Bacterial interactions in the female genital tract

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CHAPTER 7

The vaginal microbiome as a predictor for outcome of in-vitro fertilization with or without intracytoplasmic sperm injection: a prospective study

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Submitted

ABSTRACT

Background: Live birth rates for *in-vitro* fertilization (IVF) and IVF with intracytoplasmic sperm injection (IVF-ICSI) vary between 25% and 35% and it is hard to predict in advance who will get pregnant after embryo transfer (ET). Recently, the composition of the vaginal microbiota was suggested as predictor for pregnancy outcome.

Methods: In a prospective study, 303 women undergoing IVF or IVF-ICSI treatment provided a vaginal sample before commencing treatment. The vaginal microbiota composition was determined using the interspace profiling technique. Microbiome profiles were assigned to community state types based on the dominant bacterial species. The predictive accuracy of the microbiome profiles for pregnancy outcome after fresh ET was evaluated by a combined prediction model based on a small number of bacterial species. Finally, the model was validated externally in a cohort of 50 women also undergoing IVF / IVF-ICSI treatment.

Findings: In the prospective study, 192 women underwent a fresh ET of whom the vaginal microbiota profile could be analysed. Women with a low percentage of *Lactobacillus* in their sample were less likely to become pregnant. The prediction model identified a subgroup of women (17.7%, n=34) who had a low chance to become pregnant following fresh ET. This low chance was correctly predicted in 32 out of 34 women based on the vaginal microbiota composition, resulting in a predictive accuracy of 94%. Additionally, the degree of dominance of *L. crispatus* was an important factor in predicting pregnancy: women who had a favourable profile and <60% *L. crispatus* had the highest chance to become pregnant.

Interpretation: Our results indicate that vaginal microbiome profiling enables stratification of the chance to become pregnant prior to the start of an IVF or IVF-ICSI treatment. Analysis of the vaginal microbiome prior to treatment might offer an opportunity to improve the success rate of IVF or IVF-ICSI.

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INTRODUCTION

In couples trying to conceive, 10-15% are affected by subfertility – defined as one year of unprotected intercourse without achieving a pregnancy.(1) In such cases, subfertility is evaluated by means of a medical work-up of both partners, to determine whether couples have an indication for assisted reproductive technology (ART). Common causes of subfertility include ovulatory disorders, tubal disease and sperm abnormalities. In nearly one third of all cases, subfertility remains unexplained after such evaluation.(1)

Not all subfertile couples trying to conceive will benefit from an ART treatment. For example, couples with unexplained subfertility may still have a good chance of conceiving spontaneously within the first two years after they have been diagnosed. To prevent under- and overtreatment, the decision whether to wait or to start treatment requires careful consideration.

Various models have been developed for predicting the chances of a spontaneous pregnancy. For example, Hunault *et al.*(2) have developed a prediction model for spontaneous pregnancy amongst couples with unexplained subfertility. This model is based on the following predictors: duration of subfertility, woman's age, whether the infertility is primary or secondary along with the percentage of motile sperm. If the predicted chance of spontaneous pregnancy in the following year is higher than their chances with ART, couples are counselled to postpone ART treatment. If the chances are lower ART improve the chances of pregnancy.(3)

Commonly used ART procedures are *in-vitro* fertilization (IVF) and IVF with intracytoplasmic sperm injection (IVF-ICSI). These procedures are invasive, costly and do not guarantee a pregnancy. An IVF or IVF-ICSI cycle consists of several different phases, and at each phase there is a chance if it is not successfully completed the procedure will not lead to a pregnancy. First, the patient injects hormones that stimulate the ovaries. After a sufficient number of follicles develop, mature oocytes are retrieved by means of transvaginal puncture. During the *in-vitro* phase the oocytes are inseminated with sperm to allow spontaneous fertilization. In IVF-ICSI treatment, oocytes are injected with a single sperm cell due to insufficient quality of semen. If fertilization occurs and the developing embryos are of sufficient quality, one or two will be selected for embryo transfer (ET). The remaining embryos might

be cryopreserved for transfer at a later time. A complete treatment cycle includes all embryo transfers – both fresh and frozen-thawed embryos – arising from one cycle of hormonal stimulation of the ovaries.

After transfer, the embryo has to implant into the endometrium to ensure a subsequent normal pregnancy. Only 25-35% of the women become pregnant after the first embryo transfer. Currently, it is hard to predict who will and who will not have a chance of successful embryo implantation. If unsuccessful embryo implantation could be predicted prior to the start of treatment, an invasive IVF or IVF-ICSI treatment – with the accompanying physical, emotional and financial burden – could be avoided or postponed.

Over the last decade, research has shown that besides the known factors used in prediction models such as female age, sperm quality and antral follicle count, outcome of assisted reproduction might also be affected by the microorganisms of the urogenital tract.(4-8) The collection of microorganisms that live on or in the human body constitute the microbiota and their complete genetic profile is generally referred to as the microbiome. The microbiota can interact actively, both beneficially as well as detrimentally, with the internal milieu of their host.(9) The most common bacteria that inhabit the vagina belong to the genus *Lactobacillus*.(10) Studies have shown that the presence of certain *Lactobacillus* species during ART procedures may have a positive impact on outcome(4, 11, 12), whereas bacterial vaginosis is associated with poorer results.(13, 14)

The use of the vaginal microbiome as a predictor for ART outcome has not yet been investigated. We aimed to answer the following questions: First, is the presence or absence of certain vaginal bacteria associated with failure or success to become pregnant after an IVF or IVF-ICSI treatment? Second, can the composition of the vaginal microbiome be used as an independent predictor for IVF or IVF-ICSI outcome prior to treatment?

To answer these questions, we prospectively collected vaginal samples from subfertile women prior to their IVF or IVF-ICSI treatment and recorded their IVF or IVF-ICSI outcome and analysed the vaginal microbiome to determine its potential predictive value for IVF or IVF-ICSI outcome after the first fresh ET. A predictive model was built on this data of the prospective exploratory study and externally validated in a clinic outside the Netherlands.

MATERIALS AND METHODS

Two separate prospective studies were performed. First, a cohort of women from eight IVF centres in the Netherlands was used to build a prediction model for IVF and IVF-ICSI outcome of fresh ET. This model was based on a small number of vaginal bacterial species analysed by IS-pro technique. The material and methods used in this study cohort have been described previously in chapter 6.

Finally, this model was externally validated in a second study cohort in the Dutch division of the MVZ VivaNeo Kinderwunschzentrum Düsseldorf GmbH, Düsseldorf, Germany. All women that presented for IVF or IVF-ICSI treatment, regardless of cycle number or diagnosis were included sequentially over the period from March 2018 to May 2018 as part of a routine workup.

The study sponsors had no involvement in study design, collection, analysis, and interpretation of the data, in the writing of the report, and in the decision to submit the paper for publication.

RESULTS

Characteristics of the participants

In total, 303 women were included with the intention to provide a vaginal swab for bacterial profile analysis. One woman did not provide a vaginal swab and was excluded, two women were excluded by not completing the questionnaire, and three samples of women were discarded due to sampling error. Another six women were excluded by not fulfilling the inclusion and exclusion criteria: the use of more than three weeks hormonal contraceptives prior to the start of the IVF or IVF-ICSI treatment (n=5), and miscarriage in medical history (n=1). Retrospectively, 14 women collected the sample more than two months prior to the ET and for this reason these women were excluded as well from further analysis. Hence, 26 women in total were not included due to either protocol violations or selection errors.

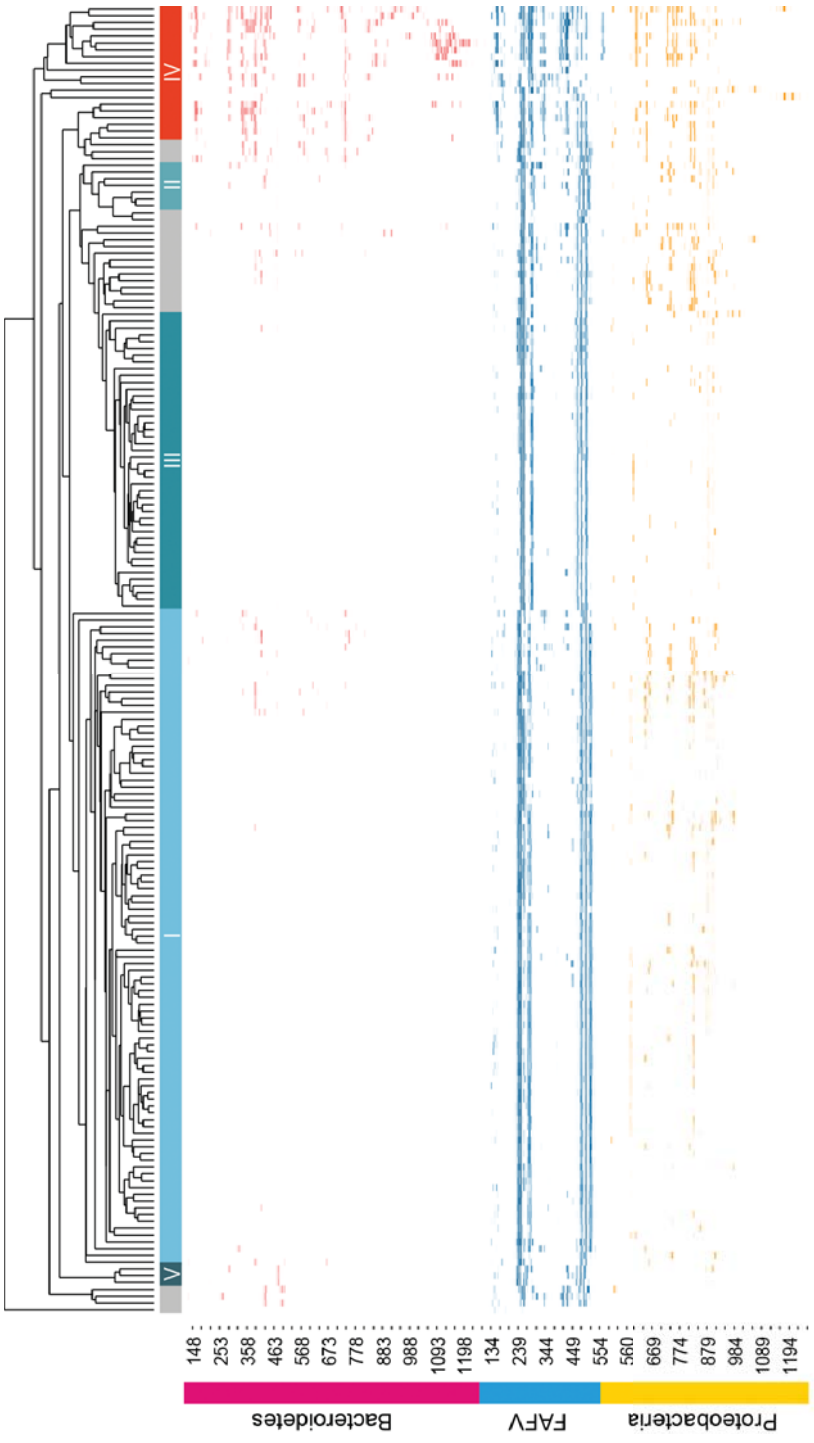

Figure 1: Heatmap of all clustered vaginal swab samples. Top section shows the clustering of all analysed microbiome profiles. The clusters that coincide with the five known community state types (CST) are shown (I-V). The columns represent the profile per individual. The rows represent the found species and the length of the interspace region in units of nucleotides (nt). This can be clustered into 3 groups at phyla level: *Bacteroidetes, FAFV, Proteobacteria*.

Of the remaining 277 women, 85 women dropped out due to the fact that there were no transferable fresh embryos after their first IVF or IVF-ICSI attempt. The various reasons for this are summarised in Supplementary table 1. Finally, data of IVF or IVF-ICSI outcome after the fresh ET was available for 192 women. There were no differences in baseline characteristics between women that were excluded compared to those that were included, except ethnicity subcategory negroid (Supplementary table 2). For the final analysis, we divided the population in two groups, 67 women that became pregnant and 125 who did not. Table 1 summarizes the baseline characteristics of these two groups.

Univariate analysis

Univariate analyses showed that there were no statistical differences between the two groups of pregnant women versus non-pregnant women (Table 1), including commonly used predictors for pregnancy outcome (e.g. age, BMI, duration of infertility). Women in the non-pregnant group had similar ages (mean ± SD age 31.92 ± 4.17 years) compared to the women in the pregnant group (age 31.16 ± 3.67 years). The median BMI in the two groups was similar (23.0 kg/m^2). The duration of infertility was the same in both groups (median (range) 2.43 (0.43-13.80) vs 2.21 (0.55-12.82) years; p=0.333). Finally, when comparing the remaining clinical characteristics of the IVF and IVF-ICSI treatments, there were no significant differences between the two groups (Supplementary table 3).

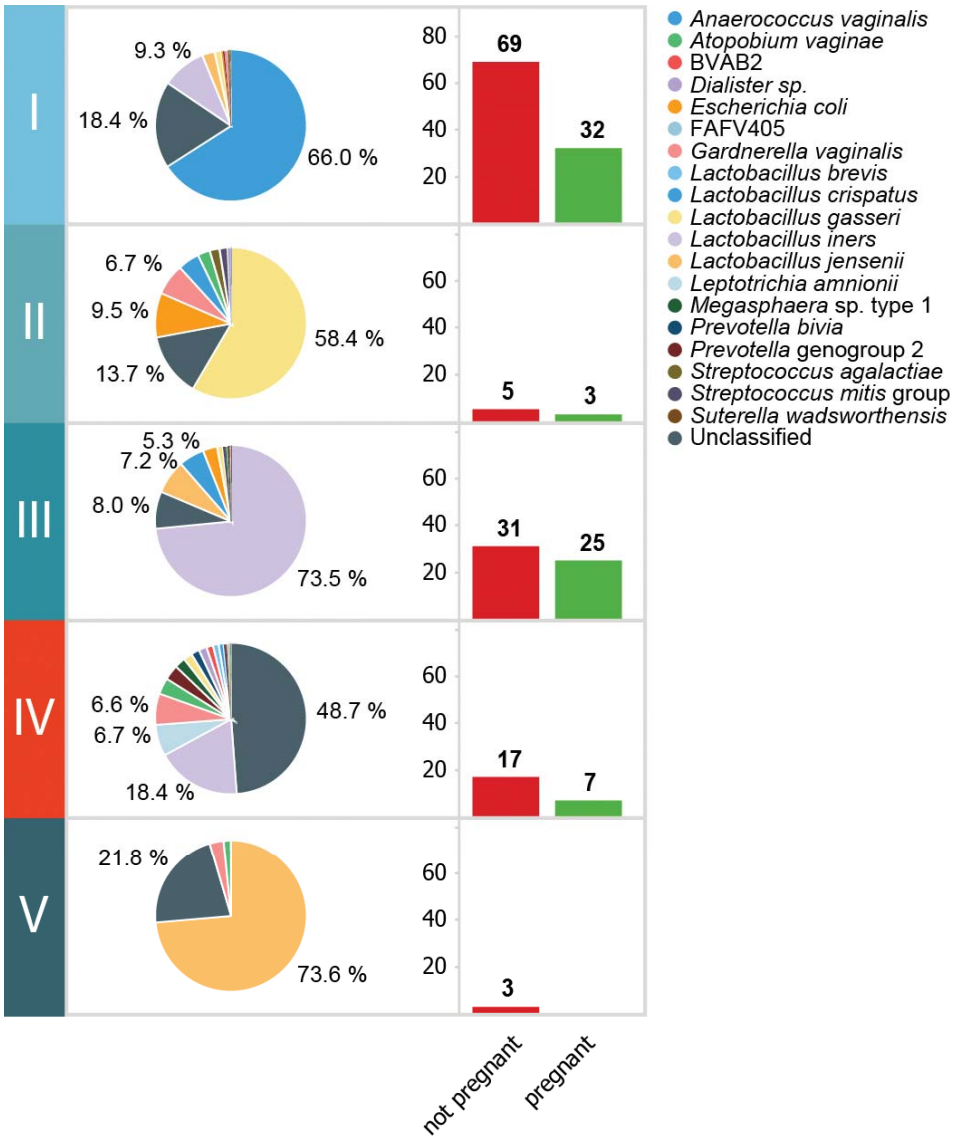

Figure 2: The community state types and the accompanying pregnancy outcome. The composition of the vaginal microbiome per community state type (CST) and the accompanying distribution of pregnancy results (not pregnant or pregnant) per CST.

Analysis of the vaginal microbiome profile

The heatmap of the microbiological data from the IS-pro technique of the vaginal samples shows a clustering into five main groups (Figure 1). These groups could be identified as the five established community state types (CSTs) earlier described by Ravel *et al.*(10)

Figure 2 shows the CSTs and the accompanying IVF or IVF-ICSI outcome in each of them. CST IV and V were enriched for women who did not become pregnant after the ET. CST IV contains 24 women, of whom 17 (70.8%) did not become pregnant. None of the women (n=3) in CST V became pregnant (100%). This proportion was lower in the other CSTs (CST I 68.3%, CST II 62.5%, CST III 55.4%).

The analyses for species content and microbial diversity per phylum showed that the absence or presence of certain bacterial species was correlated with a higher chance of not getting pregnant. A relative low load of *Lactobacillus* or a high load of *Proteobacteria* or high load of *Lactobacillus jensenii* were correlated with failure to become pregnant. We found that *Gardnerella vaginalis* strains were characterized by two distinct IS-profiles. Only one of these IS-types was correlated with low pregnancy rate, namely IS-pro type 1 (IST1). For certain bacterial groups, there was no clear correlation between individual members of the groups, however there was a correlation between the diversity of these groups and failure to achieve a pregnancy. These combined observations resulted in a predictive algorithm for failure to become pregnant with the following parameters: relative *Lactobacillus* load <20%, relative load of *Lactobacillus jensenii* >35%, presence of *Gardnerella vaginalis* IST1, or *Proteobacteria* >28% of total bacterial load. This microbiome composition is referred to as an 'unfavourable microbiome profile'.

Table 1: Univariate analysis of baseline characteristics in women who became pregnant and women who did not become pregnant.

	Pregnant		
	Yes (n=67)	**No (n=125)**	**p-value****
Age	31.16 (3.67)	31.92 (4.17)	0.214§
Ethnicity			
Caucasian	53 (79.1%)	113 (90.4%)	0.071†
Mediterranean	5 (7.5%)	3 (2.4%)	0.124‡
Hindu	3 (4.5%)	2 (1.6%)	0.339‡
Asian	2 (3.0%)	1 (0.8%)	0.268‡
African	0 (0%)	3 (2.4%)	0.552‡
Missing*	4 (6.0%)	3 (2.4%)	-
BMI	23.00 (18.00-32.04)	23.00 (18.00-42.00)	0.613¶
Menarche	13.00 (9.00-17.00)	13.00 (9.00-17.00)	0.920¶
Menstrual cycle			
Regular	53 (79.1%)	91 (72.8%)	0.331†
Mostly regular	6 (9.0%)	18 (14.4%)	0.275†
Irregular	6 (9.0%)	13 (10.4%)	0.747†
Absent	1 (1.5%)	1 (0.8%)	1.000‡
Missing*	1 (1.5%)	2 (1.6%)	-
Indication			
Male factor	48 (71.6%)	80 (64.0%)	0.196†
Idiopathic	7 (10.4%)	22 (17.6%)	0.193†
Tube factor	2 (3.0%)	5 (4.0%)	1.000‡
Cycle disorder	3 (4.5%)	4 (3.2%)	0.695‡
Uterine factor	0 (0%)	1 (0.8%)	1.000‡
Other	1 (1.5%)	3 (2.4%)	1.000‡
Combination*	4 (6.0%)	8 (6.4%)	-
Missing*	2 (3.0%)	2 (1.6%)	-
Use of medication			
Yes	15 (22.4%)	25 (20.0%)	0.680†
No	51 (76.1%)	99 (79.2%)	
Missing*	1 (1.5%)	1 (0.8%)	-
Smoking			
Yes	12 (17.9%)	18 (14.4%)	0.558†
No	53 (79.1%)	101 (80.8%)	
Missing*	2 (3.0%)	6 (4.8%)	-
Alcoholic consumption			
Yes	37 (55.2%)	82 (65.6%)	0.122†
No	28 (41.8%)	38 (30.4%)	
Missing*	2 (3.0%)	5 (4.0%)	-
Urinary tract infection in prior 3 months			
Yes	3 (4.5%)	4 (3.2%)	0.697‡
No	64 (95.5%)	121 (96.8%)	

	Pregnant		
	Yes (n=67)	No (n=125)	p-value**
Use of antibiotics in prior 3 months			
Yes	7 (10.4%)	10 (8.0%)	0.569†
No	60 (89.6%)	115 (92.0%)	
Duration of infertility	2.21 (0.55-12.82)	2.43 (0.43-13.80)	0.333¶

Values are given as mean ± SD years, number (%), and median (range). BMI= Body Mass Index (kg/m2).
† By Chi-square test.
‡ By Fisher's exact test.
§ By Independent Samples T-test.
¶ By Mann-Whitney U test.
*these subcategories are excluded from analysis.
**comparisons are made between analysed category and the other categories combined.

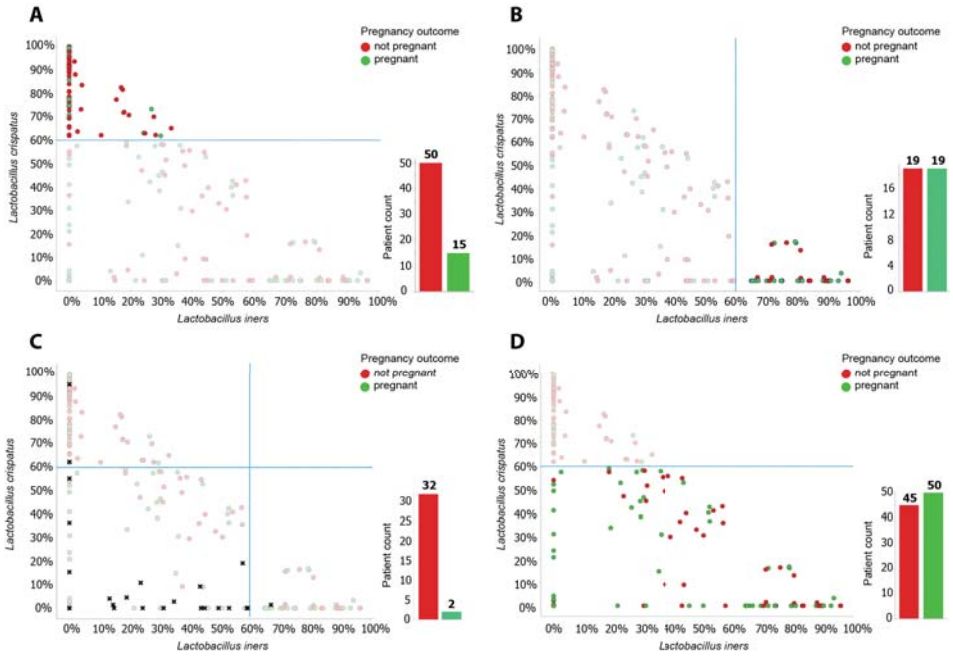

Figure 3: The role of *Lactobacillus crispatus* and *Lactobacillus iners* in predicting pregnancy outcome. Relative rates of *L. crispatus* versus *L. iners* for each patient. Each circle represents a patient, the colour of the dot shows pregnancy outcome (green – pregnant, red – not pregnant).
A: Patients with an unfavourable profile (34 of 192 women) are depicted as black crosses, they are removed from the dataset in figures B-D. **B**: Of the remaining 158 women, 63 have a relative abundance of *L. crispatus* ≥ 60%. The pregnancy rate in this group is 15/63=24%. **C**: Women with a relative abundance of *L. iners* ≥ 60% show a pregnancy rate of 19/38=50%. **D**: When taking into account *L. crispatus* abundance alone, the group of women with a *L. crispatus* abundance ≤ 60% have a pregnancy rate of 50/95=53%.

Table 2: Univariate analysis of baseline characteristics in women with a favourable or unfavourable microbiome profile.

	Microbiome profile		
	Favourable (n=158)	**Unfavourable (n=34)**	**p-value****
Age	31.75 (3.81)	31.19 (4.87)	0.461§
Ethnicity			
– Caucasian	136 (86.1%)	30 (88.2%)	0.806†
– Mediterranean	8 (5.1%)	0 (0%)	0.354‡
– Hindu	4 (2.5%)	1 (2.9%)	1.000‡
– Asian	2 (1.3%)	1 (2.9%)	0.447‡
– African	2 (1.3%)	1 (2.9%)	0.447‡
– Missing*	6 (3.8%)	1 (2.9%)	-
BMI	23.00 (18.00-42.00)	23.00 (19.00-32.00)	0.957¶
Menarche	13.00 (9.00-17.00)	13.00 (9.00-16.00)	0.589¶
Menstrual cycle			
– Regular	118 (74.7%)	26 (76.5%)	0.966†
– Mostly regular	18 (11.4%)	6 (17.6%)	0.339†
– Irregular	17 (10.8%)	2 (5.9%)	0.535‡
– Absent	2 (1.3%)	0 (0%)	1.000‡
– Missing*	3 (1.9%)	0 (0%)	-
Indication			
– Male factor	105 (66.5%)	23 (67.6%)	0.840†
– Idiopathic	24 (15.2%)	5 (14.7%)	1.000‡
– Tube factor	5 (3.2%)	2 (5.9%)	0.608‡
– Cycle disorder	7 (4.4%)	0 (0%)	0.356‡
– Uterine factor	0 (0%)	1 (2.9%)	0.176‡
– Other	4 (2.6%)	0 (0%)	1.000‡
– Combination*	9 (5.7%)	3 (8.8%)	-
– Missing*	4 (2.5%)	0 (0%)	-
Use of medication			
– Yes	32 (20.3%)	8 (23.5%)	0.696†
– No	124 (78.5%)	26 (76.5%)	
– Missing*	2 (1.3%)	0 (0%)	
Smoking			
– Yes	24 (15.2%)	6 (17.6%)	0.680†
– No	128 (81.0%)	26 (76.5%)	
– Missing*	6 (3.8%)	2 (5.9%)	
Alcoholic consumption			
– Yes	97 (61.4%)	22 (64.7%)	0.566†
– No	56 (35.4%)	10 (29.4%)	
– Missing*	5 (3.2%)	2 (5.9%)	
Urinary tract infection in prior 3 months			
– Yes	7 (4.4%)	0 (0%)	0.357‡
– No	151 (95.6%)	34 (100%)	

	Microbiome profile		
	Favourable (n=158)	Unfavourable (n=34)	p-value**
Use of antibiotic treatment in prior 3 months			
– Yes	15 (9.5%)	2 (5.9%)	0.742‡
– No	143 (90.5%)	32 (94.1%)	
Duration of infertility	2.34 (0.43-13.80)	2.37 (0.69-5.56)	0.954¶

Values are given as mean ± SD years, number (%), and median (range). BMI= Body Mass Index (kg/m2).
† By Chi-square test.
‡ By Fisher's exact test.
§ By Independent Samples T-test.
¶ By Mann-Whitney U test.
*these subcategories are excluded from analysis.
**comparisons are made between analysed category and the other categories combined.

Unfavourable microbiome profile

Amongst the 192 women with a known IVF or IVF-ICSI outcome, 34 women had an unfavourable profile. Only two of these 34 women (5.9%) became pregnant following an ET. When this unfavourable microbiome profile would be used as a predictor for the failure to become pregnant, the predictive accuracy in this group would be 94% (sensitivity 26%, specificity 97%). Univariate analyses showed that there were no statistical differences within the general characteristics and the ART procedure itself between women with a favourable and women with an unfavourable microbiome profile (Table 2 and Table 3).

Favourable microbiome profiles

In addition to profiling women with an unfavourable microbiome profile (n=34) with a low chance of pregnancy (5.9%), we could stratify women in two more groups with either an average or relatively high chance of pregnancy. This distinction could be made based on relative abundance of *Lactobacillus crispatus*: we found that women with a high relative abundance (≥60%) of *L. crispatus* (63/192 women, 33%) had a 24% chance of becoming pregnant following ET (15/63 women) (Figure 3B). In contrast, women with a high relative abundance (≥60%) of *L. iners* (38/192 women, 20%), had a 50% chance of becoming pregnant following ET (19/38 women) (Figure 3C). All women who did have a favourable profile (158/192 women, 82%) could be stratified into groups with a high and an average chance of pregnancy based on relative abundance of *L. crispatus* alone. As described above, in the group with *L. crispatus* abundance of ≥ 60%, 24% of women became pregnant. In the group with *L. crispatus* abundance <60%, 53% (50 of 95) of women became pregnant (Figure 3D). The difference in pregnancy rates between the high and average *L. crispatus* groups was highly significant (p = 0.0003).

Table 3: Univariate analysis of treatment characteristics of the IVF or IVF-ICSI procedure in women with a favourable and unfavourable microbiome profile.

	Microbiome profile		
	Favourable (n=158)	**Unfavourable (n=34)**	**p-value****
IVF or IVF-ICSI			
– IVF	53 (33.5%)	11 (32.4%)	0.875†
– IVF-ICSI	104 (65.8%)	23 (67.6%)	
– Missing*	1 (0.6%)	0 (0%)	-
Hormone downregulation			
– Decapeptyl	125 (79.1%)	26 (76.5%)	0.807†
– Lucrin	20 (12.7%)	3 (8.8%)	0.771‡
– Orgalutran	6 (3.8%)	0 (0%)	0.593‡
– Cetrotide	4 (2.5%)	3 (8.8%)	0.105‡
– Synarel	0 (0%)	1 (2.9%)	0.176‡
– Missing*	3 (1.9%)	1 (2.9%)	-
Hormone stimulation			
– Gonal-f	126 (79.7%)	28 (82.4%)	0.779†
– Menopur	21 (13.3%)	5 (14.7%)	0.787‡
– Bemfola	9 (5.7%)	0 (0%)	0.366‡
– Pregnyl	0 (0%)	1 (2.9%)	0.178‡
– Puregon	1 (0.6%)	0 (0%)	1.000‡
– Missing*	1 (0.6%)	0 (0%)	-
Endometrial thickness (mm)	10.37 (2.04)	9.97 (2.68)	0.344§
Follicle count	9.5 (2-23)	10 (2-20)	0.653¶
Number of oocytes	9 (1-29)	9.5 (1-19)	0.864¶
ET			
– SET	155 (98.1%)	34 (100%)	1.000‡
– DET	3 (1.9%)	0 (0%)	

Values are given as mean ± SD, number (%), and median (range). IVF= *in-vitro* Fertilisation; IVF-ICSI= *in-vitro* Fertilisation with Intracytoplasmic Sperm Injection; mm= millimetre; ET= embryo transfer; SET= single embryo transfer; DET= double embryo transfer.

† By Chi-square test.

‡ By Fisher's exact test.

§ By Independent Samples T-test.

¶ By Mann-Whitney U test.

*these subcategories are excluded from analysis.

**comparisons are made between analysed category and the other categories combined.

Multivariate analysis

We performed logistic regression analysis with failure to become pregnant as the dependent variable and an unfavourable microbiome profile, female BMI, female age, and duration of infertility as covariates. Twelve women with missing variables were excluded from the analysis. The results of this analysis (n=180) revealed that only an unfavourable microbiome profile showed a statistically significant change in the risk of failure to become pregnant (Table 4).

External validation

We validated the predictive model as described above in an external cohort of 50 women. Fourteen of these women had an unfavourable microbiota profile, 13 had a high *L. crispatus* abundance profile and 23 had a low *L. crispatus* abundance profile. None of the 14 women with an unfavourable profile became pregnant, four did not finish the IVF procedure due to a lack of follicles, oocytes or suitable embryo(s) for transfer. Of the 13 women with a high *L. crispatus* abundance profile, four (31%) became pregnant (three ongoing pregnancies (75%)) and four did not finish the IVF procedure. Finally, of the 23 women with a low *L. crispatus* abundance profile, 12 (52%) became pregnant (11 ongoing pregnancies (92%)) and three (13%) did not finish the IVF procedure.

Table 4: Logistic regression analysis of associations with low chance of getting pregnant in IVF or IVF-ICSI patients that underwent an embryo transfer.

Variable	OR	95% CI
Unfavourable profile	12.057	2.739-53.078
Age	1.024	0.937-1.119
BMI	1.071	0.982-1.167
Duration of infertility	1.072	0.895-1.285

OR= odds radio; 95% CI= 95% confidence interval; BMI= Body Mass Index.

DISCUSSION

In this prospective exploratory study, a subgroup of 34 women with an unfavourable microbiome profile was identified in a subfertile population undergoing their first IVF or IVF-ICSI treatment with a chance of 94% of failure to become pregnant. In addition, we found that the abundance of *L. crispatus* alone could further stratify the remaining 158 women with a favourable microbiome profile into a subgroup of 95 women with high chance (53%) of becoming pregnant and a group of 63 women with an average chance (24%) of becoming pregnant. Our results indicate that microbiome profiling using the IS-pro technique enables accurate prediction of both failure and success of fertility treatment. Women with an unfavourable profile had a seven times lower chance of achieving a pregnancy compared to the women who had a favourable vaginal microbiome profile.

The overall pregnancy rate per cycle of the cohort was 35%, which is similar to that reported by most clinics in the Netherlands in 2016 (average 34.2%).(15) Women with a low *L. crispatus*

abundance had a one and a half times higher chance to become pregnant after the first fresh ET, while women with a high *L. crispatus* profile had a third times lower chance of becoming pregnant compared to the overall pregnancy rate.

In defining the optimal parameters for stratifying patients into different pregnancy rate groups, it was found that different models can be used to predict IVF and IVF-ICSI outcome. The model described here was built with a limited number of parameters, all with well-established links to female health in order to ensure high generalizability. Relative abundance of various *Lactobacillus* species, *Proteobacteria* and *Gardnerella vaginalis* are all widely described as important parameters for vaginal health and disease. The cut-off values used for the algorithm were chosen for best predictive accuracy with the highest specificity. The model also proved to be effective in the external validation cohort, underlining its generalizability.

A possible beneficial role of *Lactobacillus* species in ART has been described in a number of recent studies. For example, the recovery of *Lactobacilli* from the vagina and the embryo transfer catheter has been associated with an increase in live-birth rate after ART.(4) A detrimental effect on pregnancy rate of non-*Lactobacillus* bacterial species has also been described. Studies using culture-dependent techniques and qPCR tests showed lower clinical pregnancy rates(6, 16), ongoing pregnancy rates(7) and implantation rates(7, 8) when abnormal vaginal microbiota were detected. In these studies, the bacterial species that defined an abnormal vaginal microbiota were *Escherichia coli, Staphylococcus* and *Streptococcus* species. In addition, a reduction in live-birth was described when *Streptococcus viridans* was recovered from the catheter tip used for ET.(4) Lower pregnancy rates were also found when patients tested positive for *Enterobacteriaceae* and *Staphylococcus*.(8) *Propionibacterium* and *Actinomyces* were also found to impair clinical outcomes.(17) Bacterial vaginosis was further associated with infertility and early spontaneous abortion.(13, 14)

Our findings show that a high abundance of *Lactobacillus* appears to be advantageous for IVF and IVF-ICSI outcome, but a high abundance (> 60%) of *L. crispatus* is not advantageous. Studies that generalize on the possible beneficial effects of *Lactobacilli* on fertility will have to be interpreted with caution. They have to take into account that different *Lactobacillus* species also have different characteristics; e.g. the ability to produce lactic acid from degradation of glycogen conversion. The acidity achieved by *L. crispatus* is the highest compared

to *L. iners, L. jensenii* and *L. gasseri.*(10) Witkin *et al.* suggests that lactic acid production may facilitate the release of innate immune system components that inhibit growth of bacteria other than *Lactobacilli.*(18) At the same time, *Lactobacilli* act as a mechanical barrier by binding to the surface of vaginal epithelial cells, preventing the binding of other bacteria that might influence these epithelial cells.

Witkin *et al.*(18) also suggest that the ability to prevent the influx of pathogenic microorganisms into the vagina without the necessity of inducing pro-inflammatory immunity would appear to be advantageous for a successful pregnancy outcome. A balance between immunogenic resistance and tolerance is of importance to conceive and establish a viable pregnancy as the implantation of the embryo in the endometrium is also dependent of the immune response in the female reproductive tract.(19, 20) However, the extent of the interactions between the microbiome and these immune responses is yet unknown.

In case of an unfavourable microbiome profile, women do not suffer from a clinically evident infection. Still, the result of the analysis of the vaginal microbiome composition is clinically meaningful for these women. Our results show that women with an unfavourable test result have a limited chance of success when transfer of a fresh embryo takes place within two months after the test was taken. Women with a low a priori chance to become pregnant might prefer to avoid the unnecessary physical and emotional burden of an IVF or IVF-ICSI treatment with a high change of failure. Whether these couples will actually discontinue the treatment at time of an unfavourable profile will require follow-up research which is ongoing at the moment. Delaying a treatment when couples have a strong desire to have children could lead to discontent when they go through years of setbacks. However, the benefits may also be that physical side effects, costs of treatment with little success and emotional burden could be saved.

Further research is needed to elucidate if women with an unfavourable microbiome profile can actually switch to a favourable profile and whether their chance of becoming pregnant subsequently increases. Earlier research investigating the stability of the vaginal microbiome revealed the dynamics of five major classes of bacterial communities (CST I-V) and showed that some communities can change markedly over short time periods, whereas the majority is relatively stable over several months.(21) However, there might also be women with fairly constant microbiota that do not change over time.

One of the potential opportunities to improve results after IVF or IVF-ICSI with the use of microbiome profiling is to counsel women to postpone ART and await a favourable profile. The women could sample the vaginal microbiome over a certain period of time awaiting a switch from an unfavourable profile into a favourable one. Another option is to postpone the first fresh ET, freeze the resulting embryos and transfer them later in unstimulated cycles with a favourable microbiome profile. It is however yet unknown how often, when and why the local microbiota composition changes spontaneously. There might also be therapeutic options aiming at modulation of unfavourable vaginal microbiota towards a more favourable profile, such as antibiotics, pre- or probiotics.(22)

Future research will hopefully elucidate whether modulation of the vaginal microbiota is possible and may indeed improve outcomes of IVF and IVF-ICSI in patients with an unfavourable profile. However, the use of antibiotics might induce transient changes in the microbiome and it is known that relapses to the initial state occur frequently.(23) More targeted options, such as vaginal administration of probiotic *Lactobacillus*, aim at specifically improving the presence of *Lactobacillus,* and may be more effective.(24)

Our suggestion is to reconsider starting the IVF or IVF-ICSI treatment, or alternatively the ET, when a woman has an unfavourable microbiome profile. Without a favourable microbiome, the implantation and subsequent embryo development seems to be compromised. Future research should aim at unravelling the complex interactions between the vaginal microbiome, the reproductive tract as well as their host and enabling meaningful interventions to improve fertility treatment outcome.

DECLARATIONS

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Declaration of interest

RK reports that she is an employee at ARTPred B.V. during her PhD at Erasmus MC.

MS reports grants from Eurostars during the conduct of the study.

SS reports no conflict of interest.

PHMS reports that he is a co-owner of IS-Diagnostics Ltd and he has a patent MICROBIAL POPULATION ANALYSIS (9506109) licensed to ARTPred B.V., a patent MICROBIAL POPULATION ANALYSIS (20170159108) licensed to ARTPred.

SAM has a 100% University appointment is co-owner of IS-diagnostics Ltd., (a spin-off company inside the VU University Medical center Amsterdam, NL), which is the company that developed the IS-pro technique.

JDdJ reports personal fees from ARTPred B.V., grants from NGI Pre-Seed, other from RedMedTech Discovery Fund, from STW Valorisation grant 1, other from Take-off early phase trajectory, grants from Innovatie Prestatie Contract, other from Microbiome Ltd., grants from MIT Haalbaarheid, grants from EUROSTARS, other from Dutch R&D tax credit (WBSO), other from Erasmus MC, during the conduct of the study; personal fees and other from ARTPred B.V., outside the submitted work; In addition, Dr. de Jonge has a patent New method and kit for prediction success of in vitro fertilization licensed to ARTPred, a patent MICROBIAL POPULATION ANALYSIS (9506109) licensed to ARTPred, a patent MICROBIAL POPULATION ANALYSIS (20170159108) licensed to ARTPred, a patent METHOD AND KIT FOR PREDICTING THE OUTCOME OF AN ASSISTEDÐEPRODUCTIVE TECHNOLOGY PROCEDURE pending to ARTPred and a patent METHOD AND KIT FOR ALTERING THE OUTCOME OF AN ASSISTED REPRODUCTIVE TECHNOLOGY PROCEDURE pending to ARTPred.

LP reports reports other from IS-Diagnostics Ltd, during the conduct of the study; other from IS-Diagnostics Ltd, outside the submitted work.

WJSSC received personal consultancy and educational fees from Goodlife Fertility B.V. His employer has in collaboration with ARTPred acquired a MIND subsidy to cover part of the costs of this collaboration project.

AEB reports that he is a co-owner of IS-Diagnostics Ltd. In addition, Dr. Budding has a patent 392EPP0 pending, has a patent MICROBIAL POPULATION ANALYSIS (9506109) licensed to ARTPred, a patent MICROBIAL POPULATION ANALYSIS (20170159108) licensed to ARTPred, a patent METHOD AND KIT FOR PREDICTING THE OUTCOME OF AN ASSISTEDÐEPRODUCTIVE TECHNOLOGY PROCEDURE pending to ARTPred and a patent METHOD AND KIT FOR ALTERING THE OUTCOME OF AN ASSISTED REPRODUCTIVE TECHNOLOGY PROCEDURE pending to ARTPred.

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NGMB is a co applicant on a Erasmus MC patent (New method and kit for prediction success of in vitro fertilization), that predicts IVF outcome based on the urinary microbiome. This particular patent is licensed to ARTPred B.V..

FJMB is a member of the external advisory board for Ferring Pharmaceuticals, Hoofddorp, The Netherlands. He receives no monetary compensation.

BJC has nothing to disclose.

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JMJSS reports personal fees and other from Merck Serono, grants and personal fees from Ferring, outside the submitted work.

The other authors declare that they have no competing interests.

RESEARCH IN CONTEXT

Evidence before this study

Several published studies have shown that *Lactobacillus* has an important role in reproductive health/outcome. These publications show that women with infertility problems have a reduced number of *Lactobacillus* compared to healthy women. Moreover, the presence of non-*Lactobacillus* dominated microbiota (e.g. presence of the genera *Gardnerella* and *Streptococcus*) seems to be associated with significant decreases in implantation, ongoing pregnancy and live birth rates and clinical pregnancy rates.

Added value of this study

The ReceptIVFity study is the first cohort study in which the potential role of the vaginal microbial composition will be used as predictor for IVF or IVF-ICSI outcome prior to the start of the treatment. Women with a low a priori chance to become pregnant might prefer to avoid the unnecessary physical and emotional burden of an IVF or IVF-ICSI treatment with a high change of failure.

Implications of all the available evidence

In the future, microbiome profiling can be a routine diagnostic test prior to IVF or IVF-ICSI treatment to prevent patients a treatment with emotional and physical burden, that has low chance of success. Predictive knowledge based on the present bacteria may enable couples to make a more substantiated decision on whether to continue fertility (IVF or IVF-ICSI) treatment or not.

REFERENCES

1. Evers JL. Female subfertility. Lancet. 2002;360(9327):151-9.
2. Hunault CC, Habbema JD, Eijkemans MJ, Collins JA, Evers JL, te Velde ER. Two new prediction rules for spontaneous pregnancy leading to live birth among subfertile couples, based on the synthesis of three previous models. Hum Reprod. 2004;19(9):2019-26.
3. Brosens I, Gordts S, Valkenburg M, Puttemans P, Campo R, Gordts S. Investigation of the infertile couple: when is the appropriate time to explore female infertility? Hum Reprod. 2004;19(8):1689-92.
4. Moore DE, Soules MR, Klein NA, Fujimoto VY, Agnew KJ, Eschenbach DA. Bacteria in the transfer catheter tip influence the live-birth rate after in vitro fertilization. Fertil Steril. 2000;74(6):1118-24.
5. Pelzer ES, Allan JA, Cunningham K, Mengersen K, Allan JM, Launchbury T, et al. Microbial colonization of follicular fluid: alterations in cytokine expression and adverse assisted reproduction technology outcomes. Hum Reprod. 2011;26(7):1799-812.
6. Egbase PE, al-Sharhan M, al-Othman S, al-Mutawa M, Udo EE, Grudzinskas JG. Incidence of microbial growth from the tip of the embryo transfer catheter after embryo transfer in relation to clinical pregnancy rate following in-vitro fertilization and embryo transfer. Hum Reprod. 1996;11(8):1687-9.
7. Fanchin R, Harmas A, Benaoudia F, Lundkvist U, Olivennes F, Frydman R. Microbial flora of the cervix assessed at the time of embryo transfer adversely affects in vitro fertilization outcome. Fertil Steril. 1998;70(5):866-70.
8. Selman H, Mariani M, Barnocchi N, Mencacci A, Bistoni F, Arena S, et al. Examination of bacterial contamination at the time of embryo transfer, and its impact on the IVF/pregnancy outcome. J Assist Reprod Genet. 2007;24(9):395-9.
9. Cho I, Blaser MJ. The human microbiome: at the interface of health and disease. Nat Rev Genet. 2012;13(4):260-70.
10. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, McCulle SL, et al. Vaginal microbiome of reproductive-age women. Proc Natl Acad Sci U S A. 2011;108 Suppl 1:4680-7.
11. Eckert LO, Moore DE, Patton DL, Agnew KJ, Eschenbach DA. Relationship of vaginal bacteria and inflammation with conception and early pregnancy loss following in-vitro fertilization. Infect Dis Obstet Gynecol. 2003;11(1):11-7.
12. Mangot-Bertrand J, Fenollar F, Bretelle F, Gamerre M, Raoult D, Courbiere B. Molecular diagnosis of bacterial vaginosis: impact on IVF outcome. Eur J Clin Microbiol Infect Dis. 2013;32(4):535-41.
13. Babu G, Singaravelu BG, Srikumar R, Reddy SV, Kokan A. Comparative Study on the Vaginal Flora and Incidence of Asymptomatic Vaginosis among Healthy Women and in Women with Infertility Problems of Reproductive Age. J Clin Diagn Res. 2017;11(8):DC18-DC22.
14. Haahr T, Zacho J, Brauner M, Shathmigha K, Skov Jensen J, Humaidan P. Reproductive outcome of patients undergoing in vitro fertilisation treatment and diagnosed with bacterial vaginosis or abnormal vaginal microbiota: a systematic PRISMA review and meta-analysis. BJOG. 2018.
15. Anovulatory infertility. The ESHRE Capri Workshop Group. Hum Reprod. 1995;10(6):1549-53.
16. Haahr T, Jensen JS, Thomsen L, Duus L, Rygaard K, Humaidan P. Abnormal vaginal microbiota may be associated with poor reproductive outcomes: a prospective study in IVF patients. Hum Reprod. 2016;31(4):795-803.
17. Pelzer ES, Allan JA, Waterhouse MA, Ross T, Beagley KW, Knox CL. Microorganisms within human follicular fluid: effects on IVF. PLoS One. 2013;8(3):e59062.
18. Witkin SS, Linhares IM. Why do lactobacilli dominate the human vaginal microbiota? BJOG. 2017;124(4):606-11.
19. Robertson SA, Chin PY, Glynn DJ, Thompson JG. Peri-conceptual cytokines--setting the trajectory for embryo implantation, pregnancy and beyond. Am J Reprod Immunol. 2011;66 Suppl 1:2-10.
20. van Mourik MS, Macklon NS, Heijnen CJ. Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment. J Leukoc Biol. 2009;85(1):4-19.
21. Gajer P, Brotman RM, Bai G, Sakamoto J, Schutte UM, Zhong X, et al. Temporal dynamics of the human vaginal microbiota. Sci Transl Med. 2012;4(132):132ra52.

22. Tachedjian G, O'Hanlon DE, Ravel J. The implausible "in vivo" role of hydrogen peroxide as an antimicrobial factor produced by vaginal microbiota. Microbiome. 2018;6(1):29.

23. Sobel R, Sobel JD. Metronidazole for the treatment of vaginal infections. Expert Opin Pharmacother. 2015;16(7):1109-15.

24. Xie HY, Feng D, Wei DM, Mei L, Chen H, Wang X, et al. Probiotics for vulvovaginal candidiasis in non-pregnant women. Cochrane Database Syst Rev. 2017;11:CD010496.

SUPPLEMENTARY TABLES

Supplementary table 1: Reasons for study participants to drop out.

Stage of treatment	Reason of drop out	Total
Hormone stimulation	Too few follicles (n=34) Too many follicles (n=10) Stagnant growth (n=2)	n=46
Puncture	Risk of ovarian hyperstimulation syndrome (n=8) Escape intrauterine insemination (n=1)	n=9
Laboratory	No oocytes (n=4) No sperm cells (n=3) Insufficient cleavage (n=1) No embryo (n=3) Total fertilisation failure (n=8)	n=19
Embryo transfer	Embryo(s) primary frozen (n=2)	n=2
Other	Intercurrent disease (n=6) Relationship ended (n=1) Spontaneous pregnancy (n=1) No IVF or IVF-ICSI treatment (n=1)	n=9

Supplementary table 2: Baseline characteristics of the study patients.

	All women (n=303)	Inclusions (n=192)	Exclusions (n=111)	p-value**
Age	31.64 (4.24)	31.65 (4.01)	31.61 (4.65)	0.940§
Ethnicity				
– Caucasian	263 (86.8%)	166 (86.5%)	97 (87.4%)	0.799†
– Mediterranean	10 (3.3%)	8 (4.2%)	2 (1.8%)	0.335‡
– Hindu	6 (2.0%)	5 (2.6%)	1 (0.9%)	0.420‡
– Asian	5 (1.7%)	3 (1.6%)	2 (1.8%)	1.000‡
– Negroid	4 (1.3%)	0 (0%)	4 (3.6%)	0.017‡
– African	4 (1.3%)	3 (1.6%)	1 (0.9%)	1.000‡
– Missing*	11 (3.6%)	7 (3.6%)	4 (3.6%)	-
BMI	23.37 (17.00-43.00)	23.00 (18.00-42.00)	24.00 (17.00-43.00)	0.229¶
Menarche	13.00 (8.00-17.00)	13.00 (9.00-17.00)	13.00 (8.00-16.00)	0.727¶
Menstrual cycle				
– Regular	227 (74.9%)	144 (75.0%)	83 (74.8%)	0.993†
– Mostly regular	32 (10.6%)	24 (12.5%)	8 (7.2%)	0.150†
– Irregular	35 (11.6%)	19 (9.9%)	16 (14.4%)	0.232†
– Absent	4 (1.3%)	2 (1.0%)	2 (1.8%)	0.625‡
– Missing*	5 (1.7%)	3 (1.6%)	2 (1.8%)	-
Indication				
– Male factor	190 (62.7%)	128 (66.7%)	62 (55.9%)	0.246†
– Idiopathic	47 (15.5%)	29 (15.1%)	18 (16.2%)	0.581†
– Tube factor	13 (4.3%)	7 (3.6%)	6 (5.4%)	0.379†
– Cycle disorder	12 (4.0%)	7 (3.6%)	5 (4.5%)	0.758‡
– Uterine factor	2 (0.7%)	1 (0.5%)	1 (0.9%)	1.000‡
– Other	6 (2.0%)	4 (2.0%)	2 (1.8%)	0.612‡
– Combination*	25 (8.3%)	12 (6.3%)	13 (11.7%)	-
– Missing*	8 (2.6%)	4 (2.1%)	4 (3.6%)	-
Use of medication				
– Yes	70 (23.1%)	40 (20.8%)	30 (27.0%)	0.188†
– No	228 (75.2%)	150 (78.1%)	78 (70.3%)	
– Missing*	5 (1.7%)	2 (1.0%)	3 (2.7%)	
Smoking				
– Yes	44 (14.5%)	30 (15.6%)	14 (12.6%)	0.441†
– No	248 (81.8%)	154 (80.2%)	94 (84.7%)	
– Missing*	11 (3.6%)	8 (4.2%)	3 (2.7%)	
Alcoholic consumption				
– Yes	180 (59.4%)	119 (62.0%)	61 (55.0%)	0.215†
– No	112 (37.0%)	66 (34.4%)	46 (41.4%)	
– Missing*	11 (3.6%)	7 (3.6%)	4 (3.6%)	
Urinary tract infection in prior 3 months				
– Yes	9 (3.0%)	7 (3.6%)	2 (1.8%)	0.497‡
– No	291 (96.0%)	185 (96.4%)	106 (95.5%)	
– Missing*	3 (1.0%)	0 (0%)	3 (2.7%)	-

	All women (n=303)	Inclusions (n=192)	Exclusions (n=111)	p-value**
Use of antibiotic treatment in prior 3 months				
– Yes	23 (7.6%)	17 (8.9%)	6 (5.4%)	0.303†
– No	277 (91.4%)	175 (91.1%)	102 (91.9%)	
– Missing*	3 (1.0%)	0 (0%)	3 (2.7%)	-
Duration of infertility	2.39 (0.00-13.80)	2.35 (0.43-13.80)	2.40 (0-12.20)	0.477¶

Values are given as mean ± SD years, number (%), and median (range). BMI= Body Mass Index (kg/m2).

† By Chi-square test.

‡ By Fisher's exact test.

§ By Independent Samples T-test.

¶ By Mann-Whitney U test.

*these subcategories are excluded from analysis.

**comparisons are made between analysed category and the other categories combined.

Supplementary table 3: Univariate analysis of clinical characteristics of the IVF or IVF-ICSI procedure in women who became pregnant and women who did not become pregnant.

	Pregnant		
	Yes (n=67)	No (n=125)	p-value**
IVF or IVF-ICSI			
– IVF	19 (28.4%)	45 (36.0%)	0.268†
– IVF-ICSI	48 (71.6%)	80 (64.0%)	
Hormone downregulation			
– Decapeptyl	51 (76.1%)	100 (80.0%)	0.440†
– Lucrin	10 (14.9%)	13 (10.4%)	0.369†
– Orgalutran	3 (4.5%)	3 (2.4%)	0.425‡
– Cetrotide	2 (3.0%)	5 (4.0%)	1.000‡
– Synarel	0 (0%)	1 (0.8%)	1.000‡
– Missing*	1 (1.5%)	3 (2.4%)	-
Hormone stimulation			
– Gonal-f	55 (82.1%)	99 (79.2%)	0.492†
– Menopur	9 (13.4%)	17 (13.6%)	0.994†
– Bemfola	1 (1.5%)	8 (6.4%)	0.167‡
– Pregnyl	0 (0%)	1 (0.8%)	1.000‡
– Puregon	1 (1.5%)	0 (0%)	0.346‡
– Missing*	1 (1.5%)	0 (0%)	-
Endometrial thickness (mm)	10.36 (2.22)	10.26 (2.15)	0.766§
OPU			
– Yes	67 (100%)	125 (100%)	-
– No	0 (0%)	0 (0%)	
Follicle count	9 (4-20)	10 (2-23)	0.140¶
Number of oocytes	8 (1-22)	10 (1-29)	0.613¶
Embryo transfer			
– Yes	67 (100%)	125 (100%)	-
– No	0 (0%)	0 (0%)	
ET			
– SET	67 (100%)	122 (97.6%)	0.553‡
– DET	0 (0%)	3 (2.4%)	

Values are given as mean ± SD years, number (%), and median (range). IVF= *in-vitro* Fertilisation; IVF-ICSI= *in-vitro* Fertilisation with intracytoplasmic sperm injection; mm= millimetre; OPU= ovum pick up; ET= embryo transfer; SET= single embryo transfer; DET= double embryo transfer.

† By Chi-square test.

‡ By Fisher's exact test.

§ By Independent Samples T-test.

¶ By Mann-Whitney U test.

*these subcategories are excluded from analysis.

**comparisons are made between analysed category and the other categories combined.