 421506 and a PhD scholarship from Aarhus University, Denmark to TH.

Research in context

Evidence before this study

Recent studies have shown an association between genital tract dysbiosis and poor reproductive outcomes in infertile patients undergoing IVF treatment. A recently updated systematic review and meta-analysis reported that IVF patients with vaginal dysbiosis have a reduced clinical pregnancy rate per embryo transfer to a relative risk of 0.82 (95%CI 0.70-0.95, N=6558 patients, 25 studies) compared to IVF patients without vaginal dysbiosis. The biological rationale may be that the dysbiotic vaginal microbiota ascends to the endometrium subsequently hampering embryo implantation and leading to implantation failure or early pregnancy loss. The most common vaginal dysbiosis is bacterial vaginosis (BV), which may be sub-clinically prevalent in approximately 20% of IVF patient and it is evident that women with BV are more likely to have endometrial bacterial colonization compared to women without BV. For many years optimal treatment of symptomatic BV has been a conundrum, however, recently it was reported that using LACTIN-V containing live *Lactobacillus crispatus* as an add-on to standard antibiotic treatment improved BV cure rates at 12- and 24-weeks follow-up.

A recent Cochrane systematic review and meta-analysis from November 2023 on the use of antibiotics prior to embryo transfer reported no studies - besides the research protocol of the present study - aiming to investigate a targeted approach of screening and treating genital tract dysbiosis in IVF patients. Thus, the hypothesis that a targeted approach of screening and treating BV-type vaginal dysbiosis might improve reproductive outcome in IVF patients is a topic in which current evidence is not sufficient to inform clinical practice. Consequently, the aim of the present study was to investigate whether treatment of BV-type vaginal dysbiosis with a combination of clindamycin and LACTIN-V would improve reproductive outcomes in IVF patients.

Added value of this study

The present randomised controlled trial systematically screened 1535 IVF patients for a BV-type vaginal dysbiosis, randomising 338 patients into three groups 1:1:1 receiving either clindamycin and LACTIN-V, clindamycin and placebo LACTIN-V or placebo/placebo. Compared to the placebo/placebo group, we report minor non-significant differences in the active treatment groups considering the reproductive outcome, including live birth rate. In contrast, significantly more patients reported abdominal pain, diarrhoea, and vaginal itching in the active treatment groups.

Implications of all the available evidence

Despite the reports of a significant association between genital tract dysbiosis and poor reproductive outcomes in IVF patients, the present study does not support a clinical benefit of treatment with clindamycin alone or in combination with LACTIN-V in IVF patients diagnosed with BV-type vaginal dysbiosis.

Introduction

A symbiotic relationship exists between reproductive age women and "normal" *Lactobacillus* dominant vaginal microbiota, reducing the acquisition of sexually transmitted infections such as *Chlamydia*, Herpes, HPV and HIV[1–4]. In contrast, the reproductive implications of subclinical non-*Lactobacillus* dominant vaginal dysbiosis is less clear. A non-*Lactobacillus* dominant vaginal dysbiosis can be defined either by molecular methods or by microscopy, predominantly including bacterial vaginosis (BV) and aerobic vaginitis (AV) type bacteria[5]. Vaginal dysbiosis is relatively common in the IVF population as seen in a recent systematic review and meta-analysis, including 26 studies, in which the prevalence of vaginal dysbiosis was 19% (95%CI 18-20%) (ref=submitted).

The underlying hypothesis of the present study is that vaginal dysbiotic microbiota ascends to the endometrium, hampering embryo implantation and early pregnancy. As evidence behind this hypothesis, it has been reported that patients with BV have an increased risk of typical BV-type bacteria (e.g. *Gardnerella*) in the endometrium[6]. In one study, the odds ratio was 5.7 (95% CI, 1.8–18.3) for endometrial bacterial colonization in women with BV as compared to women without BV[7]. Moreover, in infertile women, there may be a link between the endometrial bacterial composition and chronic endometritis – interestingly including both BV- and AV-type bacteria [8,9].

In the IVF population, a recent systematic review and meta-analysis (submitted) reported a significantly reduced clinical pregnancy rate per embryo transfer in patients with vaginal dysbiosis, RR=0.82 (95%CI 0.70-0.95, I^2=49%) as well as an increased risk of early pregnancy loss (RR 1.49 ;95%CI 1.15-1.94, I^2=38%) when compared to IVF patients without vaginal dysbiosis. Moreover, recent studies, exploring endometrial microbiota in IVF patients reported that *Lactobacillus* dominant – especially *Lactobacillus crispatus* dominant – endometrial microbiota is associated with the most

optimal reproductive outcome [10,11]. In contrast, the typical BV- and AV-type endometrial dysbiosis were associated with poor reproductive outcomes[11]. Finally, in a prospective cohort of women undergoing ultrasound scan during the first trimester, euploid pregnancy losses were associated with a *Lactobacillus* depleted vaginal microbiota [12].

In modern IVF, even young women <35 years cannot be expected to exceed implantation rates of 60-70% following euploid blastocyst transfer[13]. Moreover, it is widely accepted that the remaining approximately 30-40% of implantation failures are a black box which predominantly may be caused by uterine factors. In this aspect, one might hypothesize that genital tract dysbiosis could contribute to failed embryo implantation and early pregnancy loss. However, the evidence is inconclusive whether genital tract dysbiosis is causally involved in infertility. Apart from standard antibiotic treatment of BV, a so-called live biotherapeutic product containing live *L. crispatus* CTV-05 (LACTIN-V) has recently been proven to significantly reduce recurrent BV after 12 and 24 weeks posttreatment when used as an add-on to standard antibiotic therapy[14]. However, a potential effect on reproductive outcome of antibiotic treatment and LACTIN-V in IVF patients has not been investigated. Thus, we aimed to investigate whether diagnosis and treatment of a BV-type vaginal dysbiosis prior to embryo transfer might improve reproductive outcomes in IVF patients.

Methods

Study design

The present randomised, double-blind, parallel-group, placebo-controlled trial (RCT) was conducted at three University-affiliated fertility clinics and one private fertility clinic in Denmark. The EudraCT clinical trial identifier is 2016-002385-31; first registration day 2016-07-11. The current version of the protocol is 11, 2021-04-29. The trial protocol was published in 2020 [15]. The primary centre from which the ethical approval was accepted was Skive Regional Hospital.

159

160 *Patients*

161 Inclusion criteria were, female aged 18-42 years old, BMI <35, negative chlamydia/gonorrhoea test
162 within 6 months of IVF treatment, normal cervical smear within 3 years of IVF treatment, written
163 informed consent, and abnormal vaginal microbiota (AVM), according to criteria stated below with
164 the vaginal swab being obtained less than 90 days before the randomisation day. Exclusion criteria
165 were Hepatitis/HIV positivity, intrauterine malformations, severe concomitant disease, including
166 inflammatory bowel disease. Patients were not allowed to take vaginal probiotics, neuromuscular
167 blocking drugs, immunosuppressive medication, or investigational drug preparations other than the
168 study product. Each patient could only participate once. Patients were approached when attending
169 their first, second or third IVF stimulation cycle or frozen embryo transfer (FET) therefrom. If
170 eligible, a vaginal swab was collected by the treating physician or the patient herself using the
171 ESwab™ (Copan, Brescia, Italy); the ESwab™ was subsequently shipped for central testing at
172 Statens Serum Institut, Copenhagen where it was analysed for AVM according to criteria previously
173 reported[16].
174 Briefly, AVM is a qPCR-based diagnosis, targeting a high absolute abundance of *Gardnerella* spp.
175 and *Fannyhessea vaginae* with 93% sensitivity and 93% specificity compared to BV diagnosed by
176 Nugent score (Gold standard). Further laboratory details can be seen in the **supplement**. Patients
177 were randomised on the first day of ovarian stimulation or during the first days of elective FET
178 allowing for at least 12 days of study medication.

179

180 *Randomisation, masking and intervention*

181 The present RCT randomised three parallel groups 1:1:1. The first active treatment arm (CLLA)
182 consisted of oral clindamycin 300 mg two times daily for 7 days followed by vaginal LACTIN-V

(Osel Inc.). LACTIN-V is an investigational drug that contains *L. crispatus* CTV-05 (2×10^9 CFU/dose, 200 mg, delivered with pre-filled, single-use vaginal applicators) which was applied vaginally once daily from the last day of clindamycin treatment for a total of 7 consecutive days; thereafter twice weekly up to a total usage of 21 applicators or until completion of the clinical pregnancy scan at week 7–9.

The second active treatment arm (CLPL) consisted of oral clindamycin 300 mg, twice daily for 7 days followed by LACTIN-V placebo as in the regimen described above.

Finally, the inactive treatment arm (PLPL) consisted of identically appearing clindamycin placebo and LACTIN-V placebo. Placebo clindamycin consisted of encapsulated mannitol. The placebo LACTIN-V formulation contained the same ingredients as LACTIN-V, without *L. crispatus* CTV-05.

Randomisation code and allocation concealment was performed by the pharmacy providing the study medication, using a computer-generated code. The identical medication packs were labelled with the randomisation number and received at the IVF centres from the pharmacy in blocks of 15, five of each of the three treatments, to secure equal distribution of treatment arms at the centres. The randomisation number was continuous and unique for each patient, and it was prelabelled from the pharmacy before distribution to the clinics; thus, both patients and study personnel were blinded to the intervention. The pharmacy did not play any role in or had knowledge about the IVF treatment. The first person to investigate the unblinded dataset was an external statistician at Aarhus University, Denmark who analysed the reproductive outcome in table 2. After that, data was unblinded for the principal investigators.

Outcomes

The primary outcome was clinical pregnancy rate, defined as an ultrasound proven intrauterine foetal heartbeat during gestational week 7–9. Secondary outcomes were live birth rate, biochemical pregnancy rate (hCG positive 9–11 days after embryo transfer according to local laboratory standards), implantation rate, early pregnancy loss, preterm birth rate, birth weight and adverse events. Considering adverse events, we recorded all adverse events reported to the clinics from the day of randomisation to the day of embryo transfer, including an adverse event questionnaire on the day of embryo transfer. Patients without embryo transfer, were approached to also fill in the questionnaire.

Compliance to medication was defined as those patients reporting to take all study medicine notwithstanding those patients who took all study medicine, but inadvertently in a wrong way. Outcomes were analysed by intention to treat (ITT), modified intention to treat (mITT) and per protocol (PP). ITT included all randomised patients except those withdrawn from study within 24 hours from randomisation. For the mITT analysis, additionally, patients needed to fulfil in/exclusion criteria and to have embryo transfer less than 63 days from the active treatment start to cycle day 1 in the same menstrual cycle in which the embryo transfer was performed. For the PP analysis, additionally, all patients should adhere strictly to the protocol. Two authors (TH and MBJ) stratified patients for the mITT and PP analysis independently prior to breaking the randomization code.

Sample size

We estimated a 40% chance for clinical pregnancy per embryo transfer in the active treatment arm as compared to the placebo arm which was estimated to have a maximum of 20% chance of clinical pregnancy/transfer as based on a previous study[16]. By a two-sample proportion test with a power of 80% and an alpha at 5%, the aim was to randomise 92 patients in each group. A potential difference between the two active arms was considered exploratory and consequently this was not part of the

power calculation, but we decided to include the same number of patients in the CLLA arm to investigate a potential added benefit of LACTIN-V. An interim analysis was performed, and to adjust for this, we added 10% more patients to the 92 randomised patients as suggested in Wittes et al.[17]. We estimated that 10% of couples would have no embryos for transfer in both fresh and frozen cycles, and we adjusted for this by adding another 10% to each randomised group, that is, 19+92=111. Interim analysis was pre-planned and conducted at the time 167 patients were randomised. At this point and under the conditions described previously[15], the study board decided to continue the trial on March 12th, 2020.

Statistics

For each treatment group CLLA, CLPL and PLPL the estimated proportions, risk ratios (RR) and their confidence intervals were calculated using uni- and multivariate logistic regression analyses by generalized linear models with log-link function. The significance level for the final analysis was set at 4.9% (95.1% confidence intervals) due to the preplanned interim analysis where an alpha of 0.1% was used. The outcomes were analysed with and without adjusting for the following confounders: quality of the embryo (blastocyst/cleavage state –preimplantation genetic testing for aneuploidy (PGT-A) was not performed in this study) and female age (continuous variable) which are well-described parameters affecting pregnancy rates. It was also pre-planned to adjust for double embryo transfer and for private/public clinics, however, only five patients received double embryo transfer without achieving clinical pregnancy. Furthermore only 10 patients were included from the one participating private IVF clinic of whom only one patient had a clinical pregnancy. These numbers were not sufficient to adjust for double embryo transfer and private/public centre in the statistical model. We pre-planned to adjust for the abovementioned confounders since the primary analysis (mITT) was not performed per randomised patient, but per transferred patient. The linear relation

between the log of odds and age was evaluated using splines. To examine the sensitivity of the estimates, all the outcomes were further analysed under PP and ITT conditions. In **Tables 1** and **3**, we used the Fisher's exact test for binary variables, whereas the ANOVA was used for the continuous variables. We decided to provide a statistical test in table 1 because all patients randomised did not necessarily have an embryo transfer and as such were not eligible for mITT analysis. Safety analysis (**Table 3**) was done per ITT. All these analyses were performed in STATA version 18 (StataCorp LLC).

Data handling

Study data were collected and managed, using REDCap electronic data capture tools[18] hosted at Aarhus University and monitored by the University affiliated ICH-GCP unit. The randomization code was broken April 21, 2023, when the primary outcome was monitored, and patients had been stratified to ITT, mITT or PP analysis.

Role of the funding source

PH, TH and JSJ received—through their institutions—an unrestricted research grant from Osel, Inc., which produces LACTIN-V. A clinical trial agreement was made ensuring full data ownership and publication rights to PH. Osel inc. had inputs to study design but no role in data collection, data analysis, data interpretation, or writing of the report.

Results

Between December 7, 2017, and September 21, 2022, a total of 1535 patients were screened, and 338 patients were randomised. Failure to meet the inclusion criteria was predominantly due to absence of

AVM (N=1003). Despite being positive for AVM, a total of 19 patients declined to participate in the RCT and in addition, 36 patients became spontaneously pregnant before randomization.

For the ITT analysis, we excluded 3 patients who were withdrawn within 24 hours after randomisation in which we did not record further data in our database. One patient developed appendicitis (took one tablet clindamycin), another patient did not take any study medication at all as pills were too big to swallow, and the last patient was randomised despite not being positive for AVM because of a reading error that was discovered early.

During the time of the interim analysis, we discovered a laboratory error that resulted in five patients being incorrectly randomised and who underwent full study protocol despite of not having AVM at the time of screening. Thus, it was decided to compensate with five additional patients who had the same randomization allocation as the incorrectly diagnosed and randomised patients. Considering mITT analysis, we decided not to include the five patients who were incorrectly diagnosed and randomised, but instead included the additional five patients randomised. Moreover, four patients were randomised erroneously despite the fact that the screening vaginal swab was more than 90 days old before actual randomisation, all four patients did not have AVM at a deferred analysis of vaginal swabs from randomisation day. Finally, one patient turned out to be pregnant few days after randomisation. Thus, the abovementioned 10 patients who actually did not meet in/exclusion criteria and additionally 59 patients who did not undergo embryo transfer <63days from randomisation were not included in the mITT analysis. This resulted in 94 CLLA, 88 CLPL, and 84 PLPL patients included in the primary mITT analysis. A detailed overview can be seen in the Consort flowchart, **Figure 1**.

In **Table 1** the baseline characteristics are shown at the mITT level. There were no statistically significant differences between the three groups between any of the background variables. Most

patients (88%) were randomised in a fresh cycle and as expected, this study reports no differences between groups considering the number of oocytes retrieved and the availability of a blastocyst for transfer. A total of 14 patients (5%) received antibiotic prophylaxis at the time of oocyte retrieval due to e.g. endometriosis. Notably, prophylactic antibiotic treatment is not a standard to all patients at oocyte retrieval in the participating clinics.

In the primary analysis considering the mITT population, the crude clinical pregnancy rates per embryo transfer were 41% (95%CI 32-53%), 47% (95%CI 37-58%) and 45% (95%CI 36-57%) in the CLLA, CLPL, PLPL groups respectively. Following adjustment for embryo quality and female age the clinical pregnancy rates were 42% (95%CI 32-52%), 46% (95%CI 36-56%) and 45% (95%CI 35-56%) in the CLLA, CLPL, and PLPL groups respectively, **Table 2**. The average effect of the two active groups compared to the PLPL group was close to unity, aRR 0.98 (95%CI 0.74-1.29). Both the PP and the ITT analyses were similar to the mITT analysis and did not show any statistically significant differences between the three groups, supplementary **Table S1**.

The secondary outcomes from the mITT analysis and adjusted for female age and embryo quality can be seen in **Table 2**. The adjusted positive hCG rate per embryo transfer was 62% (95%CI 52-71%), 65% (95%CI 56-75%) and 59% (95%CI 49-69%) in the CLLA, CLPL, PLPL group respectively. The adjusted ongoing pregnancy rate was 41% (95%CI 31-51%), 45% (95%CI 35-55%) and 44% (95%CI 34-55%) in the CLLA, CLPL, PLPL group respectively. Finally, the adjusted live birth rate did not differ significantly, 40% (95%CI 30-50%), 45% (95%CI 35-55%) and 40% (95%CI 30-51%) in the CLLA, CLPL, PLPL groups respectively. There were no preterm births prior to week 34 and the number of preterm births prior to week 37 was 4 (4%), 2 (2%) and 4 (5%) in the CLLA, CLPL, PLPL group respectively, P=0.72.

The adverse events differed significantly between groups, **Table 3**. We report statistically significant increase in diarrhoea and abdominal pain in the two active clindamycin groups compared to the placebo/placebo arm, RR 2.92 (95%CI 1.26-6.76) and RR 2.19 (95%CI 1.05-4.58). Also risk of vaginal candidiasis was close to statistical significance in the two clindamycin arms. Compared to the two arms receiving placebo LACTIN-V, a significant number of patients experienced vaginal itching in the active LACTIN-V arm, RR 4.09 (95%CI 1.26-13.29). Finally, a significant number of patients suspected that they received active clindamycin in the two active clindamycin arms compared to the PLPL arm, RR 1.46 (95%CI 1.09 – 1.95).

There was nearly 100% compliance to study medication. For clindamycin, patients were asked to return the pillbox to the clinic after completion and 232/335 (69%) did whereas the remainder told the clinic that they forgot the pillbox but took all pills. Only 3 patients returned clindamycin pills due to the tablets being too big for them to swallow. Moreover, a total of seven serious adverse events were registered without being suspected as adverse reactions. The list of serious adverse events can be seen in supplementary **Table S2**.

As stated in the research protocol[15], consecutive vaginal samples were taken during the RCT; among them on the day of randomisation, embryo transfer and pregnancy scan. In the mITT population, the total number of AVM positives on the randomisation day was 78% (204/261, 95%CI 73-83%). Due to a potential misclassification bias due to randomising AVM negatives, we made a post-hoc sensitivity analysis on the mITT-level for all patients with AVM at the randomisation day. However, we found no significant differences in reproductive outcomes between the randomised groups as based on AVM at randomisation day (**Table 4)**. The number of patients who were successfully treated for AVM at embryo transfer (AVM at randomisation but not at embryo transfer) was 100% (61/61), 75% (43/57) and 36% (22/61) in the CLLA, CLPL and PLPL groups respectively,

P<0.01. All the above-mentioned treatment success rates were significantly different compared both pairwise within the respective groups to the spontaneous cure rate prior to randomisation as well as significantly different when compared independently between groups. Similarly, the cure rate of AVM present at randomisation and investigated on the day of pregnancy scan was 100% (33/33), 89% (25/28) and 58% (15/26) in the CLLA, CLPL and PLPL groups respectively. In **table 4**, we also report the reproductive outcome in patients who had AVM at embryo transfer. Notably, we did not see an overall increased risk of poor clinical pregnancy rate in case of AVM at embryo transfer, RR 1.13 (95%CI 0.81-1.56) when compared to patients not having AVM. However, comparing the AVM positives to AVM negatives at embryo transfer day within the PLPL group yielded a higher but statistically non-significant clinical pregnancy rate in the AVM positive group, RR 1.63 (95%CI 0.96-2.76). Finally, we investigated a potential differential effect between patients randomised in fresh and FET cycles, however, we observed no statistically significant differences between groups (data not shown).

Discussion

The present drug intervention trial found no evidence of an improved reproductive outcome in the two active treatment groups (CLLA and CLPL), separately or combined when compared to placebo (PLPL) in IVF patients with AVM. In contrast, patients reported significantly more adverse events such as abdominal pain, diarrhoea, and vaginal itching in the active treatment groups. Importantly, the reproductive outcome of the three groups was very close to unity despite superior treatment efficacy of AVM at the time of embryo transfer in the CLLA and CLPL group compared to the PLPL group.

The result of the present study was unexpected as our initial small association study[16] in a comparable group of IVF patients with untreated AVM had a clinical pregnancy rate per embryo

372 transfer of 9% (2/22) compared to 44% (27/62) in the PLPL group of the present study. In
373 consideration of the small sample size in the initial study, we hypothesized a more conservative
374 reduction in clinical pregnancy rate per embryo transfer of the present study in the PLPL group of
375 20% compared to 40% in the CLPL group. However, based on the results of the present study, this
376 hypothesis may now be rejected. Moreover, because we observed superior treatment efficacy of AVM
377 in the CLLA and CLPL groups compared to the PLPL group, the present study questions the
378 biological plausibility that vaginal dysbiosis may hamper reproductive outcome. As regards external
379 validity of the initial findings, a systematic review and meta-analysis was recently performed to
380 investigate the overall association between vaginal dysbiosis and reproductive outcomes in IVF
381 patients (submitted). The results of the meta-analysis were somewhat conflicting; Although the
382 vaginal dysbiosis group had lower risk of clinical pregnancy per embryo transfer (RR 0.82 ;95%CI
383 0.70-0.95, 25 studies) as well as an increased risk of early pregnancy loss (RR 1.49 ;95%CI 1.15-
384 1.94, 20 studies) compared to the non-dysbiosis group, the impact on live birth rate of both the overall
385 vaginal dysbiosis group and a sub-stratified BV-type vaginal dysbiosis group was statistically non-
386 significant, RR 0.94 (95%CI 0.76-1.16, 14 studies) and RR 0.96 (95%CI 0.76-1.21, 13 studies). Thus,
387 the true impact of vaginal dysbiosis on reproductive outcome of IVF patients may be smaller than
388 hypothesized in the power calculation of the present study. However, there was not even a trend of
389 treatment effect on reproductive outcome when comparing the CLLA and CLPL to PLPL in the
390 present study. In fact, the findings of our study are in line with the recent debate regarding treatment
391 of BV for the prevention of preterm birth; Thus, although a significant association between BV and
392 preterm birth was reported by a meta-analysis[19], the largest intervention trial[20] did not show any
393 benefit from treatment and consequently clinical guidelines[21,22] do not recommend treating BV in
394 order to reduce preterm birth.

In the present study, we report a significantly higher AVM cure rate of 36% in the PLPL group from the time of randomisation to embryo transfer as compared to the overall spontaneous AVM cure rate of 22% from screening to randomisation. This difference may indicate a positive AVM treatment effect of the LACTIN-V placebo containing mainly maltodextrin (a glucose polymer) which theoretically might have contributed to a higher pregnancy rate in the PLPL group. A study in rhesus macaques indeed showed that a vaginal gel with maltose (a dimer of glucose) significantly increased the abundance of *Lactobacillus* in the vagina[23]. However, restricting the analysis to IVF patients who remained AVM positive at embryo transfer and who were given PLPL showed a clinical pregnancy rate of 58% (23/40) as compared to PLPL patients not having AVM at embryo transfer who had a clinical pregnancy rate of 35% (12/34), RR 1.63 (95%CI 0.96-2.76). Thus, the potential treatment effect on AVM of the PLPL seems independent and not related to the pregnancy outcome. Also, we consider that if causal inference does exist between AVM and poor reproductive outcome one would still have expected the substantial number of AVM positives in the PLPL group to hamper the reproductive outcome. As based on the abovementioned lack of biological plausibility, we do not consider that an active placebo effect has impacted our result, albeit a control group of AVM positives with no intervention would have been optimal.

The most recent Cochrane systematic review and meta-analysis on the use of antibiotics prior to embryo transfer published November 2023 reported low certainty according to GRADE, considering all reproductive outcomes including clinical pregnancy rate, odds ratio 1.01 (95%CI 0.67-1.55, 2 RCTs) in the treated group compared to the untreated group[24]. The finding of the present RCT adds certainty to the effect estimates published previously, albeit it is important to note that both studies included in the Cochrane review considered IVF patients not targeted for genital tract dysbiosis/infection prior to embryo transfer. To the best of our knowledge, only a few smaller intervention studies have been published in IVF patients diagnosed with vaginal or endometrial

dysbiosis, also reporting on reproductive outcomes. Eldivan et al. [25] randomised IVF patients on the first day of ovarian stimulation to screening and subsequent treatment for BV, trichomoniasis, *Chlamydia* and Gonorrhea. The comparator was patients who were randomised to no screening for the abovementioned microorganisms but were treated as standard patients. A total of 17/45 (38%) IVF-ET patients were positive for BV using Nugent's criteria, and they received treatment with oral metronidazole 500mg x2 daily for 7 days prior to ET. Despite treatment, only 4/17 (24%) conceived (hCG positive) in the BV treated group compared to a conception rate of 12/28 (43%) in patients screened negative for BV. In the unscreened group the conception rate was 14/40 (35%). Although the results were not statistically significant, the study suggested that the poor reproductive outcome in the BV positive group persisted regardless of metronidazole treatment.

One of the primary strengths of the present study is a rigorous design, monitored according to the ICH-GCP and with adequate power to investigate the reproductive outcome in an IVF setting. We had relatively broad inclusion criteria, at large mimicking the clinical setting of daily standard IVF patients in which this intervention was intended. As an example, we included patients in both fresh and frozen embryo transfer cycles as we considered treatment prior to embryo transfer to be the primary objective and, thus, disregarded any differential effect that these two IVF treatments (fresh/frozen cycle) might have on the vaginal microbiota and the reproductive outcome. Randomising in FET cycles only would eliminate the potential bias from randomisation before oocyte retrieval, as patients in a fresh cycle may not have embryo transfer within the study intervention period. However, in the present study, we did not see any statistical difference in primary outcome across the randomised groups when comparing fresh and FET cycles, (data not shown). One of the important limitations of the present study is the spontaneous AVM cure rate of 22% from screening to randomisation. Based on recent evidence regarding temporal dynamics of the vaginal microbiota [26], this rate is probably what might be expected. Nevertheless, sensitivity analysis of AVM positive

443 patients from the vaginal swabs taken on the day of randomisation did not yield different results,
444 **Table 4**. Finally, current evidence suggests that the vaginal microbiota can be used as a proxy of the
445 endometrial microbiota, however, we cannot exclude that a specific screening and treatment for
446 **endometrial** dysbiosis would yield different results.

447 For future studies, we plan to investigate the vaginal swabs taken during the present study and
448 throughout pregnancy even further using 16S rRNA gene sequencing[15]. As an example, it has
449 become clear that the *Gardnerella* genus consists of different species which may have a different
450 pathogenicity[27]. Our qPCR probe targeted a broad selection of *Gardnerella* species, but future
451 studies may show that only select *Gardnerella* species are pathogenic. Moreover, AV-type vaginal
452 microbiota such as enterococci and streptococci may be interesting to investigate in the future.

453 In conclusion, the results of this RCT does not support a policy of screening and treating IVF patients
454 for BV-type vaginal dysbiosis prior to embryo transfer in order to improve reproductive outcomes.

455 Data sharing statement

456 In 2028, five years after study completion we are obliged to deliver the deidentified clinical trial data
457 to the Danish National Archives upon which the data is accessible for all interested parties. The
458 metadata and statistical analysis-log can be made available to reviewers upon submission.
459 Researchers interested in the individual participant data prior to 2028 may contact the first author to
460 access the data under a data sharing agreement with the Danish Data Protection Agency. The full
461 study protocol with statistical analysis plan will be shared at the institutional webpage upon
462 acceptance of this manuscript, albeit it has to a great extent already been published[15].
463

464 Declaration of interests

JSJ, TP and TH are listed as inventors in an international patent application (PCT/US2018/040882), involving the therapeutic use of vaginal lactobacilli to improve IVF outcomes. TP is an employee of Osel, Inc. TH received honoraria for lectures from Gedeon Richter. PH received unrestricted research grants outside this study from Merck, IBSA and Gedeon Richter as well as honoraria for lectures from MSD, Merck, Gedeon-Richter, and Theramex. JSJ received grants, speaker's fee and non-financial support from Hologic, speaker's fees from LeoPharma and grants from Nabriva, all outside the submitted work and serves on the scientific advisory board of Roche Molecular Systems, Abbott Molecular and Cepheid. NLCF received unrestricted research grant from Gedeon Richter and honoraria for lectures from Merck. HSN received unrestricted research grant from Ferring and honoraria for lectures from Merck, IBSA and Ferring.

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Contributors

TH wrote the first draft and made statistical analysis. NlCF was part of conceptualisation, patient recruitment, project administration, results interpretation and review and editing. AP was part of

patient recruitment, project administration, results interpretation and review and editing. MBJ was part of conceptualisation, patient recruitment, project administration, results interpretation and review and editing. HOE was part of conceptualisation, patient recruitment, project administration, results interpretation and review and editing. BA was part of conceptualisation, patient recruitment, project administration, results interpretation and review and editing. RL was part of conceptualisation, patient recruitment, project administration, results interpretation and review and editing. LP was part of patient recruitment, project administration, results interpretation and review and editing. HSN was part of conceptualisation, patient recruitment, project administration, results interpretation and review and editing. VH was part of conceptualisation, patient recruitment, project administration, results interpretation and review and editing. TRP was part of project administration, data curation and review and editing. ASH was part of literature search and review and editing. JSJ was part of conceptualisation, methodology, supervision, project administration, data curation, funding acquisition, results interpretation and review and editing. PH was part of conceptualisation, methodology, supervision, project administration, data curation, funding acquisition, results interpretation and review and editing. All authors had access to the study data and had final responsibility for the decision to submit for publication. PH and TH directly accessed and verified the underlying data reported in the manuscript.

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