The impact of bacterial and uterine factors on subfertility

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The impact of bacterial and uterine factors on subfertility

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INTRODUCTION

Gynaecology and obstetrics are amongst the oldest disciplines in healthcare. As with many other healthcare disciplines, gynaecology and obstetrics experience significant changes due to lifestyle transformations in society. In the last few decades, women have increasingly pursued their own careers, with more flexibility in family planning due to contraception methods. This has led to an increased age for couples receiving their first child, with women in the Netherlands reaching an average age of 31.6 years in 2020 for their first child. Increased age is highly associated with a loss of ovarian reserve and oocyte quality, leading to an increase in subfertility rates. Another well-known example of societal change is the increasing incidence of obesity and smoking, leading to fertility issues and pregnancy complications as well. The introduction of fertility treatments such as ovulation induction, intrauterine insemination (IUI) and in vitro fertilization (IVF) meant a breakthrough and greatly influenced the practice of gynaecology [1]. In 2020 in the Netherlands, 21.507 embryo transfers were performed, which resulted in 4644 ongoing pregnancies. This means that 1 in 30 babies born is conceived by IVF. In contrast, in 2000 12.266 embryo transfers were performed, which resulted in 2984 ongoing pregnancies, which is less than 1 in 50 babies (CBS/degynaecoloog.nl).

The initial indication for IVF treatment was blocked fallopian tubes, as women with this condition were unable to conceive spontaneously. Nowadays, IVF is used for various indications, such as unexplained subfertility and diminished ovarian reserve. However, advanced techniques such as IVF cannot treat all these causes for subfertility. 50% of couples below 35 years old conceive after IVF treatment (CDC data 2020). Prediction models for the success of IVF are based on factors such as women's age, body mass index and subfertility diagnosis, among others, but it remains difficult to provide an accurate prediction [2]. The more information we have about all factors influencing treatments, the closer answers will be to improve success rates.

In this introduction we outline the standard fertility treatments in the Netherlands and important lifestyle interventions in fertility treatment. The subsequent focus will be on two phenomena still under research, believed to influence subfertility: bacterial vaginosis and a previous delivery by caesarean section [3,4]. Investigating the connection between subfertility, medical history and lifestyle provides insights into multi-faceted aspects to improve fertility outcomes. An introductory overview is provided about bacterial vaginosis, caesarean section, and a rationale on why a caesarean delivery and bacterial vaginosis can be a combined issue in subfertility.

Fertility treatments

In the Netherlands, couples who do not conceive after one year often seek assistance from a fertility specialist. The prevalence of subfertility is around 15% among couples trying

to conceive (FMS richtlijnendatabase, Oriënterend fertiliteitsonderzoek). During initial fertility assessment both man and woman are evaluated for factors such as the ovulation, tubal patency, sexual activity, and semen quality. Different treatments can be advised based on these assessed factors. Ovulation induction is started in cases of an irregular menstrual cycle. Intrauterine inseminations (IUI) can be started in mild male factor. IVF or intracytoplasmic sperm injection (ICSI) is started, for example, in cases of tubal pathology, severe endometriosis, or severe male factor.

Approximately 30% of the subfertile couples are considered as having unexplained subfertility. In case of unexplained subfertility (and the woman's age is below 38 years), the Hunault model is used to estimate the chance of a spontaneous conceived pregnancy the following year based on woman's age, motility of the sperm cells, duration of subfertility and history of previous pregnancy. When the Hunault prediction score indicates a chance of spontaneous conception above 30% in the following year, fertility treatment is believed not to add any extra value. Couples are then advised to have expectant management for six to twelve months before initiating fertility treatment [5,6].

If the Hunault prediction score is below 30%, fertility treatment is often initiated. First-line treatment consists of IUI with controlled ovarian stimulation for four to six consecutive attempts when tubes are patent. If couples do not conceive after IUI, IVF is started [5,6]. During IVF, women receive medication for ovarian stimulation, followed by oocyte retrieval. The collected eggs are fertilised with the partners semen in the laboratory, and three to five days later, a single embryo is transferred into the uterus. A woman can only receive two embryos if she has undergone multiple IVF attempts or if her age is above 38 years.

Fertility treatments are expensive and have a high psychological burden on couples. Total average costs of one year of IVF, including 1.5 cycles and subsequent freeze/thaw attempts, are estimated at €6595 [7]. In the Netherlands three rounds of IVF with oocyte retrieval are covered by the basic health insurance. Also, there are indirect costs to society that should be taken into consideration. Costs of productivity losses due to hospital visits for IVF are on average 596 euro per cycle per woman [8,9].

Lifestyle intervention

Lifestyle is becoming increasingly crucial during fertility assessment. The rise in the population's body mass index (BMI) has led to a shift in the upper limit of BMI for eligibility to initiate fertility treatments. A raised BMI is associated with a threefold risk of infertility, mainly because of anovulation and menstrual cycle alterations. In IVF a higher BMI is associated with lower live birth rates and higher miscarriage rates [10]. The upper BMI

limit varies among hospitals, generally within the BMI-range of 32-35. Couples above this limit are encouraged to lose weight.

There is also evidence that smoking is detrimental on the chance to conceive, with an increased amount of fertility treatments needed. The impact of smoking on live birth rates in IVF is comparable with an increased age of > 10 years from age 20 to 30 years [11,12]. Couples are strongly advised to quit smoking, and in certain hospitals, it is mandatory to quit smoking before commencing treatment.

Nevertheless, a robust framework to motivate couples towards a healthier lifestyle is not incorporated in the standard assessment and is often not covered by health insurance. If prevention could limit the number of fertility treatments required, it could also reduce costs for society [9].

Unexplained factor 1: Bacterial vaginosis

The link between bacterial vaginosis and subfertility was already studied as early as 1997 [4], but the actual understanding about the etiology between the two is still unknown [13]. Bacterial vaginosis (BV) is an imbalance of the vaginal microbiome, where there are fewer *Lactobacilli*. An abnormal vaginal microbiome is therefore also a commonly used term to refer to BV. Half of the persons with BV have symptoms, such as odorous discharge complaints, while the other half are asymptomatic. The incidence of BV can be up to 30% in various (ethnic) populations [14]. Different lifestyle factors are associated with BV, including smoking, contraceptives, obesity, sexual activity and more [15]. BV is associated with sexual transmitted diseases, premature delivery, and other serious complications [16,17]. Bacterial vaginosis treatment is possible with antibiotics and/or probiotics, but the recurrence rate remains high [18]. Vaginal discharge complaints during fertility treatment will be treated if mentioned, but asymptomatic bacterial vaginosis is not routinely screened for in the Netherlands unless in a research setting. Lifestyle is closely linked to the gut and vaginal microbiome, but there is insufficient evidence regarding lifestyle recommendations to advise the optimal regimen currently.

There are different methods to test for BV. An accessible method for general practitioners is the Amsel criteria, consisting of pH-testing, KOH-testing and microscopic evaluation of a discharge sample. The gold standard remains the Nugent score, in which a microbiologist performs a gram stain. Both methods are partly subjective, and time consuming, and new techniques based on deoxyribonucleic acid (DNA) sequencing are increasingly used, such as polymerase chain reaction (PCR) or sequencing the 16s ribosomal ribonucleic acid (rRNA) to define the composition of the microbiome. Quantitative PCR (qPCR) is believed to be more accurate in diagnosing BV than Nugent score [19].

The microbiome consists of all microbes, such as bacteria and fungi that live in our bodies, with different compounds in multiple organs, for example the gut or the vagina. A healthy vaginal microbiome is mainly dominated by *Lactobacillus* species. qPCR assays analyse the balance between the quantity of *Lactobacilli* and anaerobics, such as *Gardnerella* and *Fannyhessia*. 16s rRNA based sequencing can be used to identify the bacteria present in a sample to the genus level (for example *Lactobacilli*). Only a limited number of bacteria can be identified down to the species level (such as *L. crispatus* and *L. iners*) due to the lack of resolution of the 16s rRNA gene [20]. The vaginal microbiome can be classified in five community state types (CST). CST I is dominated by *Lactobacillus crispatus*, and respectively CST II by *L.gasseri*, CST III by *L. iners*, CST IV by non-lactobacilli and CST V by *L. jensenii*. CST IV is the microbiome community state type associated with bacterial vaginosis. Shotgun metagenomics sequencing is the latest and most precise method, capable of sequencing entire DNA, including fungi and viruses. However, as these methods become more advanced, they also become more expensive. With the increasing accessibility of DNA sequencing and PCR techniques, it is possible to test the microbiome of the endometrium and vagina. This may offer insights into why some subfertile persons encounter challenges in conceiving.

In the literature, the focus is primarily on describing the relationship between BV and IVF pregnancy outcomes. Haahr et al. demonstrated a detrimental effect of BV, diagnosed by Nugent score and qPCR, with only a 9% pregnancy rate in individuals with BV compared to a 44% pregnancy rate in individuals with a normal microbiome [21]. Koedooder et al. employed an algorithm to categorize different prognosis groups based on their vaginal microbiome, revealing a 5.9% pregnancy rate in the worst microbiome group and up to a 54% pregnancy rate in the most favourable microbiome group [22].

For this thesis, our studies not only focused on IVF treatment but also explored the role of BV in a general subfertile population and during intrauterine insemination. To enhance comparability between studies, a combination of tests to diagnose BV was used, including pH, qPCR, and microbiome analysis. While many studies only report the pregnancy results of one embryo transfer, we believe it is important to account for multiple attempts. Live birth was considered as the most important primary outcome, an outcome many earlier studies did not report. However, there is a consensus that this is the most important outcome in fertility treatment studies [13,23].

Unexplained factor 2: Caesarean section scar

Due to the increasing women's age for receiving their first child, obesity and the more scheduled and risk-averse nature of society, the number of caesarean sections has also risen globally, exceeding 40% in some countries [24–26]. The scar can make subsequent

pregnancies and deliveries more complicated, leading to placental disorders and higher maternal morbidity. Population-based studies showed a lower number of pregnancies after caesarean sections. This could be a sign of subfertility; however, it could also be attributed to the traumatic experience of an emergency delivery setting and anxiety for a second pregnancy [3]. In persons with a history of caesarean section embryo transfer during IVF is more difficult, due to the need to transfer beyond the scar. The flexible tip of the catheter used for embryo transfer can more readily pass into the site of the scar than into the uterine cavity. This significantly prolongs the time necessary for the embryo transfer and increases the likelihood of blood or mucus on the tip of the catheter [27].

Another complication that could arise from a caesarean section is called a niche. This is an indentation in the myometrial lining, which could be filled with fluid or blood. A niche could cause menstrual complaints such as postmenstrual spotting. The perfect technique for a caesarean section to decrease the chance of a niche forming is still unknown [28,29]. Recently, a consensus has emerged about the definition of a niche (at least 2mm in size) and about certain symptoms (such as subfertility) secondary to a niche [30]. A hysteroscopic or laparoscopic repair of the niche could relieve complaints of spotting, however there is little evidence to indicate the effectiveness of niche surgeries for pregnancy results [31,32].

To study the impact of the scar caused by the caesarean section on fertility, it is more accurate to assess the impact of the scar during fertility treatments. Psychological factors of not desiring a subsequent child are not of concern during fertility treatments in contrast to population-based studies. Also, the presence of a niche can be assessed, to study if individuals with a niche have more fertility problems than individuals with a caesarean scar without a niche.

Scary bacteria

It is hypothesised that fluid within a niche could create an embryo-toxic environment, similar to a hydrosalpinx (a fallopian tube filled with fluid, sometimes caused by a *Chlamydia* infection) [33]. Certain studies suggest a connection between tubal factor infertility and bacterial vaginosis [4,21]. Both hydrosalpinx and the niche could result in endometrial fluid within the uterine cavity, impacting the success rates of fertility treatment [34]. In cases of endometrial fluid within the uterine cavity there are different approaches on embryo transfer during IVF [35]. It is not known if certain bacteria or micronutrients might be more prevalent in endometrial or niche fluid, causing the embryotoxic environment. It is postulated that the accumulation of blood may lead to degradation of hemoglobin, which increases iron exposure. Another cause could be continuous flow of intrauterine

fluid interfering with the implantation of an embryo. A hypothesis could be that the cause for the fertility issues after caesarean section may not be the niche itself, but perhaps the embryotoxic fluid.

Aim and outline of this thesis

This thesis tries to answer whether live birth rates in subfertile couples are influenced by an abnormal vaginal microbiome or a caesarean section scar, and whether there is a link between these two.

The BIFI (Bacterial vaginosis In Fertilllty) study was a prospective cohort study. This study investigated if (asymptomatic) bacterial vaginosis had a negative effect on live birth rates in a subfertile population. While collecting study data, particular emphasis was placed on ensuring the completion of information on confounding lifestyle factors. In ***chapter 2*** the role of bacterial vaginosis during IUI or IVF treatments is investigated. Different methods as pH, qPCR and 16s rRNA sequencing were used, to increase comparability with other literature, as there is no consensus on which method to use.

Measurements of pH and qPCR are less expensive and less time-consuming testing methods compared to 16s rRNA sequencing, so could potentially more easily be implemented in clinical practice. Therefore, in ***chapter 3*** pH testing is analysed compared to qPCR testing.

The effect of the fertility treatments on the vaginal microbiome (tested with 16s rRNA sequencing) over time was evaluated (***chapter 4***), because bacterial vaginosis could be influenced by hormonal status. In ***chapter 5*** the role of bacterial vaginosis at initial fertility assessment (also including persons with ovulation induction and expectant management) is studied and how this influences time to pregnancy leading to live birth in different patient groups.

The SCAR (Sectio Caesarea and Assisted Reproduction) study (***chapter 6***) was a retrospective study and assessed the pregnancy rates during IVF after a caesarean section compared to a vaginal delivery. A systematic review (***chapter 7***) tried to answer if subfertility after a caesarean section is caused by the niche. During the BIFI study the idea grew that maybe the fluid formed inside a niche after a caesarean section could be colonised with abnormal bacteria. This second hypothesis was also included in the systematic review.

A summary and in-depth discussion of the main research findings, implications and directions for future research is discussed in ***chapter 8***.

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2

BACTERIAL VAGINOSIS IN A SUBFERTILE POPULATION UNDERGOING FERTILITY TREATMENTS: A PROSPECTIVE COHORT STUDY

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Abstract

Purpose

This study investigates the role of bacterial vaginosis (BV) on pregnancy rates during various fertility treatments. BV is known to influence several obstetric outcomes, such as preterm delivery and endometritis. Only few studies investigated the effect of BV in subfertile women, and studies found a negative effect on fecundity especially in the in vitro fertilisation population.

Methods

Observational prospective study, 76 couples attending a fertility clinic in the Netherlands between July 2019 and June 2022, undergoing a total of 133 attempts of intrauterine insemination, in vitro fertilization or intracytoplasmatic sperm injection. Vaginal samples taken at oocyte retrieval or insemination were analysed on qPCR BV and 16S rRNA gene microbiota analysis of V1-V2 region. Logistic regression with a Generalized Estimated Equations analysis was used to account for multiple observations per couple.

Results

A total of 26% of the 133 samples tested positive for BV. No significant differences were observed in ongoing pregnancy or live birth rates based on BV status (OR 0.50 (0.16-1.59), aOR 0.32 (0.09-1.23)) or microbiome community state type. There was a tendency of more miscarriages based on positive BV status (OR 4.22 (1.10-16.21), aOR 4.28 (0.65-28.11)) or community state type group III and IV. On baseline qPCR positive participants had significantly higher body mass index and smoked more often. Odds ratios were adjusted for smoking status, body mass index and socioeconomic status.

Conclusion

Bacterial vaginosis does not significantly impact ongoing pregnancy rates but could affect miscarriage rates.

Introduction

Between 8-12% of couples worldwide are affected by subfertility. There are many causes of subfertility, such as ovulatory dysfunction or male factor, but 15% of couples have no identifiable cause [1,2]. Bacterial vaginosis (BV) might be a reason (or a cue) for infertility, which is defined as dysbiosis of the vaginal microbiome. It is characterized by the loss of lactic acid-producing bacteria and an increase in the number and diversity of anaerobic bacteria (such as *Gardnerella vaginalis*). The etiology and pathogenesis of BV is still unknown, but it is associated with certain lifestyle factors [3]. BV can cause symptoms such as abnormal discharge and fishy odour, however half of patients with BV are asymptomatic. Various reports measure an incidence of 10-32% of BV among the total population. The incidence of BV is influenced by ethnicity and is shown to be higher in subfertile populations [4,5]. BV is associated with a higher risk of infections (including sexually transmitted ones), tubal factor infertility, and obstetric complications, such as preterm delivery and endometritis [6,7].

Some studies have found a negative effect of BV on pregnancy rates among (sub)fertile women, which was primarily studied in an IVF population [6,8,9] and only in two studies in a population undergoing intrauterine insemination [10,11]. Only in recent years, the more sensitive methods like qPCR and microbiota analysis have been used to diagnose BV [12,13]. Most studies investigate the vaginal microbiome, and some also the endometrial microbiome. There is evidence of a continuum between these two microbiotas [14].

It is suggested BV influences the endometrium, and thus, the implantation rate. Meta-analysis showed more early pregnancy loss in BV positive women [13,15]. Furthermore, higher prevalence of unfavourable microbiome was found in multiple studies investigating recurrent implantation failure (RIF) or recurrent miscarriage, except for one study with a larger study size [16,17].

Therapies for BV include metronidazole and clindamycin, but the recurrence rate within a year remains high [18,19]. There are also promising data about new treatment options, for example with probiotics [20]. Up till now there are no studies with good quality evidence that treatment of BV increases pregnancy rates.

Since evidence on the relation between BV and a subfertile population undergoing intrauterine insemination (IUI) and IVF/ICSI is insufficient, our aim is to investigate the influence of BV on pregnancy rates in this study population. It is hypothesized that BV may have a negative impact on ongoing pregnancy rates in both the IUI and IVF/ICSI population. Additionally, BV could be possibly associated with higher rates of early pregnancy loss.

Materials & methods

This is a prospective single centre cohort study in the Haaglanden Medical Center (HMC) in the Hague, the Netherlands. This fertility clinic is a transport clinic to the IVF laboratory of the Leiden University Medical Center. Microbiological assessment of vaginal swabs was done by external laboratory of Eurofins NMDL and DDL Diagnostic Laboratory in Rijswijk, The Netherlands. Participants (18 years and older) undergoing fertility treatments (intra uterine insemination (IUI), in vitro fertilization (IVF) and intracytoplasmatic sperm injection (ICSI)) were included between July 2019 and June 2022. Exclusion criteria were inability to understand Dutch or English language, persons with three or more miscarriages, and those on prophylactic antibiotic treatment. There were no couples treated with donor sperm or donor oocytes. Before starting IUI or IVF/ICSI-treatment couples were required to cease smoking.

Sample size calculation was performed before starting the study. To detect a difference of 15% in pregnancy rate assuming a 30% prevalence of BV 372 fertility treatments should be included to reach a power of 90% (alpha 0.05, percentages based on the results of the study of Haahr) [6].

Patient recruitment, sample and data collection

Persons were asked to join the study at their initial fertility assessment (IFA). In the Netherlands persons need to have a referral (for example from a general practitioner) for an IFA. When participants gave informed consent, the first vaginal swab (e-swab, Copan Italia SpA, Breschia, Italy) and pH measurement (pH-Fix 4.0-7.0, ref 92137, Macherey-Nagel, Düren, Germany) were taken from the posterior fornix after inserting a speculum. The measurements were not performed if the woman was menstruating or post-coital. Participants and fertility doctors were blinded for the outcome of the swab. If participants had subsequent IUI or IVF/ICSI treatment the sampling was performed again, prior to the oocyte pick up or insemination, up to a maximum of 4 procedures, to obtain an accurate BV status for each individual IUI and IVF/ICSI attempt. During oocyte pick up, antibiotics are not routinely administered, but only given for certain indications (for example with endometriomas). In case of a frozen embryo cycle, the sampling was performed at the last ultrasound before transfer. After an ongoing pregnancy no new swabs were collected. The vaginal samples were frozen within 24 hours in the HMC laboratory and later transported to the external laboratories for molecular analysis. If participants had complaints of BV which required treatment, they were tested separately from our study. When tested positive for BV, they were treated according to the standard protocol.

Blood samples for hCG measurement were collected two weeks after insemination, oocyte pick up or embryo transfer. Follow up ended at cessation of treatment, end of study (August 2022) or live birth (follow up until June 2023). Information about patient characteristics, fertility treatment and pregnancy outcomes were extracted from the electronic patient files. This information was managed using Castor EDC, a cloud-based clinical data management service.

BV qPCR

Extracted DNA from all swabs was tested with a CE-IVD marked multiplex quantitative PCR assay (AmpliSens® Florocenosis/Bacterial vaginosis-FRT PCR kit, InterLabService, Moscow, Russia) according to the manufacturer's instructions. Based on the presence of *Lactobacillus* species, *Gardnerella vaginalis*, *Atopobium vaginae* (recently reclassified as *Fannyhessea vaginae*) [21] and total amount of bacteria, swabs were categorised as BV positive (amount of *G. vaginalis* and/or A. vaginae is almost equal or exceeds the amount of *Lactobacillus spp.),* BV negative (*G. vaginalis* and/or *A. vaginae* are absent or its amount is substantially less than the *Lactobacillus spp.* amount), unspecified dysbiosis (amount of *Lactobacillus spp.* is reduced relative to the total amount of bacteria, whereas *G. vaginalis* and/or *A. vaginae* are absent or its amount is substantially less than total amount of bacteria) or suspected dysbiosis (amount of *G. vaginalis* and/or *A. vaginae* is similar to the amount of *Lactobacillus spp.* but does not exceed the limit value) using the software tool provided by the kit manufacturer. Unspecified dysbiosis and suspected dysbiosis were regarded as qPCR BV positive.

Microbiota analysis

Microbiota analysis was performed on the extracted DNA of swabs of participants who started directly with IUI or IVF. Microbiota analysis was done on their IFA swab, and on their subsequent swabs during IUI or IVF/ICSI treatment until pregnant or until switch from IUI to IVF/ICSI. A fragment of ~421bp of the V1-V2 region of the 16S rRNA gene was amplified using the primers described by Ravel, et al. (2011) and Walker, et al. (2015) with Illumina overhang adaptor sequences added[22,23]. Results were classified in one of five vaginal microbiome community state types (CST), as described by Ravel, et al. (2011). CST I is dominated by *L. crispatus*, and respectively CST II by *L.gasseri*, CST III by *L. iners*, CST IV by non-lactobacilli, CST V by *L. jensenii* [22]. For more details on the nucleic acid extraction and microbiota analysis see supplementary file 1.

Outcomes

Primary endpoint of the study was ongoing pregnancy rate at 12-weeks' gestation. Secondary endpoints were miscarriages (including biochemical pregnancy and clinical pregnancy with or without fetal heartbeat at 7-weeks' gestation), live birth, ectopic pregnancy, and preterm delivery (<37 weeks).

Compliance with Ethical Standards

This study was approved by the Medical Ethics Committee of Leiden Den Haag Delft, reference Z21.031. All participants gave their informed consent to involve in this study.

Statistical analysis

IBM SPSS statistics for Macintosh, version 27, was used for all analysis. Continuous parametric variables were analysed using an unpaired t test. Continuous non-parametric variables are analysed using the Mann-Whitney-U test. Categorical variables are examined using the Chi^2 or Fischer's exact test. To account for the repeated measurements per case a general estimating equations analysis (GEE regression model) is used, with an exchangeable correlation matrix.

Results

Description of included participants

Figure 1 show a flow diagram of included couples. Some couples started IUI or IVF/ICSI treatment but had no subsequent swabs done, and therefore were excluded from the analysis. In total 76 couples could be included, of which 68 couples underwent fertility treatment with subsequent vaginal swabs. Couples conceiving spontaneously before fertility treatment and couples starting fertility treatments after expectant management or ovulation induction were included for analysis. Microbiome CST was analysed in the subgroup of direct indication for fertility treatment, with eight couples conceiving spontaneously before fertility treatment started.

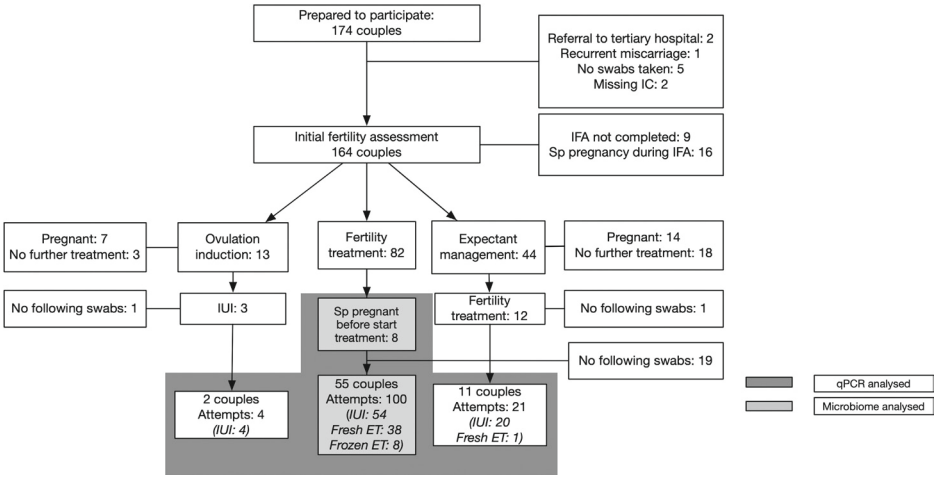

Fig. 1 Flowchart of included participants

Table 1 describes baseline characteristics of the couples based on BV qPCR results at IFA. One participant had no start qPCR result, but two subsequent negative qPCR results, and was regarded as BV negative at IFA.

The maximum age of participants was 42 years. Almost no discharge complaints were reported, only one participant was treated for discharge complaints. BV positive participants at baseline have a significant higher BMI and a higher percentage of smokers. The measured pH was significantly higher in the BV qPCR positive group.

Separate analysis comparing ongoing pregnant versus non-pregnant participants or participants experiencing a miscarriage versus no miscarriage did not show significant differences for BMI or smoking (data not shown).

Table 1 Baseline characteristics

Baseline IFA	**BV positive**	**BV negative**	**p-value**
Participants	17	59	
Age (at IFA) (mean, SD)	34 (4.3)	35 (4.3)	0.52
pH (median, IQR)	5.3 (5-5.5)	4.1 (4-5)	**<0.001***
Discharge complaints (at IFA) n(%)	0	3 (5%)	1.00
BMI (at IFA) (mean, SD)	27.3 (5.6)	24.6 (4.2)	**0.04***
Smoking (at IFA) n(%)	5 (29%)	4 (7%)	**0.01***
Alcohol (≥ 1 glas per week) n(%)	6 (35%)	14 (24%)	0.36
Druguse (on regular basis) n(%)	2 (12%)	2 (3,5%)	0.22
Medication use (for comorbidities)	3 (17,5%)	10 (17%)	1.00
Chlamydia antibodies positive n(%)	2 (12%)	5 (9%)	0.49
Ethnicity n(%) **			0.22
Caucasian	7 (41%)	38 (64%)	
African	1 (5,8%)	0	
Antillean	2 (12%)	2 (3,5%)	
Asian	2 (12%)	6 (10%)	
Moroccan	1 (5,8%)	4 (7%)	
Hindu	2 (12%)	4 (7%)	
Turkish	0	1 (1.5%)	
Other	1 (5,8%)	4 (7%)	
Missing	1 (5,8%)	0	
Socioeconomic status n(%)***			0.08
Low	2 (12%)	1 (1.5%)	
Middle	6 (35%)	13 (22%)	
High	8 (47%)	44 (76%)	
Missing	1 (6%)	1 (1.5%)	
Regular cycle n(%)	16 (94%)	54 (92%)	0.73
HPV positive last year n(%)	3 (17,5%)	8 (14%)	0.60

Table 1 Baseline characteristics (continued)

Baseline IFA	**BV positive**	**BV negative**	**p-value**
Gravidity n (%)			0.47
0	12 (70,5%)	38 (64%)	
1	2 (12%)	15 (25%)	
2	3 (17,5%)	5 (9%)	
3	0	1 (2%)	
History of preterm birth	0	1	1.00
History of c-section	0	7	0.28
Subfertility duration in years (median, IQR)	2 (1-2.5)	1 (1-2)	0.48
Cause of subfertility n (%)****			0.07
Male factor	2 (12%)	20 (34%)	
Tubal factor	3 (17,5%)	3 (5%)	
Hormonal	2 (12%)	5 (8,5%)	
Endometriosis	0	9 (15%)	
Unknown	10 (58,5%)	20 (34%)	
Other	0	2 (3,5%)	
First treatment			0.07
Expectant management	1 (6%)	10 (17%)	
Spontaneous pregnancy	4 (23%)	4 (7%)	
IUI	9 (53%)	23 (39%)	
IVF	2 (12%)	21 (35%)	
Ovulation induction	1 (6%)	1 (2%)	

** p-value ≤0.05 considered significant*

*** other mostly Hispanic persons*

**** as defined by education status*

***** hormonal: premature ovarian insufficiency or anovulation, other: uterine myomas, uterus anomaly, sexual disfunction*

Pregnancy outcomes

Pregnancy results are shown in Table 2, based on BV status at the time of attempt. In total 133 attempts were included, of which 26% of samples tested positive on BV. One of those attempts had a total fertilization failure (IVF-procedure), so the participant could not receive a fresh ET. Ongoing pregnancy and live birth rates were lower in BV qPCR positive attempts, however not significant (20% vs 11%, OR 0.50). Table 2 shows a higher percentage of miscarriages in qPCR BV positive attempts. Analysed on number of miscarriages in pregnant participants, this also was significant (25% vs 60%, OR 4.22). To adjust for confounders, correction was applied for BMI, smoking and SES. The adjusted odds ratios were in line with the non-adjusted odds. Cause of subfertility and age had no significant influence and were therefore excluded from the model.

Separate analysis of the IVF/ICSI attempts are in line with data above, however, data did show an on average stronger effect of BV positivity than in IUI attempts (data not shown).

Four participants had a preterm delivery, three of whom delivered between 32-and 37 weeks' gestation. One participant delivered at 24 weeks' gestation after a weeklong episode of cramps and vaginal blood loss, which required admission to the hospital. The neonate died 7 days postpartum due to its prematurity. This participant tested negative for BV qPCR with every IUI attempt. However, the microbiota analysis of this participant at the start of treatment showed microbiome CST IV and later CST III. One of four preterm delivered participants tested BV positive and was in preterm labor around 32 weeks, although delivered eventually at 36 weeks. One participant had a termination of pregnancy around 20 weeks' gestation because of a serious birth defect (Noonan's syndrome, BV qPCR negative, CST III). One pregnancy of unknown location was reported.

Table 2 Pregnancy results by qPCR (GEE analysis)

Outcomes	BV positive	BV negative	OR (CI 95%)	p-value	aOR*** (CI 95%)	p-value
Attempts n(%)	35 (26%)	98 (74%)				
Outcome/attempt						
Ongoing pregnancy n(%)	4 (11%)	20 (20%)	0.50 (0.16-1.59)	0.24	0.32 (0.09-1.23)	0.10
Live birth n(%)	4 (11%)	19 (19%)	0.53 (0.17-1.70)	0.29	0.34 (0.09-1.31)	0.12
of which premature (n (% of live birth))	1 (25%)	3 (17%)				
Pregnancy n(%)	10 (29%)	27 (28%)	1.05 (0.44-2.50)	0.91	0.63 (0.26-1.52)	0.30
Miscarriage n(%)	6 (17%)	7 (7%)	2.70 (0.92-7.92)	0.07	2.03 (0.62-6.71)	0.24
of which early miscarriage/ biochemical* n(%)	2 (6%)	3 (3%)				
Outcome/pregnancy						
Miscarriage (% of pregnancy)	60%	25%	4.22 (1.10-16.22)	**0.04****	4.28 (0.65-28.11)	0.13

** miscarriage without heartbeat measured*

*** p-value ≤0.05 considered significant*

**** adjusted for smoking/bmi/ses*

Table 3 shows pregnancy results based on microbiome community state type (CST) at the moment of IUI/IVF/ICSI attempt. In total, 95 attempts were analysed on microbiome CST. CST II and CST V together were detected in only 5 attempts and were not included in the statistical analysis. CST I (*L. crispatus*) has a tendency of higher chance of an ongoing pregnancy compared to CST III and CST IV (32% in CST I vs 13% in CST III vs 22% in CST IV). Live birth rate in CST III was even lower because of one termination of pregnancy. CST III (*L. iners*) and CST IV has a tendency of higher chance of miscarriage (5% in CST I vs 13% in CST III and 22% CST IV). Numbers were too small for a clinically significant effect.

Table 3 Pregnancy results by microbiome CST (GEE analysis)

Outcomes	CST I (reference)	CST III	CST IV	other CST
Attempts	19	48	23	5
Ongoing pregnancy n(%)	6 (32%)	6 (13%)	5 (22%)	0
OR (CI 95%)	1	0.29 (0.07-1.13)	0.57 (0.13-2.51)	
Live birth n(%)	6 (32%)	5 (10%)	5 (22%)	0
OR (CI 95%)	1	0.24 (0.06-0.99)	0.56 (0.13-2.48)	
Pregnancy n(%)	7 (37%)	12 (25%)	10 (43%)	0
OR (CI 95%)	1	0.46 (0.13-2.61)	1.19 (0.29-4.89)	
Miscarriage n(%)	1 (5%)	6 (13%)	5 (22%)	0
OR (CI 95%)	1	2.29 (0.25-21.32)	4.66 (0.47-46.52)	
Miscarriage (% of pregnancy)	14%	50%	50%	
OR (CI 95%)	1	5.46 (0.56-53.52)	5.46 (0.56-53.52)	

Description of microbiome results

A total of 161 samples were tested with BV qPCR and microbiota analysis (including swabs at IFA and at insemination or IVF/ICSI procedure). The microbiome was analysed in a subgroup of the study, as seen in Figure 1. These 161 samples belonged to 73 different people, so a median of 2 samples per person. At species level all five CST classes are present. CST group II and V had only few samples as these *Lactobacilli* types are less common. One participant could not be classified with the classification system of Ravel (with dominating *L. johnsonii* en *L. ultunensis*). Of these 161 samples, 40 (25%) were tested positive for BV qPCR (Supplemental Figure 1). Shannon Diversity Index was significantly higher in the BV qPCR positive samples (2.62 vs 0.95, $p<0.001$). Group IV, the type of microbiome described as the most abnormal in literature, contained 37 samples (23% of total number of samples). 33 of the 37 CST IV samples tested positive for BV qPCR (89%, $p<0.001$). Supplementary file 2 shows a more detailed description about different bacteria found.

Out of the in total 73 participants, 15 persons had no subsequent attempts. The microbiota of all the swabbed IUI/IVF/ICSI attempts of in total 58 persons is shown in Figure 2a. The samples are ordered per class, with the highest percentage of *Lactobacilli* shown first. Shannon Diversity Index did not significantly differ between samples whether a pregnancy was established or not ($p=0.07$).

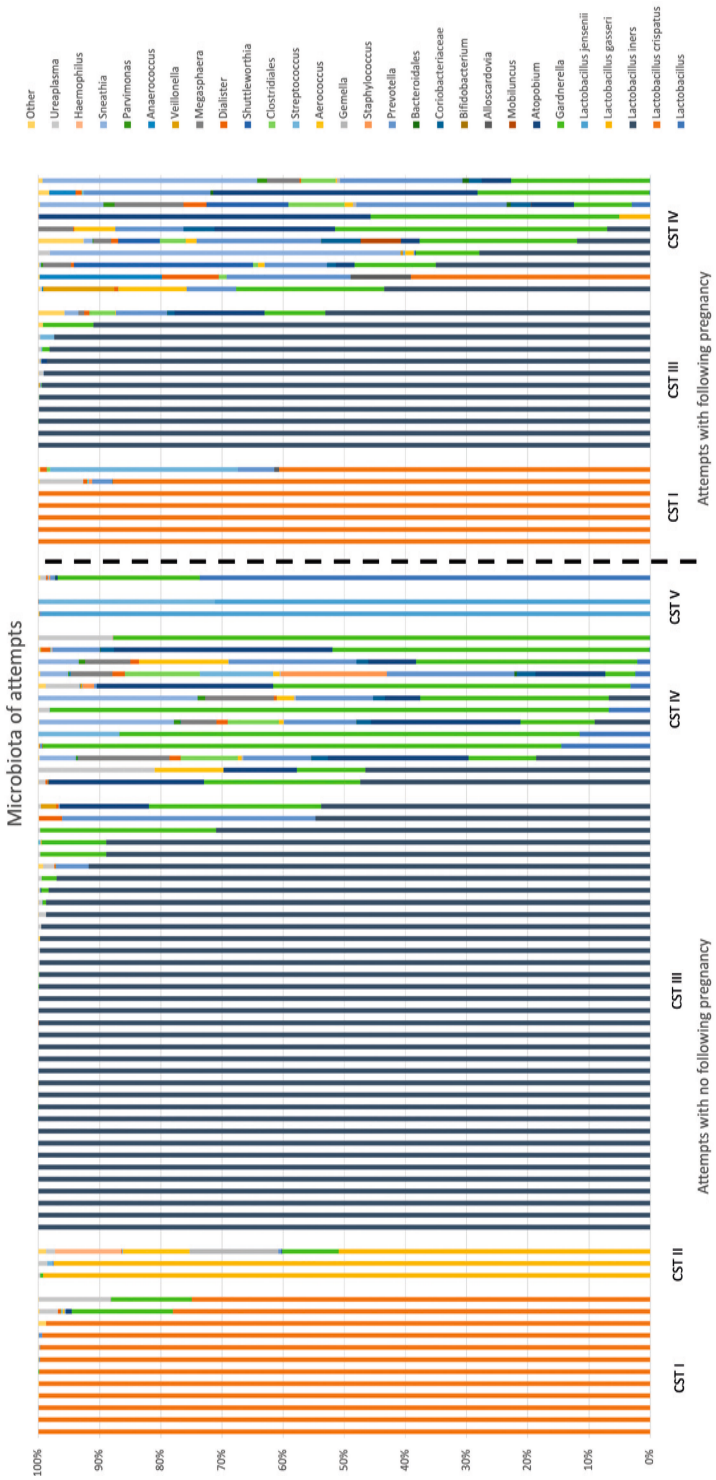

Fig. 2a overview of microbiota of swabbed attempts arranged on non-pregnant vs pregnant

When zooming in on the 29 microbiome samples on which a pregnancy was directly established (Figure 2b), CST I, III, and IV are present in both groups. The only CST I sample with a subsequent miscarriage contained 61% of *L. crispatus*. The samples with ongoing pregnancy contained on average 74% *Lactobacilli* and samples with subsequent miscarriage contained on average 59% *Lactobacilli* (p=0.33). Shannon Diversity Index did not significantly defer between samples with subsequent ongoing pregnancies or miscarriages (p=0.16).

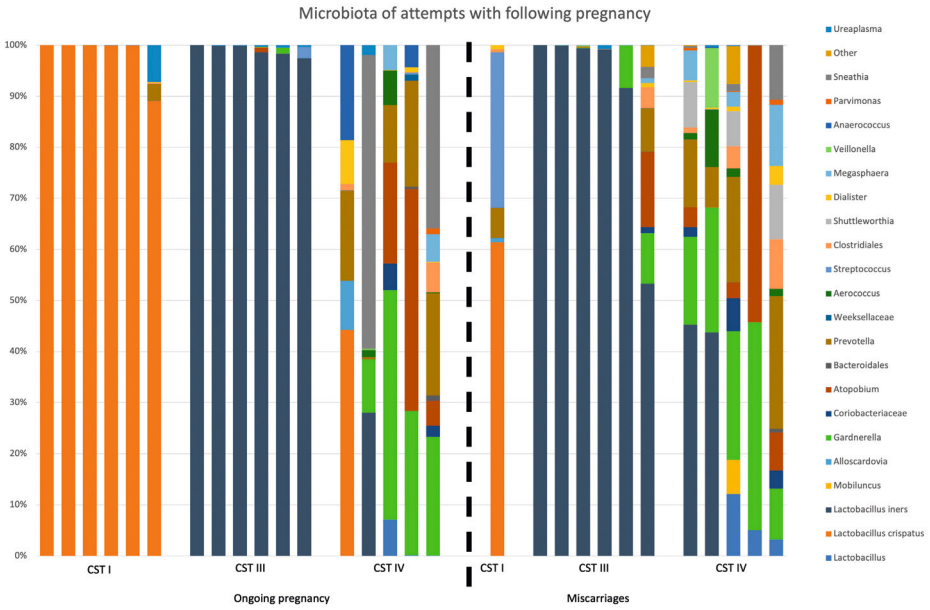

Fig. 2b overview of microbiota of swabbed attempts arranged in ongoing pregnant vs miscarriage

Discussion

This is one of the first studies to date researching the effect of bacterial vaginosis (diagnosed by qPCR) in a broader population than solely IVF patients. This study shows an increase in miscarriages due to qPCR BV positive status or microbiome CST III and IV during IUI or IVF/ICSI. A slight decrease in ongoing pregnancies due to qPCR BV positivity was observed, however not significant. The CST I (*L. crispatus*) group showed a small (not significant) increase in ongoing pregnancies compared to other community state types.

Previous studies had a very specific IVF population [6,8,9], with probably a higher significant influence of BV status on pregnancy outcomes then this relative 'good prognosis' study group treated with also IUI. Haahr et al. described a difference in IVF pregnancy results

of 35% based on qPCR [6]. Koedooder et al. reported a 5.9% IVF pregnancy rate when using the specific unfavourable microbiome algorithm, whereas they observed a 54% pregnancy rate in their most favourable group with less than 60% *L. crispatus* [9]. This could suggest that BV only has a significant impact in a certain (poor prognosis) population. There was a difference on smoking status and BMI between groups in our study. Smoking was measured as smoking at moment of sampling. Other studies do not mention the definition of smoking or classify smoking as 'has ever smoked'. Probably BMI and currently smoking (and thereby the immune system) play a role in BV status [3]. Participants had a relatively high socioeconomic status (SES), which could result in bias. However, a slightly lower SES in participants with BV was shown. This could indicate a relationship between lifestyle and dietary intake on the microbiome [24]. Most other studies did not record the SES thoroughly, so possibly the effect of BV lies more in being a proxy for a healthy lifestyle.

A consensus should be reached on how to define and measure an abnormal microbiome or BV status to minimize heterogeneity in further studies. The predictive model of Koedooder et al. (2019) described a negative impact on pregnancy outcomes when there was a high percentage of *Proteobacteria* and *L. jensenii* [9]. However, in this study, there were almost no samples with these percentages of *Proteobacteria* or *L. jensenii* (detailed in supplementary file 2). This study showed that CST III and CST IV have similar pregnancy results. The similarity of CST III *(L. iners)* and CST IV is previously described [6]. Since treatment of CST III is not possible yet, qPCR testing could be sufficient in future clinical settings. In the absence of evidence that treatment of BV leads to better pregnancy outcomes, testing of asymptomatic subfertile individuals for BV (qPCR or microbiome sequencing) should be discouraged at this moment.

A strength of this study is to analyse several consecutive attempts of IUI or IVF/ICSI instead of only one attempt, which could show an effect if it takes longer to become pregnant with a certain condition. Another strength is that the follow-up period was long enough to report live birth rates. Thirdly, this study compares qPCR BV and (partly) the microbiota analysis. This was also suggested by Skafte-Holm, to make it easier to compare studies in the future [13]. Sample outcomes of BV qPCR were in concordance with 16S microbiota analyses. This suggests that qPCR analysis is probably sufficient for a classification in certain patients. Low rates of lactobacilli are correlated with lower ongoing pregnancy rates, and this is exactly what the BV qPCR tests. The incidence of bacterial vaginosis is high in this multi-ethnic subfertile group, which aligns with other literature. The pathogens and vaginal CST classes identified in earlier research were also seen in this study. However, one participant's vaginal microbiome was dominated by *L. johnsonii/L. ultunensis*, which could not be classified into the known CST classes.

A limitation of this study is not reaching the sample size needed for the power calculation. The inclusion rate was much lower than expected, mainly due to a temporary closure of the fertility department during the COVID pandemic, and individuals being less willing to participate in scientific studies. Another limitation is that microbiota analysis was performed in a selected group of samples. Microbiota data of all samples might have resulted in stronger evidence of the outcome results, as seen in other literature [9]. Furthermore, there was no data on the microbiome of participants' partners' semen. The male partner's microbiome could also play an interacting role on influencing the vaginal microbiome and pregnancy outcomes.

Further research, especially a randomized trial, should be performed to see if treatment or lifestyle interventions, based on BV qPCR status as a marker, improves pregnancy outcomes. Factors such as smoking, BMI, SES, and ethnicity should be reported to investigate if there is a causal effect of BV on pregnancy results. Further research about BV in persons with RIF or recurrent miscarriage is necessary, and the same kind of randomized trial should be performed in this patient group as well. This study suggests that an unfavourable microbiome or BV could predict for a miscarriage in a population with (yet) no RIF or recurrent miscarriages. In the future, it could be a possibility to wait for a third or last IVF treatment until a more favourable microbiome is reached, to lower the chances of having a miscarriage. It is not clear in current literature if BV is the causal reason for unfavourable pregnancy outcomes or a marker for a certain lifestyle. Testing BV positive could be used as an additional reason to encourage couples to adopt a healthier lifestyle to increase the success of having a healthy baby with fertility treatment.

Conclusions

This study suggests an increased risk in miscarriages in BV qPCR positive IUI/IVF/ICSI attempts (OR 4.22 (1.10-16.22), aOR 4.28 (0.65-28.11)) and in the microbiome CST III and CST IV group (in a subcohort of the study). BV qPCR positive IUI/IVF/ICSI attempts had a slight lower ongoing pregnancy rate, however not significant (OR 0.50 (0.16-1.59), aOR 0.32 (0.09-1.23)).

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Supplement 1 – extension on materials and methods

Nucleic acid extraction

DNA was extracted from 200 µl sample and eluted in a final volume of 100 µl with the MagNA Pure 96 instrument using the MagNA Pure 96 DNA and Pathogen Universal small Volume Kit and the Pathogen Universal protocol (Roche Diagnostics, Basel, Switzerland).

Detailed description of microbiota analysis

A fragment of ~421bp of the V1-V2 region of the 16S rRNA gene was amplified using the primers described by Ravel, et al. (2011) and Walker, et al. (2015) with Illumina overhang adaptor sequences added. Each 50 µL PCR reaction contained 5 µL (10x) Expand High Fidelity Buffer with 15 mM MgCl2 (Roche), 2.6 U Expand High Fidelity Enzyme mix (Roche), 0.2 mM of each dNTP (Roche), various primer concentrations and 10 µL of extracted DNA. The PCR was run for 2 min at 94°C followed by 35 cycles of 94°C for 15 sec, 55 °C for 30 sec and 72 °C for 1 min and a final extension step at 72 °C for 7 min. The PCR products with a visible band of ~421bp on gel were subsequently purified and quantified using AMPure XP Beads (Agencourt Bioscience Corporation, Beverly, USA) and the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Paisley, UK), respectively. After library preparation using the Nextera XT kits (Illumina, San Diego, USA), sequencing was performed with the MiSeq desktop sequencer using the MiSeq Reagent Kits v2 500-cycles (Illumina). In each run, a negative control (PBS) and a positive control (Microbial Community Standard of ZymoBIOMICS) was included to monitor quality of the procedure. Samples should have a minimum number of 80,000 reads per sample and at least 75% of the reads should have an average quality score (Phred) ≥ Q30 to continue with data analysis. Sequencing data was processed following the QIIME pipeline (Hall & Beiko, 2018). Open reference operational taxonomic units clustering of high-quality sequences (≥ 100bp in length with a quality score ≥ Q20) was conducted at a 97% similarity level against a pre-clustered version of the Augustus 2013 GreenGenes database. No low abundance filtering was used. Instead OTUs were checked for relevance per sample. Low abundance OTUs that were not relevant for any sample were included in the group "others".

Determining the CST-classification

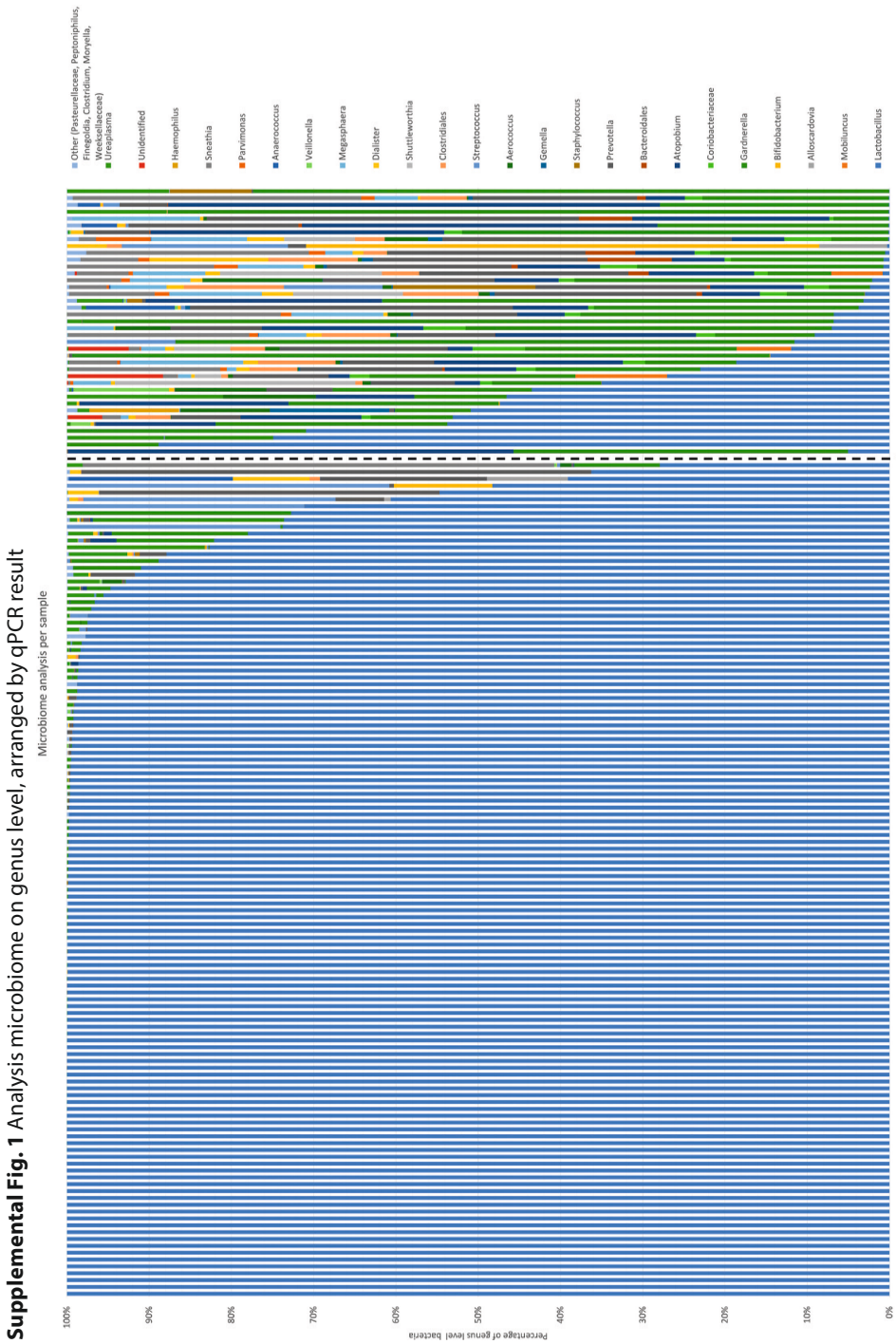
The highest percentage of type of Lactobacillus was chosen to determine to which CST the sample belonged. For example, if a sample contained 70% of *L. iners* and 20% of *L. jensenii*, the sample was classified to CST III (*L. iners*). Samples having less than 50% *Lactobacilli* were classified as CST IV.

Supplement 2

Detailed description of comparing microbiome results with qPCR data

Most qPCR BV positive diagnosed samples had less than < 50% *Lactobacilli*. Four samples were tested BV negative and had <50% *Lactobacilli,* in those samples were a great amount of *Sneathia, Prevotella, Anaerococcus tetradius* or *Streptococcus Agalactiae* respectively. Six samples were classified BV positive and had > 50% *Lactobacilli*, all these samples had a high amount of *G. vaginalis* (or too lesser extent, *A. vaginae (Fannyhessea vaginae)*). Two of these six positive qPCR regarded results, had officially the label 'suspected dysbiosis'. A few samples in the CST IV group contained *L. acidophilus*.

Comparing our microbiome findings with the algorithm used by Koedooder et al., only five samples in this study were defined with the described percentage of > 35% *L. jensenii*. There were no samples with >28% *Proteobacteria* (*Haemophilus/Pasteurellacea*)). Only two samples had <60% *L. crispatus*, one was followed by an ongoing pregnancy and the other was followed by a miscarriage.



Supplemental Fig. 1 Analysis microbiome on genus level, arranged by qPCR result

3

THE RELATIONSHIP BETWEEN VAGINAL PH AND BACTERIAL VAGINOSIS AS DIAGNOSED USING QPCR IN AN ASYMPTOMATIC SUBFERTILE POPULATION

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Abstract

Purpose

Bacterial vaginosis (BV) is a dysbiosis of the vaginal microbiome and a condition found in 20-30% of all women. Literature describing the possible link between BV and subfertility is increasing. Newer techniques such as quantitative polymerase chain reactions (qPCR) detect BV more accurately than traditional methods but come with high costs. The association between pH and BV as diagnosed using traditional methods is well-established in a symptomatic population. This study is the first to investigate the association between pH and BV diagnosed by qPCR in an asymptomatic subfertile population and to examine the usefulness of pH as a means of cost reduction.

Methods

Data of 170 pH – qPCR combinations were used from a prospective cohort study examining bacterial vaginosis in a subfertile population. 102 women received a vaginal swab and pH measurement at baseline and subsequent advanced reproductive technology (ART) treatments. The swabs are analysed using the AmpliSens®Florocenosis/Bacterial vaginosis-FRT qPCR kit.

Results

pH is strongly associated with BV as diagnosed by qPCR (OR 3.06, p=0.000, CI: 1.65 -5.68). The cut-off point for pH ≥ 4.7 maximised diagnostic performance (AUC 0.74 (CI: 0.66-0.83), sensitivity 76%) and reduced costs by 60%.

Conclusion

This study shows that the vaginal pH for a multi-ethnic, asymptomatic population of women attending fertility clinics is strongly associated with BV qPCR outcome. Using the cut-off of pH of 4.7 has a high sensitivity for diagnosis of BV by qPCR and can be achieved at a cost reduction of 60%.

Introduction

Subfertility is a relatively common and increasing problem over the last decades [1]. In The Netherlands, subfertile couples are eligible for advanced reproductive technologies (ART) such as in-vitro fertilisation (IVF) or intrauterine insemination (IUI) when the one-year prognosis for spontaneous pregnancy is under 30% [2]. For many people, having children constitutes a major part of their existence and an inability to do so can be a traumatic experience [3]. In recent years, dysbiosis of the vaginal microbiome or bacterial vaginosis (BV) has been associated with preterm birth, endometritis, subfertility and the success rates of fertility treatments [4-7]. The mechanism behind the effect on subfertility is still unclear. Some studies report that BV has no effect on conception rates but is significantly associated with early pregnancy loss (RR 1.68, 95% CI 1.24-2.27), indicative of a problem related to implantation [8,9].

BV is characterised by a shift in the vaginal microbiome from a *Lactobacillus* dominated profile to a more variable profile with anaerobic and facultative anaerobes. The overgrowth of these anaerobes causes symptoms of abnormal discharge, fishy odour, and itching. However, half of the BV cases are asymptomatic. Typical treatment consists of a seven-day course of either oral or vaginal clindamycin or metronidazole and is known to have high recurrence over 50% of cases within one year [10]. The exact aetiology of BV is unclear but influencing factors include: vaginal douching, ethnicity, obesity, smoking, sexually transmissible diseases or unprotected sexual behaviour [11-13].

The golden standard for the diagnosis of BV is the Nugent score utilising gram-stained smears. The technique requires a skilled microbiologist and is time-consuming. The Amsel criteria are an alternative due to their simplicity and comparable performance but still require a microscope. Both methods are quite cumbersome and the Amsel criteria, by virtue of using clinical criteria, favours symptomatic BV cases. The use of the newer quantitative polymerase chain reactions (qPCR) methods allows for a more objective and accurate diagnosis of BV [4] [14]. A recent study compared the Nugent score and a BV qPCR assay to a full microbiome analysis using 16s ribosomal DNA-sequencing. The Nugent score attained a sensitivity for BV of 63.9%, while the BV qPCR assay achieved 80.6%, both methods had a specificity of ≥ 92.4%. This indicates a substantial performance gap between traditional methods and qPCR [14].

A disadvantage of using qPCR-tests is that they are expensive. Measuring a vaginal pH (as used in the Amsel criteria) is a simple and cheap procedure. pH is a good predictor for BV diagnosed by the Nugent score, but it is unknown whether this holds true for asymptomatic cases detected by qPCR [15] [16].

In some fertility clinics, commercial qPCR or microbiome testing is already available for subfertile couples. Searching for the cause of their subfertility, couples are willing to pay a high price for extra examinations, even when evidence is still insufficient [17]. The goal of this study is to examine the association of pH value with asymptomatic BV as diagnosed using qPCR, in a bid to potentially drive down costs related to using qPCR or microbiome testing in fertility clinics.

Materials and Methods

Data were extracted from the database of an ongoing prospective cohort study examining the impact of BV in fertility patients (approved by medical ethics committee Leiden-Delft-Den Haag, reference Z21.031). Inclusions for this prospective study were ongoing at the time of writing. Couples visited the fertility outpatient clinic of The Haaglanden Medical Center (The Hague, The Netherlands). Eligible women undergoing an initial fertility assessment (IFA) were included and followed for up to five ART treatments.

Participants: Women of 18 years and older were eligible for inclusion. After the initial assessment, participants were either managed expectantly or treated using IVF, IUI or ovulation induction depending on the cause and duration of subfertility. Eligible women were measured at baseline and those treated with IVF or IUI received further swabs and pH measurements. The exclusion criteria were a history of three or more miscarriages, an inability to speak neither Dutch nor English, the use of antibiotics in the previous month or the use of prophylactic antibiotics in general. Incomplete combinations of pH measurements and vaginal swabs were excluded from the analysis.

Data collection: An e-swab (Copan Italia SpA, Breschia, Italy) was taken from the posterior fornix after inserting a speculum while wearing gloves. The pH measurements were performed using a pH-Fix 4.0-7.0 (ref 92137, Macherey-Nagel, Düren, Germany). The pH strip measured in the following increments: 4, 4.4, 4.7, 5, 5.3, 5.8, 6.1, 6.5, and 7.0. The measurements were not performed if the participant was menstruating or post-coital. For the subsequent ART procedures, the combination of swab and pH measurement was performed prior to the oocyte pick up or insemination.

The swabs were analysed by an external laboratory (NMDL & DDL laboratory, Rijswijk, the Netherlands), using a CE-IVD marked multiplex quantitative PCR assay, the AmpliSens® Florocenosis/Bacterial vaginosis-FRT PCR kit (InterLabService, Moscow, Russia). Based on the presence of *Lactobacillus* species, *Gardnerella vaginalis*, *Atopobium vaginae (*recently reclassified as *Fannyhessea vaginae*[18]*)* and total amount of bacteria, swabs were categorised as BV positive (amount of *G. vaginalis* and/or *A. vaginae* is almost equal or

exceeds the amount of *Lactobacillus* spp.), BV negative (*G. vaginalis* and/or *A. vaginae* are absent or its amount is substantially less than the *Lactobacillus* spp. amount), unspecified dysbiosis (amount of *Lactobacillus* spp. is reduced relative to the total amount of bacteria, whereas *G. vaginalis* and/or *A. vaginae* are absent or its amount is substantially less than total amount of bacteria) or suspected dysbiosis (amount of *G. vaginalis* and/or *A. vaginae* is similar to the amount of *Lactobacillus* spp. but does not exceed the limit value) using the software tool provided by the kit manufacturer. All swab results other than BV negative were classified as a BV positive qPCR result for this study [14]. Relevant information about patient characteristics and treatments (such as age, duration of subfertility, antibiotic use) was extracted from the electronic patient dossiers. This information was managed using Castor EDC (electronic data capture, 2021), a cloud-based clinical data management service.

Outcome: The main outcome was the association between a positive qPCR result and the vaginal pH value. The secondary outcomes of interest were the test characteristics of each pH value for BV namely, sensitivity, specificity, area under the curve (AUC) and diagnostic odds ratio (OR).

Statistical analysis: IBM SPSS statistics for Macintosh, Version 27, released 2020, was used for all analysis. Continuous parametric variables were analysed using an unpaired t-test. Continuous non-parametric variables were analysed using the Mann-Whitney-U test. Categorical variables were analysed using the Chi ^{2} or Fisher's exact test. To account for the repeated measurements per case a general estimating equations analysis (GEE) was used. ROC curves were made to find the cut-off that maximises sensitivity and specificity. Contingency tables were used to assess the performance of each pH value for predicting BV.

Results

A total of 102 eligible women were included. In total 170 pH and qPCR swab combinations were available for analysis. Figure 1 shows the included number of combinations for each timepoint (IFA and ART treatments). One participant contributed 5 combinations, and four participants contributed four combinations. All other participants contributed three or less combinations (data not shown).

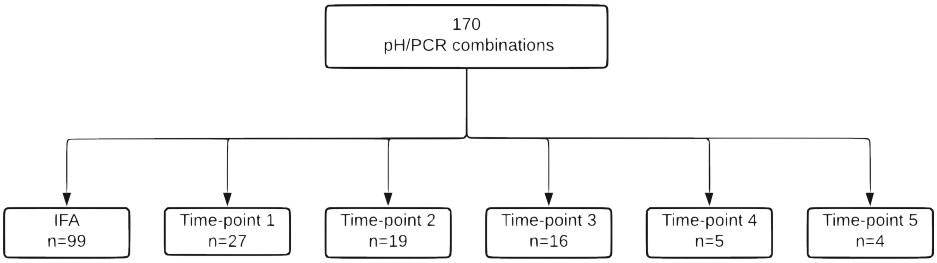

Fig. 1 Flowchart detailing the number of pH-qPCR combinations per sequential timepoint

The baseline characteristics of participants with at least one set of pH and BV measurement are shown in table 1. The total number of participants is 102 instead of 99 to account for three participants who had an incomplete measurement set at baseline but contributed valid pH-swab combinations at following measurements. Only eight participants mentioned mild discharge complaints, with no need to treat with antibiotics. Most of the population were of Caucasian decent (45% non-Caucasian) and had a high social economic status (62% of total). Most participants were expectantly managed or conceived spontaneously. Of the 29 participants who were expectantly managed, seven switched to IUI after several months. Of the nine participants receiving ovulation induction, one switched to IUI. One woman changed after one IUI procedure to IVF. One woman had an escape IUI because of poor response during IVF treatment.

In some participants, their qPCR results differed over time. Seven participants who received IVF, and two participants who received IUI showed a negative qPCR result at baseline but became positive during their treatment. In one of them, the qPCR result changed to positive at the second IUI attempt, after using antibiotics for a urinary tract infection. Two participants (one IUI, one IVF treatment) changed from positive, to negative, to positive again.

Table 1 Baseline characteristics per participant at initial fertility assessment

Baseline IFA	**BV negative**	**BV positive**	**p-value***
Participants (n)	73	26	
Age (mean, SD)	34.9 (4.6)	34.0 (4.1)	0.38
pH (median, IQR)	4.4 (4.0-4.9)	5.5 (5.0-5.8)	**0.00**
BMI (mean, SD)	24.5 (4.3)	25.7 (5.4)	0.30
Discharge complaints n (%)	5 (7%)	3 (12%)	0.40
Smoking n (%)	4 (5%)	4 (15%)	0.20
Alcohol n (%)	18 (25%)	6 (23%)	1.00
Chlamydia antibodies positive n (%)	9 (12%)	3 (12%)	1.00
Ethnicity n (%)			0.57
Caucasian	43 (59%)	12 (46%)	
African	2 (3%)	1 (4%)	
Antillean	2 (3%)	2 (8%)	
Asian	6 (8%)	2 (8%)	
Maroccan	4 (6%)	0	
Hindu	6 (8%)	3 (11%)	
Other	8 (11%)	4 (15%)	
Turkish	1 (1%)	0	
Missing data	1 (1%)	2 (8%)	
Social economic status n (%)			0.36
Low	3 (4%)	3 (11.5%)	
Middle	19 (26%)	5 (19%)	
High	47 (64%)	15 (58%)	
Missing data	4 (6%)	3 (11.5%)	
Subfertility duration in years (median, IQR)	1.0 (1.0-2.0)	2.0 (1.0-2.0)	0.19
Gravidity n (%)			0.73
0	41 (56%)	17 (65%)	
1	24 (33%)	6 (23%)	
2	7 (10%)	3 (12%)	
5	1 (1%)	0	
Cause of subfertility n (%)			0.16
Unknown cause	30 (41%)	14 (53%)	
Andrologisch	18 (25%)	2 (8%)	
Endometriosis	9 (12%)	0	
Tubal factor	3 (4%)	3 (12%)	
Hormonal	9 (12%)	5 (19%)	
Other	2 (3%)	1 (4%)	
Missing data/not fully analysed	2 (3%)	1 (4%)	
First treatment after IFA n (%)			0.23
Expectant management	22 (30%)	7 (27%)	
Spontaneous pregnancy	9 (12%)	7 (27%)	
IUI	12 (17%)	5 (19%)	
IVF	19 (26%)	3 (12%)	
OI	5 (7%)	4 (15%)	
unknown/not started yet	6 (8%)	0	

**p-value ≤0.05 considered significant*

The main outcomes are shown in tables 2 and 3. Of all 170 swabs, 125 (74%) were classified as BV negative, 37 as BV positive, five as 'unspecified dysbiotic' and three as 'suspected dysbiosis'. Therefore 45 (26%) were considered a positive qPCR result. A significant relationship between pH and qPCR outcome was found (p=0.000, OR 3.06; 95% Confidence Interval (CI) 1.65 -5.68) (table 2).

A pH value ≥4.7 was the best predictor for a positive qPCR result (OR 7.8; 3.35-18.20, p-value <0.001). The cut-off for pH of 4.7 has the highest AUC (AUC 0.74, 95% CI: 0.66-0.83) compared to other cut-off values (pH >4.4, >5, >5.3 and higher). The cut-off for pH of 4.7 has a sensitivity of 76% and specificity of 73% for BV positive swabs (table 3). The optimal cut-off value of 4.7 is also reflected in the ROC curve analysis (figure 2). Performing a qPCR-test after a pH-value >4.7 results in the utilisation of 60% fewer qPCR's overall (table 4). This method could reduce the price per correct BV diagnosis from €188,89 to €100,50 and total costs of this study from €8500,00 to €3417,00 (calculations shown in table 4).

Table 2 Main results, mean pH for qPCR result

qPCR	subset	n (%)	pH (mean IQR)	p-value	OR (CI)
Total		170		0.000	3.06 (1.65 -5.68)
BV negative		125 (74%)	4.4 (4.0-4.7)		
BV positive		45 (26%)	5.2 (4.6-5.7)		
	positive	*37*			
	dysbiosis	*3*			
	unspecified	*5*			

IQR = interquartile range

Table 3 Testresults for each pH cut-off point

	Sensitivity	Specificity	AUC	OR*	p-value	PPV	NPV		
pH cut-off	**(%)**	**(%)**	**(CI 95%)**	**(CI 95%)**	**	**(%)**	**(%)**	**PLR**	**NLR**
pH ≥4.4	87	58	0.73 (0.64-0.81)	8.20 (2.69-25.03)	<0.001				
pH ≥4.7* **	**76**	**73**	**0.74 (0.66-0.83)**	**7.80 (3.35-18.20)**	**<0.001**	**50**	**89**	**1.04**	**0.90**
pH ≥5	64	81	0.73 (0.63-0.82)	7.10 (3.21-15.68)	<0.001				
pH ≥5.3	51	89	0.70 (0.60-0.80)	7.61 (3.20-18-08)	<0.001				
pH ≥5.8	24	95	0.60 (0.50-0.70)	5.55 (2.10-14.69)	<0.001				
pH ≥6.1	16	96	0.56 (0.46-0.66)	3.85 (1.28-11.58)	0.016				

**Odds ratio (OR) calculated with GEE-analysis.*

***Sensitivity/specificity/Area Under the Curve (AUC) only shown for significant (p ≤ 0.05) pH cut-off values.*

****Positive predictive value (PPV)/Negative predictive value (NPV)/Positive Likelihood ratio (PLR)/Negative Likelihood Ratio (NLR) only shown for best pH cut-off value.*

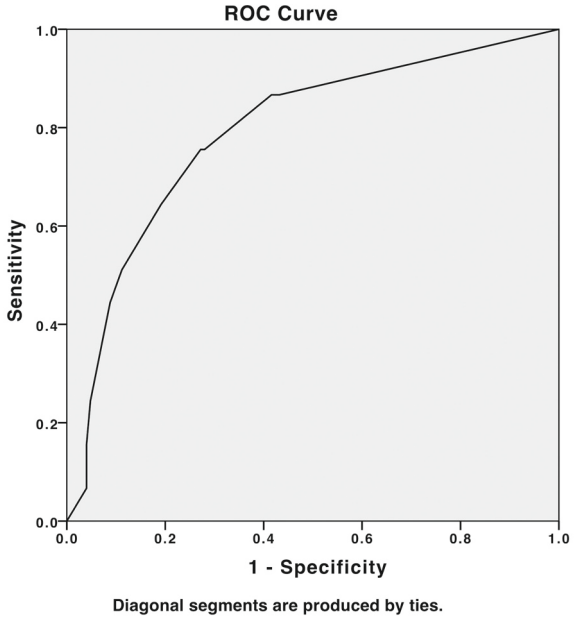

Fig. 2 ROC curve for prediction of BV by pH

Table 4 Contingency table for pH ≥4.7

	qPCR positive	qPCR negative	total
≥4.7	34	34	68 (40%)
<4.7	11	91	102 (60%)
total	45	125	170

Cost qPCR: 170x €50 = €8500
Cost pH≥4.7 + qPCR: (170 x €0.1) + (68 x€50) = €3417
Price per diagnosis using qPCR: €8500/45= €188,89
Price per diagnosis using pH≥4.7 + qPCR: €3417/34 = €100,50

Discussion

To our knowledge, this is the first study that investigates the association between pH and BV as diagnosed by qPCR in an asymptomatic subfertile population. Additionally, this study explores the utility of pH as a cost-reducing measure in this population. The estimated price of €50,- per qPCR forms a barrier to its clinical adoption despite superior performance versus traditional methods, especially given the backdrop of rising healthcare costs in the Netherlands. Using a value pH ≥ 4.7 as a step-up for qPCR succeeds at lowering total study

costs by 60%. The cut-off pH ≥ 4.7 was chosen because it maximised diagnostic value and is in line with previous research on the use of pH as an indicator for BV diagnosed using more traditional methods [15] [16].

This study confirms the high prevalence of BV in a multi-ethnic subfertile population (45% non-Caucasian) with 26% of the participants testing positive by qPCR. This percentage is in accordance with the studies of Haahr et al. and Borgdorff et al. These authors report a BV prevalence of 28% and 32% in a respectively 10% non-Caucasian and 90% non- Caucasian population [4,12].

Lifestyle factors such as smoking, diet, vaginal douching and multiple sexual partners are known to effect BV. No information about vaginal douching and multiple sexual partners have been gathered in this study [13] [17]. Measured factors (smoking, BMI) in this study did not significantly alter the association between pH and BV, probably because of the small study size. Furthermore, hormonal treatment could be of influence on BV status as suggested in a few prior studies investigating the microbiome during IVF [19] [20]. In this study, 9 participants became positive during treatment, an interesting finding requiring further investigation.

Unexplained subfertility had a possible association with a positive qPCR (p=0.16), suggesting that BV might be connected to subfertility in this group. Other studies describe that BV might be an unrecognised factor behind unexplained subfertility, but further research on this topic is necessary [7,21]. If there is a strong association between BV and unknown causes of subfertility, treatment of BV in these cases could be fundamental and potentially lead to more successful outcomes.

A limitation of this study is the sample size, which was too small to determine the influence of relevant factors as ethnicity or cause of subfertility on the cut-off pH value. More than half of the population was of high social economic status, which could indicate self-selection bias. A second limitation could be the measurement tool for pH only registered values ranging from 4.0 to 7.0. Physiological vaginal pH values can go lower than 4.0 and in some extreme cases of BV surpass 7.0 [22]. Capping the extreme pH values leads to an underestimation of the association between pH and BV. Thirdly, measurement errors might be introduced due to a degree of subjectivity in the visual colour coding of the pH strips.

The utility of these findings to the clinical practice is not yet fully known. Trials should further investigate the possible causal link between BV and fertility outcomes and whether treatment of BV leads to better fertility outcomes. The results of a prospective cohort study about the influence of BV in a general subfertility population (undergoing ART treatment)

of this study group are soon to be expected. Another component of uncertainty is the absence of effective methods for the treatment of BV and a lack of knowledge about whether treatment leads to improved live birth rates. New treatment options for BV need to be investigated as well, such as novel anti-microbial agents, concomitant use of vaginal acidification and the use of probiotics or vaginal flora transplantation.

If treatment of BV will improve fertility outcomes, pH and qPCR testing will become a standard part of the initial fertility assessment. At this point, commercial BV or microbiome testing is already a daily practice in some fertility clinics, at high costs. If more evidence underwrites this association, the investment of a qPCR is small relative to the total cost of a failed IVF treatment. Nevertheless, pH could be used as a step up for qPCR to reduce costs even further or as a screening method in the initial fertility assessment. This study details a simple method in which the diagnostic power provided by qPCR can be leveraged at a 60% reduced cost, potentially removing some future hurdles for the implementation of qPCR in the daily fertility practice [17].

Conclusion

This study shows that the vaginal pH for a multi-ethnic, asymptomatic population of women attending fertility clinics is strongly associated with BV qPCR outcome. Using the pH cut-off point of 4.7 has a high sensitivity for the diagnosis of BV and can be achieved at a cost reduction of 60%.

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THE VAGINAL MICROBIOME CHANGES DURING VARIOUS FERTILITY TREATMENTS

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Abstract

This study aimed to investigate the influence of hormonal treatment on the vaginal microbiome during fertility treatments. Bacterial vaginosis (BV) could affect fecundity, particularly in the in vitro fertilization (IVF) population, where negative effects on pregnancy outcomes have been reported. It is hypothesized that the hormone treatment during fertility treatments could influence the abundance of *Lactobacilli*, with negative effects on the pregnancy results.

A total of 53 couples attending a fertility clinic in the Netherlands between July 2019 and August 2022 were included in this prospective cohort study. Vaginal samples were collected at start of treatment, oocyte retrieval or insemination from subjects undergoing intrauterine insemination (IUI) with mild ovarian stimulation, IVF or intracytoplasmatic sperm injection (ICSI) with controlled ovarian hyperstimulation. AmpliSens® Florocenosis/Bacterial vaginosis-FRT qPCR and 16S rRNA gene-based amplicon sequencing were performed on all samples.

In total 140 swabs were analysed, with a median of two swabs per person. 33 swabs (24%) tested qPCR BV positive. *Lactobacilli* percentage decreased during fertility treatments, leading to changes in the vaginal microbiome. Shannon Diversity Index was not significantly different. Of the total of 53 persons, 9 switched from qPCR BV negative to positive during treatment. The persons switching to qPCR BV positive had already a (not significant) higher Shannon Diversity Index at start of treatment. If the vaginal microbiome of persons deteriorates during fertility treatments, timing of following treatments, lifestyle modifications or a freeze all strategy could be of possible benefit.

Introduction

The vaginal microbiome is known to change over time, depending on menstrual cycle, suggesting that the vaginal microbiome is influenced by hormones. *Lactobacilli* are bacteria known to be dependent on hormonal status, especially levels of oestrogen, in women. Lack of *Lactobacilli* is associated with an abnormal microbiome, also called bacterial vaginosis. Incidence of bacterial vaginosis (BV) can be as high as 29%, with a significant portion of persons being asymptomatic [1,2].

It is hypothesized that the hormones used during assisted reproduction could influence the vaginal microbiome through glycogen accumulation, which is utilized by *Lactobacillus*. [2,3] Recent studies in persons undergoing in vitro fertilization (IVF) have shown a negative effect of an abnormal microbiome on pregnancy outcomes [4,5]. Over the past decade, more research has been published on the endometrial and vaginal microbiome, with a growing understanding of a continuum between the vaginal and endometrial microbiome [6].

Only a few studies have investigated the influence of external hormones on the microbiome over time. One study found no effect of vaginal progesterone treatment during pregnancy on vaginal microbiome [2] and another study reported no microbiome changes during IVF treatment [7]. Other studies reported alterations in the vaginal microbiome during IVF; however, these studies were small [3,8]. Carosso reported a small decrease in *Lactobacilli* and higher bacterial diversity during the first IVF treatment session compared to pretreatment. Hyman found changes in microbiome status during IVF, however all subjects were treated with antibiotics, which could also influence their microbiome. There is currently no literature on the impact of lower-dose hormonal stimulation during intrauterine insemination (IUI) cycles on the vaginal microbiome.

This is the first study monitoring the vaginal microbiome using qPCR and sequencing during multiple consecutive fertility treatments (pre-treatment and around ovulation) in a subfertile population undergoing IUI, IVF and intracytoplasmic sperm injection (ICSI). It is hypothesized that *Lactobacilli* will decrease over time, potentially leading to a higher incidence of bacterial vaginosis.

Materials & Methods

This study was performed on a prospective single center cohort in the Haaglanden Medical Center (HMC) in the Hague, the Netherlands. The fertility department of the HMC collaborates with the IVF laboratory of the Leiden University Medical Center. Persons (18 years or older) undergoing fertility treatments directly after fertility assessment were

included between July 2019 and June 2022. Exclusion criteria were inability to understand Dutch or English language, 3 or more miscarriages, and prophylactic antibiotic treatment. No couples were treated with donor sperm or donor oocytes. Microbiological assessment of vaginal swabs was done by external laboratory of Eurofins NMDL and DDL Diagnostic Laboratory in Rijswijk, The Netherlands.

Sample and data collection

When persons signed informed consent at their initial fertility assessment (intake), the first vaginal swab (e-swab, Copan Italia SpA, Breschia, Italy) was taken from the posterior fornix after inserting a speculum. No measurements were performed if the participants were menstruating, on hormonal therapy or post-coital. At consecutive IUI or IVF/ICSI procedures the sampling was performed prior to the oocyte pick up or insemination, up to a maximum of 4 procedures. The vaginal sampling was performed at moment of the last ultrasound before transfer in case of a frozen embryo cycle. No new swabs were collected after an ongoing pregnancy or when switched from IUI to IVF/ICSI. The swabs were used for BV qPCR and microbiota analysis. Fertility doctors and study participants were blinded for the results of the swab. Participants with symptoms of BV were tested separately and treated according to the standard protocol when tested positive.

Follow up ended at cessation of treatment, ongoing pregnancy or at end of study (August 2022). Data about patient characteristics and fertility treatments were extracted from the electronic patient files. A cloud-based clinical data management service, Castor EDC, was used to collect all data.

Fertility treatments

All couples starting with IUI or IVF/ICSI at the HMC hospital need to stop smoking before starting treatment and need to have a body mass index (BMI) below 35. Fertility treatment is initiated if indicated based on the results of the fertility assessment. Couples with unexplained infertility started with IUI with mild ovarian stimulation or IVF based on female age. For IVF/ICSI protocols both long and short agonist and antagonist protocols were applied. Oestrogen (E2) levels are measured in blood in most cases before oocyte pick up is scheduled. During oocyte pick up antibiotics are not routinely administered, but only given for certain indications (for example with endometriomas).

Nucleic acid extraction

In the HMC laboratory the vaginal samples were frozen within 24 hours and afterwards transported to the external laboratory for molecular analysis. DNA was extracted from 200 µl sample and eluted in a final volume of 100 µl with the MagNA Pure 96 instrument using the MagNA Pure 96 DNA and Pathogen Universal small Volume Kit and the Pathogen

Universal protocol (Roche Diagnostics, Basel, Switzerland). Appropriate positive and negative control samples were included in testing, which were evaluated in the BV qPCR.

BV qPCR

The extracted DNA was tested according to the manufacturer's instructions with a CE-IVD marked multiplex quantitative PCR assay (the AmpliSens® Florocenosis/Bacterial vaginosis-FRT PCR kit InterLabService, Moscow, Russia). Based on the presence of *Lactobacillus* species, *Gardnerella vaginalis*, *Atopobium vaginae* (recently reclassified as *Fannyhessea vaginae*)[9] and total amount of bacteria, swabs were labeled as BV positive (amount of *G. vaginalis* and/or *A. vaginae* is almost equal or exceeds the amount of *Lactobacillus*), BV negative (*G. vaginalis* and/or *A. vaginae* are absent or its amount is considerable less than the *Lactobacillus* amount), unspecified dysbiosis (amount of *Lactobacillus* is reduced relative to the total amount of bacteria, whereas *G. vaginalis* and/or *A. vaginae* are absent or its amount is considerable less than total amount of bacteria) or suspected dysbiosis (amount of *G. vaginalis* and/or *A. vaginae* is similar to the amount of *Lactobacillus* but does not exceed the limit value) using the software tool provided by the kit manufacturer.

Microbiota analysis

Microbiota analysis was performed on the extracted DNA of swabs of persons with repeated measurements of vaginal microbiome. A fragment of ~421bp of the V1-V2 region of the 16S rRNA gene was amplified using the primers described by Ravel, et al (2010) and Walker, et al. (2015) with Illumina overhang adaptor sequences added [10,11]. Outcomes were categorized in one of five vaginal microbiome community state types (CST), as reported by Ravel et al. CST I is dominated by *L. crispatus,* and respectively CST II by *L. gasseri,* CST III by *L. iners*, CST IV by non-lactobacilli, CST V by *L. jensenii* [11]. Previously described literature on this subject also used these CSTs. More details on the microbiome analysis are described in the supplement.

Outcomes

Primary outcome were *Lactobacilli* amount and Shannon Diversity Index. Secondary outcomes were change of qPCR BV status or microbiome CST. Shannon Diversity Index (SDI) is a commonly used weighted measure to analyse the various types of bacteria in a particular environment. The SDI is zero when there is only one type of bacteria present and there is no diversity[12]. Sample size was not calculated upfront. For statistical analysis IBM SPSS statistics for Macintosh, version 27, was used. Main outcome parameters were analyzed using a paired t test.

Results

Population

In total 82 couples were eligible for inclusion, however of 21 participants there were no following swabs, and 8 participants became pregnant spontaneously before starting fertility treatment. In Figure 1 the included participants are shown. The remaining couples were multi-ethnic and most had a high socioeconomic status (Table 1). Only two persons had discharge complaints. 22 persons were treated with IVF and 31 persons treated with IUI. In total 53 participants had 87 consecutive treatment cycles (in total 140 samples), with a median of 2 samples per person.

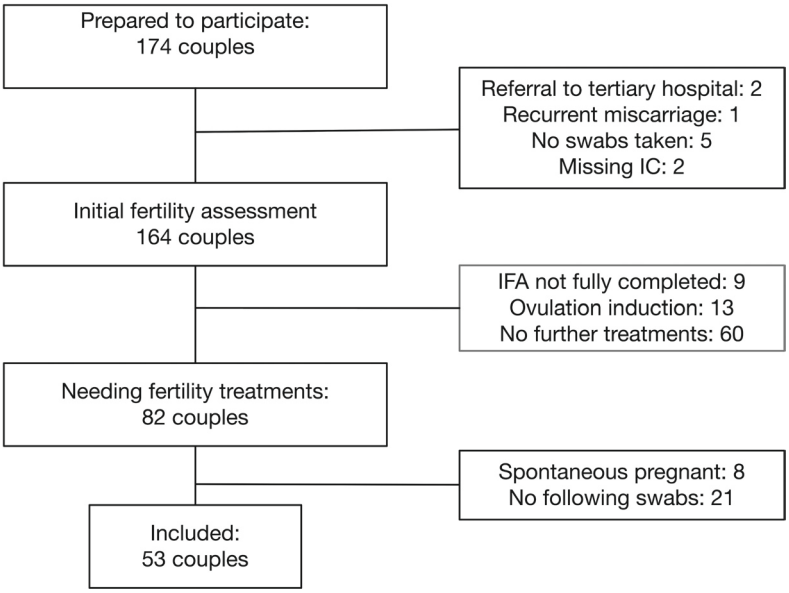

Fig. 1 Flowdiagram of included participants

Table 1 Baseline characteristics of participants at intake

Table 1	
Participants (descriptives at time of intial fertility assessment)	53
Age (mean, SD)	34.9 (4.3)
Discharge complaints n(%)	2 (4%)
Antibiotic/antifungal treatment n(%)	1 (2%)
BMI (mean, SD)	25 (4.3)
Smoking n(%)	5 (9%)
Alcohol (≥ 1 glass a week) n(%)	16 (30%)
Druguse (on regular basis) n(%)	3 (6%)
HPV positive in past year n(%) (only tested when indicated)	9 (17%)
Medication use (for comorbidities)	6 (11%)
Ethnicity n(%) *	
Caucasian	30 (57%)
Hindu	4 (7,3%)
African	1 (2%)
Antillean	4 (7,3%)
Asian	6 (11%)
Moroccan	3 (6%)
Turkish	1 (2%)
Other	4 (7,3%)
Socioeconomic status n(%)**	
Low	0
Middle	15 (28%)
High	37 (70%)
Missing	1 (2%)
Regular cycle n(%)	49 (92%)
Subfertility duration in years (median, IQR)	1 (1-2)
Cause of subfertility n(%)***	
Male factor	19 (36%)
Tubal factor	6 (11%)
Hormonal	3 (6%)
Endometriosis	7 (13%)
Unexplained	16 (30%)
Other	2 (4%)
Fertility treatment n(%)	
Intrauterine insemination (IUI)	31 (58%)
In vitro fertilization (IVF)	22 (42%)

** other mostly Hispanic participants*

*** as defined by education status*

**** hormonal: premature ovarian insufficiency or anovulation, other: uterine myomas, uterus anomaly, sexual disfunction*

Microbiota over time

Of the 140 samples, 33 (24%) were tested positive for BV qPCR. At intake 10 of 53 (19%) persons tested BV qPCR positive, and during treatment 17 of the 53 (32%) tested BV qPCR positive. The 140 samples were also tested with the microbiota analysis (Supplemental figure 1). The details about the microbiome results and the comparison with qPCR are described in another paper [13].

On average the number of *Lactobacilli* decreased 4.6% from intake to last treatment, and 8.9% when comparing the intake swab with the lowest following swab during treatment. This decrease in *Lactobacilli* was significant (p=0.01, Figure 2). Shannon Diversity Index (SDI) was not significantly different from first sample (p=0.59). During fertility treatment more than 10% decrease of *Lactobacilli* can be seen in 17 of the 53 persons (and only a 10% increase in 4 persons). This appeared in all community state types (CST). E2 levels were measured in most IVF participants but did not show a specific association with changing microbiome (data not shown).

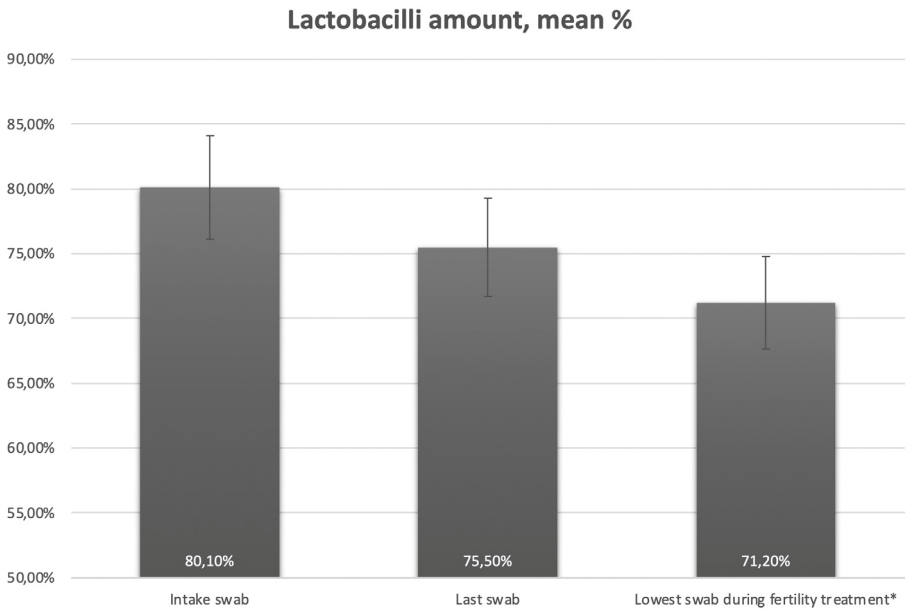

Fig. 2 Decrease of *Lactobacilli* during fertility treatment (*p=0.01)

Of the 52 participants, in total 27 persons had a more than 10% change in *Lactobacilli*, *Gardnerella* or *Atopobium/Fannyhessiae* over time (Figure 3). Of these 27 persons, 8, 2, 7 and 9 persons were classified as CST I, II, III and IV at intake, respectively. One participant had a microbiome dominated by *L. ultenesis/L. johnsonii* and could not be classified in the regular CST (in Figure 3 personID 27).

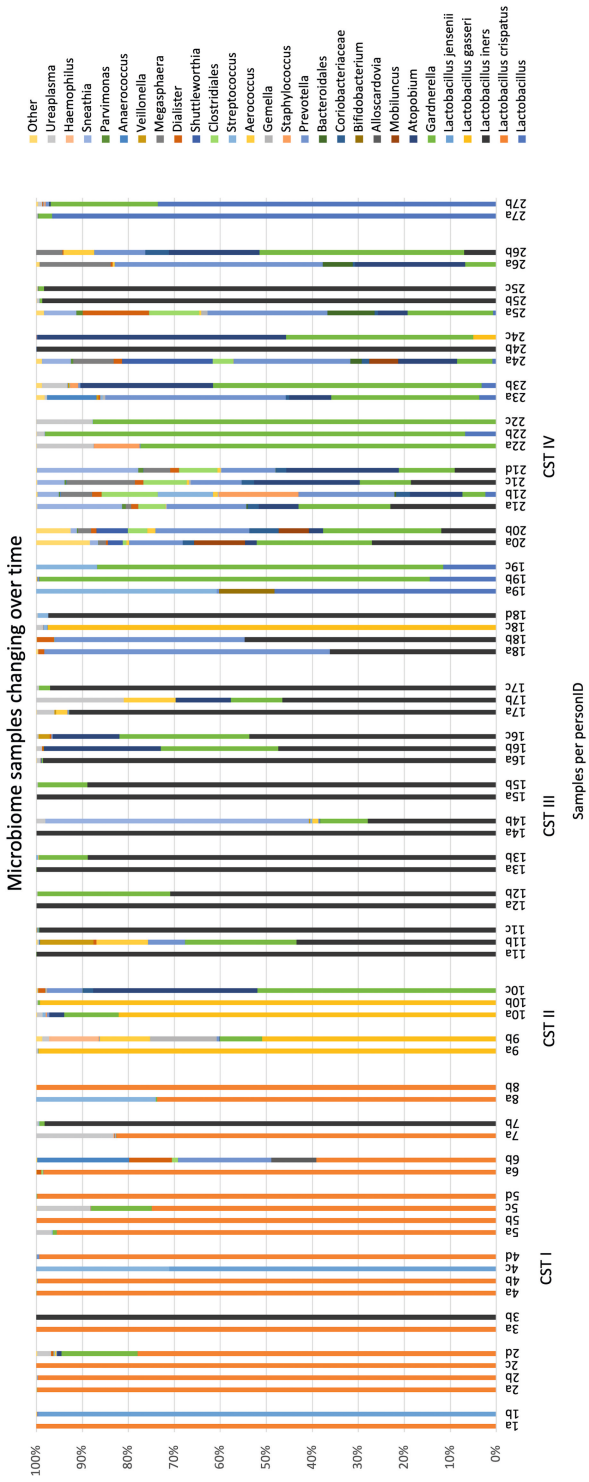

Fig. 3 The microbiome of 27 persons with a more than 10% change in *Lactobacilli*, *Gardnerella* or *Atopobium/Fannyhessiae* over time. Samples are arranged per person, categorized on community state type microbiome on basis of the first sample at intake.

Deterioration of the microbiota

In total thirteen persons had a deterioration of qPCR or CST result (including switching to less favourable *L. iners*) as shown in Table 2 (extended descriptions of which bacteria leaded to a change is shown in Supplement Table 1). Nine of these persons had IVF/ICSI-treatment and four had IUI treatment. The decrease of *Lactobacilli* resulted in a switch from BV negative to BV positive in nine persons (personID 10 at second treatment). There were no differences seen compared to the whole group based on ethnicity, reason of subfertility, gonadotropin dose or IVF protocol (not all data shown). However, five of 14 negatively changed persons had human papillomavirus (HPV) infection last year. Also, two persons were (former) smokers. In swabs that switched from qPCR BV negative to positive, there were already a small number of non-lactobacilli bacteria present in the vaginal microbiome at baseline. The mean Shannon Diversity Index was 1.02 in the qPCR BV negative baseline samples who switched and 0.87 in the baseline samples that remained qPCR negative (not significant p=0.46). The main non-lactobacilli bacteria found in these swabs were *Gardnerella* or *Atopobium/Fannyhessiae*.

In one person (personID 12) *Staphylococcus* and *Ureaplasma* were found and *Ureaplasma* and *Gardnerella* increased during treatment. In another person (personID 19) mainly *Bifidobacterium, Streptococcus* and *Prevotella* were found, and *Gardnerella* was increased during treatment. One subject changed to qPCR BV positive during one of the IUI cycles (personID 5) just after an antibiotic treatment for a urinary tract infection. One person (personID 17) had a dramatic decrease of *Lactobacilli* during IVF treatment and switched to qPCR BV positive. This person turned back to qPCR BV negative during her frozen embryo transfer in her natural cycle. In total eleven persons switched from microbiome CST. Two persons (personID 3 and 7) who changed from CST I to CST III had already a low amount of *L. iners* in the first swab, one of them stopped smoking (personID 7).

Improvement of the microbiota

Three persons had an improvement of their microbiome. Two persons switched from qPCR BV positive to negative during IUI treatments. One (personID 18) switched to qPCR BV negative because of treatment with antibiotics for discharge complaints. The other person (personID 25) stopped smoking before starting treatment. One person switched (personID 24) from qPCR BV positive to negative during first IVF treatment. The person lost some weight prior to start with IVF (BMI below 35 at start IVF). With the second IVF stimulation this person switched back to qPCR BV positive again. A different stimulation protocol with urinary FSH was used this second time, compared to recombinant FSH the first stimulation.

Table 2 Detailed description of persons with changed microbiome

PersonID	qPCR at intake	CST at intake	Day of menstrual cycle	SDI at intake	Treatment	2nd qPCR	2nd CST	Day of menstrual cycle 2	Gonadotropin dose (IU)	E2 level (pmol/L)	3rd qPCR	3rd CST	Day of menstrual cycle 3	Gonadotropin dose2 (IU)	E2 level2 (pmol/L)	Related factors
1	neg	I	28	1.69	IVF	neg	V	11	300	3632						HPV +
Deteriorated microbiota																
3	neg	I	14	0.63	IUI	neg	III	15	37.5							
5	neg	I	9	1.55	IUI	pos	I	12	62.5		neg	I	12	62.5		antibiotics for UTI, HPV +
6	neg	I	15	1.25	IVF	neg	IV	22	112.5	7073						stopped smoking, HPV +
7	neg	I	13	2.07	ICSI	neg	III	14	225	8201						stopped smoking, HPV +
9	neg	II	8	1.39	IVF	pos	II	18	225	4848						
10	pos	II	2	2.55	IUI	neg	II	13	50		pos	IV	12	62.5		
11	neg	III	8	0.23	ICSI	pos	IV	15	300	6133	neg	III		300	6154	HPV +
12	neg	III	14	0.78	ICSI	pos	III	14	150	7650						
14	neg	III	21	0.6	IVF	neg	IV	17	150	4149						
15	neg	III	16	0.12	ICSI	pos	III	27	112.5							HPV +
16	neg	III	8	0.14	IUI	pos	IV	13	50		pos	III	11	75		
17	neg	III	4	0.52	IVF	pos	IV	13	200	2879	neg	III	13	n.a., frozen embryo transfer		
19	neg	IV	6	1.46	IVF	pos	IV	12	225	4521	pos	IV	13	225		
Improved microbiota																
18	neg	IV	13	2.43	IUI	neg	III	11	75		neg	II	9	87.5		antibiotics for discharge
24	pos	IV	13	4.15	IVF	neg	III	22	150	1866	pos	IV		urinary FSH 225	3711	decreased BMI
25	pos	IV	15	3.85	IUI	neg	III	12	62.5		neg	III	8	62.5		stopped smoking

SDI Shannon Diversity Index, CST community state type, E2 estrogen, UTI urinary tract infection, HPV human papilloma virus

Discussion

This is the first study investigating the impact of hormones during multiple fertility treatments on the vaginal microbiome. This study showed that fertility treatment resulted in a decrease in the rate of *Lactobacilli,* ranging from 4.6% to 8.9% in the worst case. Shannon Diversity Index did not show a significant difference, which suggests that the decrease in *Lactobacilli* is most prominent in samples that were already more diverse at intake. At intake 10 of 53 (19%) persons tested BV qPCR positive, and during treatment 17 of the 53 (32%) tested BV qPCR positive. In total thirteen persons showed a deterioration in their BV PCR and/or microbiome result (including changing to less favorable *L. iners*). In contrast, only three persons showed an improvement of their microbiome status. This could be probably intentional because these persons were trying to improve their lifestyle.

A strength of this study are the multiple observations during consecutive treatment cycles. Earlier studies only examined the microbiome prior to IVF treatment and during the first treatment, with less than 30 participants [3,7,8]. Carosso et al. did find a decrease of *Lactobacilli*, however this was not statistically significant. Possibly *Lactobacilli* decrease more pronounced after undergoing multiple treatments. Another strength is the heterogenous population studied, which includes non-Caucasian persons and a mix of IUI and IVF/ICSI treatment protocols. This enhances the applicability of the findings and allows for a broader understanding of the potential impact of fertility treatments on the vaginal microbiome.

This decrease in *Lactobacilli* and negative change of microbiome status could have a negative effect on ongoing pregnancy rates. Another paper by this research group showed that samples with a consecutive miscarriage had 15% less *Lactobacilli* compared to samples with a consecutive ongoing pregnancy (however not significant) [13]. The numbers in this study were too low to calculate specific pregnancy outcomes for changing qPCR or microbiome status compared to persons with a stable microbiome. It should be further investigated which amount of decrease of *Lactobacilli* is clinically relevant. Understanding the fluctuations of the vaginal microbiome and knowing which persons are at risk, could improve fertility treatments in the future.

This study is the largest study conducted on this subject to date, however the numbers of subjects is still limited because this study is a subanalysis of a larger prospective study. This study did not consider sexual intercourse and the seminal microbiome around time of sampling, which could influence the change of vaginal microbiome as well. Another limitation could be the variability in hormonal treatment protocols utilized. The most common stimulation was with recombinant gonadotropins, and an agonist protocol in IVF.

However, the decrease of *Lactobacilli* was observed in both IUI and IVF, suggesting that it is not solely depending on specific protocols (recombinant or urinary FSH, antagonist or agonist protocol) or on E2 levels, as previously suggested by Hyman et al.

The change of vaginal microbiome could be related to overall health status or the immune system. The persons with a deteriorated microbiome during treatment were found to be more frequently smokers and/or tested positive for HPV (human papillomavirus). The correlation between HPV infections and abnormal microbiota has already been reported in literature before [15]. Healthier lifestyle might have a positive impact on the microbiome. Three cases presented here show this positive effect; however, it is important to note that these examples are anecdotal, and this is insufficient evidence for a broader conclusion. If lifestyle changes could reduce recurrence of BV after treatment or prevent the worsening of the microbiome during fertility treatment, this could serve as additional motivation for persons to adopt healthier lifestyle before conception.

Vaginal microbiome testing to monitor changes in *Lactobacilli* levels during IUI or IVF treatment could provide valuable insights. Future research should focus on the microbiome and pregnancy results in the context of multiple sequential fertility treatments. This study showed one case in which the vaginal microbiome returned to normal during frozen embryo transfer (personID 17). If future research can show a clinically relevant association between the vaginal microbiome and pregnancy outcomes, it may lead to different treatment strategies. Treatment options such as freeze all strategy, introducing pauses between treatments, vaginal probiotics and lifestyle interventions could be investigated further to improve pregnancy outcomes.

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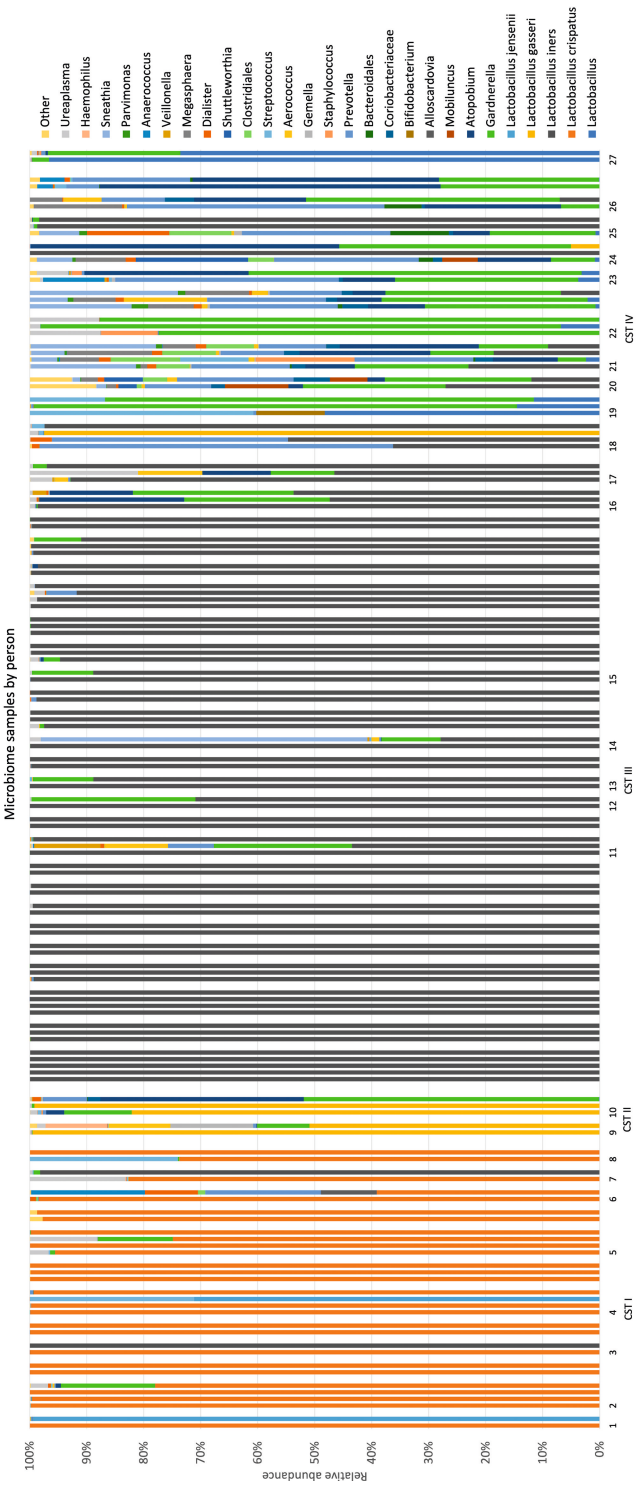
Supplement 1 – extension on materials and methods

Each 50 µL PCR reaction contained 5 µL (10x) Expand High Fidelity Buffer with 15 mM MgCl2 (Roche), 2.6 U Expand High Fidelity Enzyme mix (Roche), 0.2 mM of each dNTP (Roche), various primer concentrations and 10 µL of extracted DNA. The PCR was run for 2 min at 94°C followed by 35 cycles of 94°C for 15 sec, 55 °C for 30 sec and 72 °C for 1 min and a final extension step at 72 °C for 7 min. The PCR products with a visible band of ~421bp on gel were subsequently purified and quantified using AMPure XP Beads (Agencourt Bioscience Corporation, Beverly, USA) and the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Paisley, UK), respectively. After library preparation using the Nextera XT kits (Illumina, San Diego, USA), sequencing was performed with the MiSeq desktop sequencer using the MiSeq Reagent Kits v2 500-cycles (Illumina). In each PCR run, a microbial community standard (ZymoBIOMICS) was included as positive control and PBS as negative control. Both controls were assessed on gel and included in the sequencing run to assess the quality. Samples should have a minimum number of 80,000 reads per sample and at least 75% of the reads should have an average quality score (Phred) ≥ Q30 to continue with data analysis. The QIIME pipeline was used to process sequencing data. [13]Open reference operational taxonomic units clustering of high-quality sequences (≥ 100bp in length with a quality score ≥ Q20) was conducted at a 97% similarity level against a pre-clustered version of the Augustus 2013 GreenGenes database. No low abundance filtering was used. Instead OTUs were checked for relevance per sample. Low abundance OTUs that were not relevant for any sample were included in the group "others".

Determining the CST-classification

The highest percentage of type of Lactobacillus was chosen to determine to which CST the sample belonged. For example, if a sample contained 60% of *L. iners* and 30% of *L. jensenii*, the sample was classified to CST III (*L. iners*). Samples having less than 50% *Lactobacilli* were classified as CST IV.



Supplemental Fig. 1 all microbiome samples arranged per person, categorized on community state type microbiome on basis of their first sample at intake. Persons used in Figure 3 are marked with their personID in this figure as well.

Supplemental table 1 detailed description of persons with changed microbiome

PersonID	qPCR at intake	CST at intake	Changing bacteria	2nd qPCR	2nd CST	Time between 1st and 2nd swab (months)
1	neg	I	*L. gasseri*	neg	V	7
Deteriorated microbiota						
3	neg	I	*L. iners*	neg	III	4
5	neg	I	*Gardnerella/Ureaplasma*	pos	I	4
6	neg	I	*Prevotella/Anaeroccocus*	neg	IV	9
7	neg	I	*L. iners*	neg	III	3
9	neg	II	*Gardnerella/Gemella/ Haemophilus/Aerococcus*	pos	II	4
10	pos	II	*Gardnerella*	neg	II	1
11	neg	III	*Gardnerella*	pos	IV	3
12	neg	III	*Gardnerella/Ureaplasma*	pos	III	3
14	neg	III	*Sneathia*	neg	IV	2
15	neg	III	*Gardnerella*	pos	III	2
16	neg	III	*Gardnerella/Atopabium*	pos	IV	3
17	neg	III	*Ureaplasma*	pos	IV	3
19	neg	IV	*Gardnerella*	pos	IV	5
Improved microbiota						
18	neg	IV	*Prevotella*	neg	III	1
24	pos	IV	*Gardnerella/Atopabium/ Prevotella*	neg	III	12
25	pos	IV	*Prevotella/Gardnerella*	neg	III	3

5

TESTING ON BACTERIAL VAGINOSIS IN A SUBFERTILE POPULATION AND TIME TO PREGNANCY: A PROSPECTIVE COHORT STUDY

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Abstract

This study aimed to investigate the influence of bacterial vaginosis on time to pregnancy in subfertile couples. Couples attending a teaching hospital in the Netherlands having an initial fertility assessment (IFA) between July 2019 and June 2022 were included in this prospective study, with follow up of pregnancies until June 2023. Vaginal samples at IFA were analysed on pH, qPCR BV and 16S rRNA gene microbiome analysis of V1-V2 region. Main outcome measures were time from initial fertility assessment to ongoing pregnancy at 12 weeks and live birth, analysed by Kaplan Meier and Cox regression with adjustment for potential confounders. At IFA 27% of 163 included participants tested positive for BV. BV-status had no influence on time to ongoing pregnancy (HR 0.98, 0.60-1.61, aHR 0.97, 0.58-1.62). In persons with unexplained subfertility positive BV-status had a tendency of longer time to pregnancy. When persons had an indication for fertility treatment positive BV-status (HR 0.21, 0.05-0.88, aHR 0.19, 0.04-0.85) and microbiome community state type III and type IV had significant longer time to pregnancy. This study indicates that BV may have a potential negative impact on time to live birth pregnancy in subfertile persons with an indication for fertility treatment. This study did not find an association between BV and time to live birth pregnancy in a general group of subfertile couples or in unexplained subfertility. More research should be done in persons with unexplained subfertility and if treatment improves time to pregnancy.

Introduction

Up to 1 in 8 couples experience subfertility, and among them, 25% will be diagnosed with unexplained subfertility [1]. Subfertility could form a psychologic burden on couples, but also poses an economic burden on society due to substantial costs [2,3].

In the Netherlands, couples are typically referred for an initial fertility assessment (IFA) with a gynaecologist after experiencing one year of subfertility. During the IFA, the Hunault prediction model is used to determine whether fertility treatment is necessary or expectant management is a viable option [4]. Expectant management is often chosen for unexplained subfertility.

A possible link between unexplained subfertility and bacterial vaginosis (BV) has been mentioned in previous studies [5]. BV refers to a dysbiosis of the vaginal microbiome. While BV can cause discharge problems with a fishy odour, approximately half of BV-positive persons are asymptomatic. Several studies have shown an incidence rate of 10-32% of BV, with a higher occurrence among subfertile women and certain ethnicities [6,7]. The diagnosis of BV can be made using the Nugent score, but qPCR has been shown to be more sensitive in detecting BV [8]. However qPCR and microbiome testing have so far mainly be used in in vitro fertilization (IVF) populations to investigate the impact of BV on pregnancy rates. In the IVF population BV was associated with early pregnancy loss and lower clinical pregnancy rates [9]. Only two studies have investigated BV in a preconception population cohort and found a probable effect on fecundity using the Nugent score or a 16S rRNA gene microbiome analysis to diagnose BV [10,11].

Metronidazole and clindamycin are commonly used therapies for BV, but the recurrence rate within a year remains high [12]. Certain lifestyle factors have been associated with BV [13], which might influence the treatment of BV as well as pregnancy outcomes. It is also suggested BV changes over time, and timing of fertility treatments is crucial [14]. However, the exact causal relation between BV and subfertility is still unknown, and it remains uncertain which approach improves outcomes. Having a more precise understanding of the direct correlation between BV and pregnancy outcomes may offer insights for potential future treatments.

This study investigates the effect of BV in a subfertile population diagnosed by qPCR at the time of initial fertility assessment on time to live birth pregnancy. It is hypothesised that persons who test positive for BV may experience a longer time interval to pregnancy,

particularly in participants with unexplained subfertility or those with a direct indication for fertility treatments such as intrauterine insemination (IUI), IVF or intracytoplasmic sperm injection (ICSI).

Materials and Methods

Patient recruitment, sample and data collection

This is a prospective single center cohort study in the Haaglanden Medical Center (HMC, a teaching hospital) in the Hague, the Netherlands. Persons above 18 years old were included after informed consent at the initial fertility assessment (IFA) between July 2019 and June 2022. Persons were excluded when they had a history of three or more miscarriages, could not understand Dutch or English, or used prophylactic antibiotic treatment. This study was designed as part of a prospective cohort study about BV and pregnancy results during IUI and IVF/ICSI treatment [18], therefore no separate power analysis was conducted.

The vaginal swab (e-swab, Copan Italia SpA, Breschia, Italy) and pH measurement (pH-Fix 4.0-7.0, ref 92137, Macherey-Nagel, Düren, Germany) were taken from the posterior fornix after inserting a speculum. Assessment of vaginal swabs for BV was done by external laboratories NMDL and DDL, Rijswijk, The Netherlands. Study participants and their doctors were blinded for the outcome of the swab. If study participants had symptoms of BV at any point, they underwent additional testing according to the standard protocol and treated (with clindamycin or metronidazole) if they tested positive for BV.

Follow up ended at the point of not wishing to conceive, live birth, end of relationship, age 43 years old or end of study (October 2022). Follow up of established pregnancies in this period was continued until it was known whether the pregnancy resulted in a live birth. Information about patient characteristics, fertility treatment and pregnancy outcomes were collected from the electronic patient dossiers. To minimise loss to follow up, a survey was sent to study participants if they did not return to the fertility clinic or gave birth elsewhere. This information was managed using Castor EDC, a cloud-based clinical data management service.

BV qPCR and microbiome analysis

The vaginal samples were frozen within 24 hours after collection and transported to the external laboratory (NMDL and DDL, Rijswijk, the Netherlands) for molecular analysis. DNA was extracted from 200 µl sample and eluted in a final volume of 100 µl with the MagNA Pure 96 instrument using the MagNA Pure 96 DNA and Pathogen Universal small Volume Kit and the Pathogen Universal protocol (Roche Diagnostics, Basel, Switzerland). The extracted DNA of all obtained vaginal swabs was tested with a CE-IVD marked

multiplex quantitative PCR assay, the AmpliSens® Florocenosis/Bacterial vaginosis-FRT PCR kit (InterLabService, Moscow, Russia) according to the manufacturer's instructions. Based on the presence of Lactobacillus species, *Gardnerella vaginalis*, *Atopobium vaginae* (recently reclassified as *Fannyhessea vaginae*)[15] and total amount of bacteria, swabs were categorised as BV positive (amount of *G. vaginalis* and/or *A. vaginae* is almost equal or exceeds the amount of *Lactobacillus* spp.), BV negative (*G. vaginalis* and/or *A. vaginae* are absent or its amount is substantially less than the *Lactobacillus* spp. amount), unspecified dysbiosis (amount of *Lactobacillus* spp. is reduced relative to the total amount of bacteria, whereas *G. vaginalis* and/or *A. vaginae* are absent or its amount is substantially less than total amount of bacteria) or suspected dysbiosis (amount of *G. vaginalis* and/or *A. vaginae* is similar to the amount of *Lactobacillus* spp. but does not exceed the limit value) using the software tool provided by the kit manufacturer. Unspecified dysbiosis and suspected dysbiosis were classified as BV qPCR positive.

In a subgroup of participants, the microbiota composition was determined. A fragment of ~421bp of the V1-V2 region of the 16S rRNA gene was amplified using the primers described by Ravel, et al (2011) and Walker, et al. (2015) with Illumina overhang adaptor sequences added [16,17]. Results were classified in one of five vaginal microbiome community state types (CST), as described by Ravel et al. CST I is dominated by *L. crispatus*, and respectively CST II by *L. gasseri*, CST III by *L. iners*, CST IV by non-lactobacilli, CST V by *L. jensenii*. More detailed information on the microbiota analysis is described in supplement 1.

Outcomes

Primary endpoint of the study was time until live birth pregnancy (time calculated from date of initial fertility assessment swab until positive pregnancy test leading to live birth). Secondary endpoints were time until ongoing pregnancy rate at 12 weeks' gestation, miscarriage - and preterm birth rates.

Statistical analysis

IBM SPSS statistics version 27 was used for all analysis. Continuous variables were compared between participants with and without BV using an unpaired t-test or a Mann-Whitney-U test in case of skewed distributions. Categorical variables are compared using the Chi^2 or Fischer's exact test. Kaplan Meier curves with the log rank test were used to compare the time to pregnancy between the two groups. Time to pregnancy was measured between date of IFA swab until date of positive pregnancy test. We performed Cox proportional hazard analysis was used to analyse time to pregnancy and to adjust for body mass index (BMI) and age which were considered the most important confounders. Because of low number of pregnancies, it was not possible to adjust for more than two confounders.

Subgroup analysis was performed for unexplained fertility, Caucasian descent, and direct indication for IUI or IVF/ICSI treatment, because literature suggests a different impact of BV in these groups.

Results

Study population

A total of 163 persons were included at the initial fertility assessment (Figure 1). 82 of included participants needed direct IUI or IVF/ICSI treatment. One person was excluded because after follow-up of expectant management a complete tubal factor was encountered. Fourteen participants conceived during their initial fertility assessment work up. 83 of the included couples eventually had a live birth.

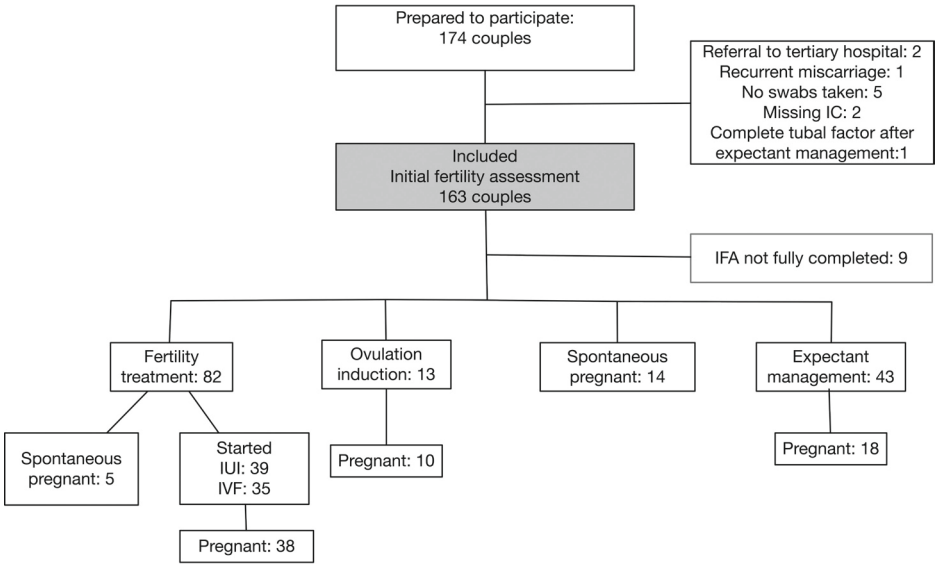


Fig. 1 flow diagram of included couples and all first follow up treatments

Of the total of 163 participants, 27% tested positive on BV qPCR at initial fertility assessment (Table 1). For one participant, no qPCR result was available at IFA, but vaginal swabs collected during two follow up treatments were tested BV qPCR negative. Therefore, this participant was regarded as BV qPCR negative. Maximum age at IFA was 42 years. BMI and pH were significantly higher in the BV qPCR positive group. BMI was not available for seven participants. Twelve participants reported discharge problems. Three of them were treated with antibiotics for respectively a yeast infection after hysterosalpingogram, BV or for urinary tract infections. The duration of subfertility of BV qPCR positive participants was slightly

longer before visiting the outpatient clinic (difference of 6 months, p=0.09). Male factor was more often the reason of subfertility in BV qPCR negative participants (25% versus 12%) and participants with endometriosis never tested BV qPCR positive, while BV qPCR positive participants had more often a hormonal factor or tubal factor as reason for subfertility. The follow up survey was sent to 50 participants, of which 40 replied (which included all pregnant participants).

Table 1 Baseline characteristics

Baseline IFA	**BV pos**	**BV neg**	**p-value**
Participants	44	119	
Age (at IFA) (mean, SD)	33 (4.0)	34 (4.4)	0.27
pH (median, IQR)	5.5 (5.0-5.8)	4.4 (4.0-4.7)	**<0.001***
Discharge complaints (at IFA) n(%)	4 (9%)	8 (7%)	0.52
Antibiotic/antifungal treatment	1	2	1.00
BMI (at IFA) (mean, SD)	26 (4.8)	24 (4.0)	**0.03***
Smoking (at IFA) n(%)	8 (18%)	11 (9%)	0.11
Alcohol (≥ 1 glas per week) n(%)	14 (32%)	44 (37%)	0.52
Druguse (on regular basis) n(%)	4 (9%)	5 (4%)	0.26
Medication use n(%)	5 (11,5%)	28 (24%)	0.09
Chlamydia antibodies positive n(%)	6 (14%)	14 (12%)	0.74
Ethnicity n(%) **			0.11
Caucasian	22 (50%)	78 (65%)	
African	1 (2%)	2 (1,5%)	
Antillean	7 (16%)	3 (2,5%)	
Asian	2 (5%)	7 (6%)	
Moroccan	2 (5%)	7 (6%)	
Hindu	5 (11%)	8 (7%)	
Turkish	0	3 (2,5%)	
Other	4 (9%)	9 (8%)	
Missing	1 (2%)	2 (1,5%)	
Socioeconomic status n(%)***			0.14
Low	4 (9%)	4 (3%)	
Middle	13 (30%)	31 (26%)	
High	25 (56%)	83 (70%)	
Missing	2 (5%)	1 (1%)	
Regular cycle n(%)	35 (80%)	101 (85%)	0.42
HPV positive last year n(%)	4 (9%)	13 (11%)	1.00
Gravidity n(%)			0.72
0	26 (59%)	69 (58%)	
1	10 (23%)	35 (29%)	
2	7 (16%)	13 (11%)	
3	1 (2%)	1 (1%)	
5	0	1 (1%)	
History of preterm birth	0	4	0.57
History of c-section	1	9	0.27

Table 1 Baseline characteristics (continued)

Baseline IFA	**BV pos**	**BV neg**	**p-value**
Subfertility duration in years (median, IQR)	2 (1-2)	1.5 (1-2)	0.09
Cause of subfertility n(%)****			0.07
Male factor	5 (11,5%)	29 (24,5%)	
Tubal factor	5 (11,5%)	7 (6%)	
Hormonal	11 (25%)	17 (14%)	
Endometriosis	0 (0%)	10 (8,5%)	
Unexplained	19 (43%)	48 (40%)	
Other	1 (2%)	5 (4%)	
Missing	3 (7%)	3 (3%)	
First treatment n(%)			0.29
Expectant management	9 (20,5%)	34 (29%)	
Spontaneous pregnancy	9 (20,5%)	15 (13%)	
IUI	11 (25%)	28 (23%)	
IVF	6 (14%)	29 (24%)	
Ovulation induction	5 (11%)	8 (7%)	
IFA not finished	4 (9%)	5 (4%)	

** p-value ≤0.05 considered significant*

*** other mostly Hispanic participants*

**** as defined by education status*

***** hormonal: premature ovarian insufficiency or anovulation, other: uterine myomas, uterus anomaly, sexual disfunction*

Pregnancy results

Out of 163 couples, 85 had an ongoing pregnancy (Table 2). Median follow up time without an occurring pregnancy was 8 months (IQR 5-16). No significant differences were found for BV qPCR status on time to ongoing pregnancy rates (aHR 0.94, 0.57-1.57) or time to live birth pregnancy rates (aHR 0.97, 0.58-1.62) (Table 2 and Figure 2a). Two participants had a termination of pregnancy because of Noonans syndrome or because of a serious neural tube defect. Twelve participants (two (5%) BV qPCR pos and 10 (8%) BV qPCR neg) delivered prematurely, of which eleven between 31- and 37-weeks' gestation, and one participant delivered at 24 weeks pregnant, after which the newborn died seven days postpartum due to its prematurity. The two premature deliveries in the BV qPCR positive group occurred at 36 weeks.

In the subgroup of 67 participants with unexplained fertility a tendency to a longer time to live birth pregnancy was observed in the BV qPCR positive group, but differences were not statistically significant (aHR 0.63, 0.28-1.45) (Figure 2b). Data about only Caucasian or non-Caucasian population and time to pregnancy based on BV-status did not show any differences (see Supplement Figure 4a/b and Supplemental Table 1).

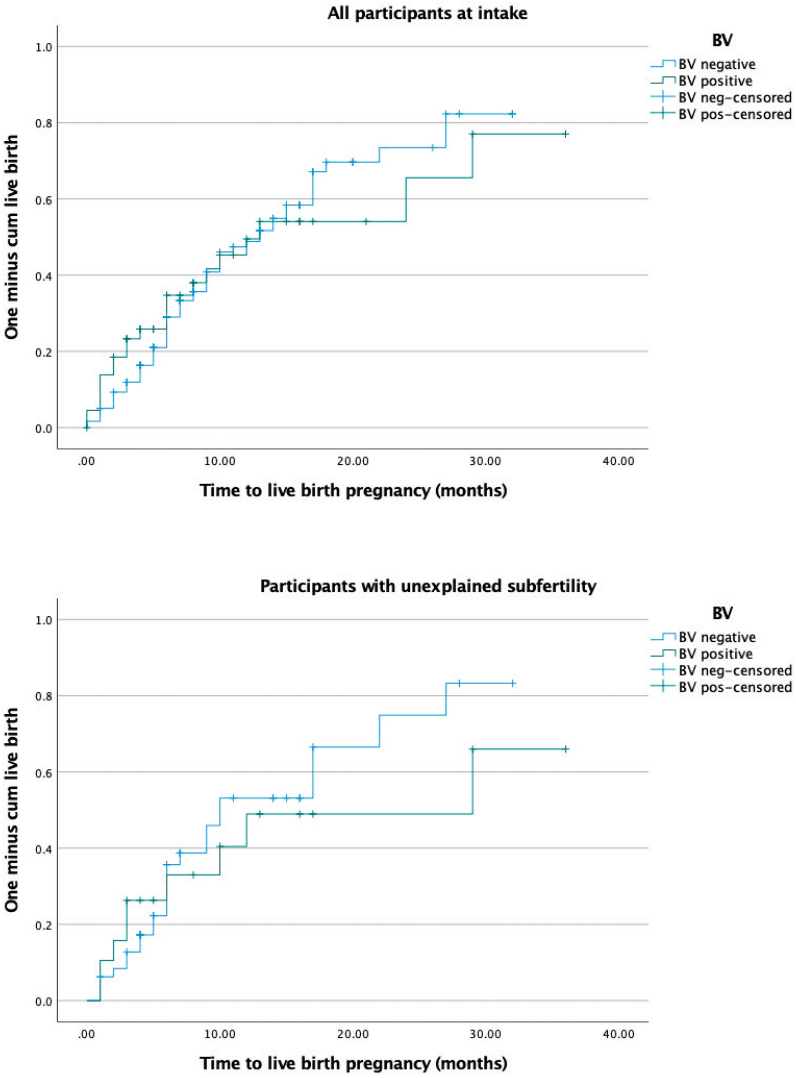


Fig. 2 Kaplan Meier curves for time to live birth pregnancy based on qPCR status at IFA for (a) all participants and (b) participants with unexplained subfertility

Table 2 Pregnancy results by BV qPCR status

Outcomes	BV pos	BV neg	HR (CI 95%)	p value*	aHR (CI 95%)**
Participants	44	119			
Ongoing pregnancy n(%)	21 (48%)	64 (54%)	0.95 (0.58-1.56)	0.85	0.94 (0.57-1.57)
Live birth n(%)	21 (48%)	62 (52%)	0.98 (0.60-1.61)	0.94	0.97 (0.58-1.62)
Premature birth n(%)	2 (5%)	10 (8%)			
Miscarriage in follow up period* **	7 (16%)	22 (18%)		0.70	
Persons with unexplained subfertility	19	48			
Ongoing pregnancy n(%)	9 (47%)	24 (50%)	0.78 (0.35-1.70)	0.52	0.63 (0.28-1.45)
Live birth n(%)	9 (47%)	24 (50%)	0.78 (0.35-1.70)	0.52	0.63 (0.28-1.45)
Miscarriage in follow up period* **	5 (26%)	8 (17%)		0.37	
Persons starting IUI/IVF	21	61			
Ongoing pregnancy n(%)	8 (38%)	35 (57%)	0.52 (0.23-1.17)	0.11	0.49 (0.20-1.15)
Live birth n(%)	8 (38%)	33 (54%)	0.54 (0.24-1.23)	0.14	0.50 (0.21-1.19)
Miscarriage in follow up period* **	5 (24%)	14 (23%)		0.94	
Ongoing pregnancy/Live birth (after 6 months)			0.21 (0.05-0.88)	**0.03**	0.04-0.85)

**p-value ≤0.05 considered significant*

*** adjusted for BMI and age*

**** Persons experiencing one or more miscarriages*

Pregnancy results in participants with a direct IUI or IVF/ICSI indication

82 participants had an indication of treatment by IUI or IVF/ICSI based on the IFA. The Kaplan Meier curve showed longer duration until live birth pregnancy in the BV qPCR positive group (Figure 3a) in particular after 6 months. The adjusted hazard ratio was 0.50 (0.21-1.19) in the total period, and 0.19 (0.04 -0.85) in the period after 6 months.

The percentages of occurrence of one or more miscarriages were the same for both groups. Three BV qPCR negative tested persons experienced two or more miscarriages. One BV qPCR positive tested person experienced three miscarriages.

To study effect of microbiome community state types (CST) on time to live birth pregnancy rates, 16S rRNA gene microbiota analysis was performed on vaginal swabs obtained at IFA for 72 of these 82 participants. Of the 72 tested participants, 18, 3, 33, 17 and 1 participants were classified as CST I, II, III, IV and V, respectively (Table 3a). A significant negative effect on time to live birth pregnancy rates was observed in CST group III dominated by *L. iners* (HR 0.45, 0.22-0.96) and CST group IV dominated by non-lactobacilli (HR 0.39, 0.16-0.98) (Table 3b). Here, numbers were too low to perform accurate adjusting for BMI and age. A significant longer time to live birth pregnancy interval was observed for CST III and IV combined compared to CST I-II-V combined (Figure 3b).

Table 3a Description of pregnancy results per community state type (CST)

Outcomes	CST I	CST II	CST III	CST IV	CST V
Persons starting IUI/IVF	18	3	33	17	1
Ongoing pregnancy n(%)	14 (77%)	2 (66%)	16 (48%)	7 (41%)	1
Live birth n(%)	14 (77%)	2 (66%)	14 (42%)	7 (41%)	1
Premature birth	4	0	1	2	0

Table 3b Pregnancy results by CST, CST I (*L. crispatus*) used as reference CST

	Ongoing pregnancy		Live birth		Live birth	
Coxregression	**HR (CI 95%)**	**p-value**	**HR (CI 95%)**	**p-value**	**aHR (CI 95%)****	**p-value**
CST I	1		1			
CST II	0.69 (0.16-3.03)	0.62	0.69 (0.16-3.06)	0.63	0.50 (0.11-2.27)	0.37
CST III	0.52 (0.25 -1.07)	0.08	0.45 (0.22 -0.96)	**0.04***	0.45 (0.21-0.96)	**0.04***
CST IV	0.40 (0.16-1.00)	**0.05***	0.39 (0.16-0.98)	**0.05***	0.34 (0.13-0.90)	**0.03***
CST V	1.63 (0.21-12.60)	0.64	1.71 (0.22-13.22)	0.61	3.19 (0.38-26.99)	0.29

**p-value ≤0.05 considered significant*

*** adjusted for BMI and age*

Discussion

This is the first study to provide more insight into time to pregnancy interval in couples attending a fertility clinic, based on qPCR and microbiota testing. This study did not find an association between BV and time to live birth pregnancy in a general group of subfertile couples or in unexplained subfertility. In the subgroup of couples with an indication for IUI of IVF/ICSI, BV or an abnormal microbiota is possibly associated with a longer time to live birth pregnancy interval.

The effect of BV qPCR positivity on time to pregnancy was only observed after 6 months. This can be explained by the fact that it usually takes a few months to start IUI of IVF/ICSI treatment, and the couples who did not conceive spontaneously during this period have a poorer prognosis. Previous studies in IVF, focusing on the microbiome, have not conducted survival analyses before. The use of survival analysis is essential to gain better understanding of the implications of an abnormal microbiome in relation to pregnancy outcomes.

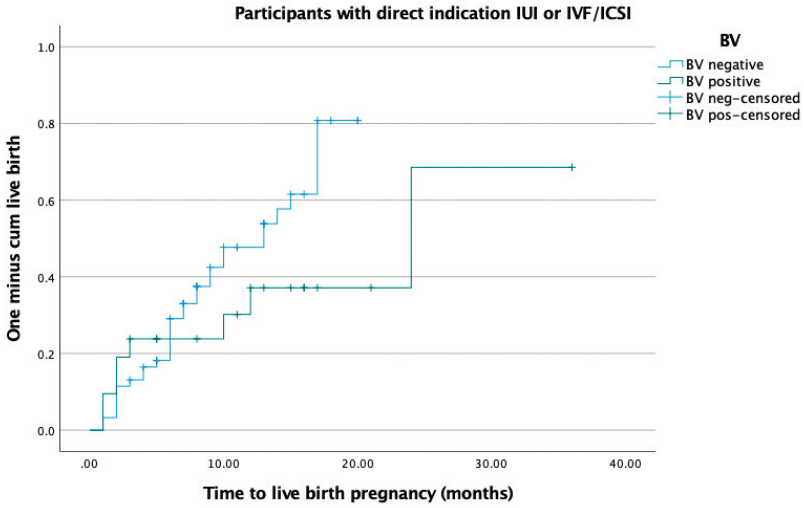
The follow-up period after start of pregnancy was long enough to report live birth rates, and there were no losses to follow up among known pregnant participants, which are strengths of this study. Another strength of this study is that microbiome analysis is additionally used in a subgroup to enhance comparability of results with future studies.

This study could be influenced by information bias of the follow-up survey. Ten participants did not reply to our survey, so the follow-up time for these participants ended at their last visit or contact in the hospital, when they were not pregnant. Another limitation of this study is the small study size, especially of the subgroups. It was designed as part of a prospective cohort study about IUI and IVF/ICSI treatment [18], so no power analysis was conducted upfront and microbiome analysis was not available for every participant.

A study showed a high incidence of an endometrial Gardnerella biofilm in cases of non-viable pregnancy curettage [19]. Also a meta-analysis indicated more early pregnancy losses in BV-positive persons [9]. This was not observed in our study, but a slight indication of more miscarriages was observed in the subgroup with unexplained infertility (26% vs 17%, p=0.37). Experiencing a miscarriage could lead to delay in trying to conceive for a subsequent pregnancy. In our study, BMI was significantly larger for participants with BV. Obesity is associated with a deterioration in the vaginal microbiome, with reduced *Lactobacillus* dominance and increased diversity of bacteria [20]. A higher BMI has been associated with a longer time to pregnancy interval, so it could be confounding this study [21]. However, when adjusting for BMI, it did not change the hazard ratios.

There is a possibility that factors such as BV in unexplained subfertility could lead to better management of treating unexplained subfertility in the future. Even though this study had a limited number of persons with unexplained subfertility to provide a definite answer, we found a suggestive negative effect of BV in this group. More research in this specific group could lead to better understanding of why these couples do not timely conceive. Hopefully, in the future, these persons could be treated with more personalised medicine such as lifestyle interventions or for example vaginal microbiome transplantation.

As microbiota testing becomes more widely known, couples can ask for commercially available tests during their fertility treatment. This study could offer couples an answer, eliminating the need to test for BV at their initial fertility assessment. It could be a cost-effective indicator to check only the vaginal pH value at IFA if couples want to know about asymptomatic BV or are willing to try lifestyle interventions [22]. For persons starting with fertility treatments, BV qPCR testing could be considered to optimise treatment. It is shown in literature that the microbiome can change over time, but optimal timing of testing for BV is not yet known (at IFA or during fertility treatments). However, as there are currently no treatment options available for CST III and no evidence of improved pregnancy outcomes by treating CST IV, microbiota testing should at this moment be preserved for study purpose only.



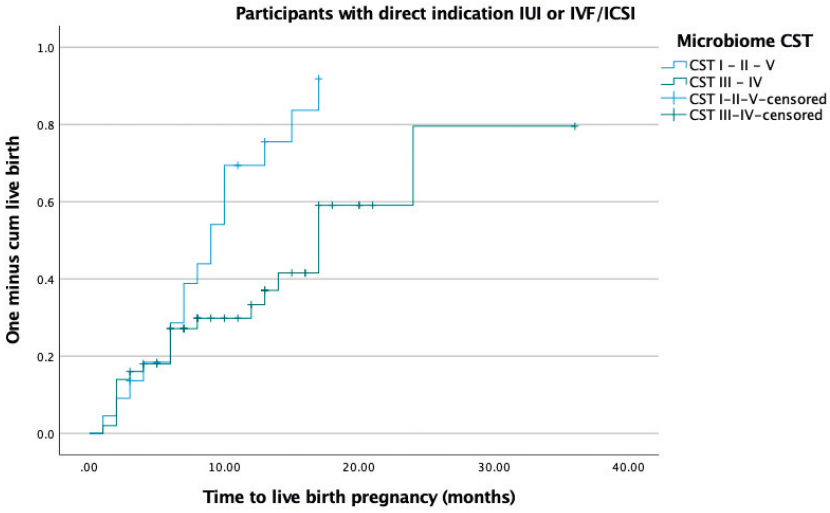


Fig. 3 Kaplan Meier curves for time to live birth pregnancy when IUI or IVF/ICSI treatment is indicated by (a) BV qPCR and (b) microbiome analysis

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Supplement 1 – Extension on materials & methods

Detailed description of microbiota analysis

A fragment of ~421bp of the V1-V2 region of the 16S rRNA gene was amplified using the primers described by Ravel, et al. (2011) and Walker, et al. (2015) with Illumina overhang adaptor sequences added. Each 50 µL PCR reaction contained 5 µL (10x) Expand High Fidelity Buffer with 15 mM MgCl2 (Roche), 2.6 U Expand High Fidelity Enzyme mix (Roche), 0.2 mM of each dNTP (Roche), various primer concentrations and 10 µL of extracted DNA. The PCR was run for 2 min at 94°C followed by 35 cycles of 94°C for 15 sec, 55 °C for 30 sec and 72 °C for 1 min and a final extension step at 72 °C for 7 min. The PCR products with a visible band of ~421bp on gel were subsequently purified and quantified using AMPure XP Beads (Agencourt Bioscience Corporation, Beverly, USA) and the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Paisley, UK), respectively. After library preparation using the Nextera XT kits (Illumina, San Diego, USA), sequencing was performed with the MiSeq desktop sequencer using the MiSeq Reagent Kits v2 500-cycles (Illumina). In each run, a negative control (PBS) and a positive control (Microbial Community Standard of ZymoBIOMICS) was included to monitor quality of the procedure. Samples should have a minimum number of 80,000 reads per sample and at least 75% of the reads should have an average quality score (Phred) ≥ Q30 to continue with data analysis. Sequencing data was processed following the QIIME pipeline(21) Open reference operational taxonomic units clustering of high-quality sequences (≥ 100bp in length with a quality score ≥ Q20) was conducted at a 97% similarity level against a pre-clustered version of the Augustus 2013 GreenGenes database. No low abundance filtering was used. Instead OTUs were checked for relevance per sample. Low abundance OTUs that were not relevant for any sample were included in the group "others".

Determining the CST-classification

The highest percentage of type of Lactobacillus was chosen to determine to which CST the sample belonged. For example, if a sample contained 70% of *L. iners* and 20% of *L. jensenii*, the sample was classified to CST III (*L. iners*). Samples having less than 50% *Lactobacilli* were classified as CST IV.

Supplemental table 1

Supplemental table 1 Pregnancy results by qPCR based on ethnicity

Time to live birth pregnancy	BV neg	BV pos	HR (CI 95%)	p-value*	aHR (CI 95%)**	p-value
Caucasian descent	78	22	1.22 (0.64-2.31)	0.55	1.24 (0.63-2.45)	0.54
non-Caucasian descent	39	21	0.98 (0.42-2.31)	0.97	0.90 (0.39-2.11)	0.81

** p-value ≤0.05 considered significant*

***adjusted for BMI and age*

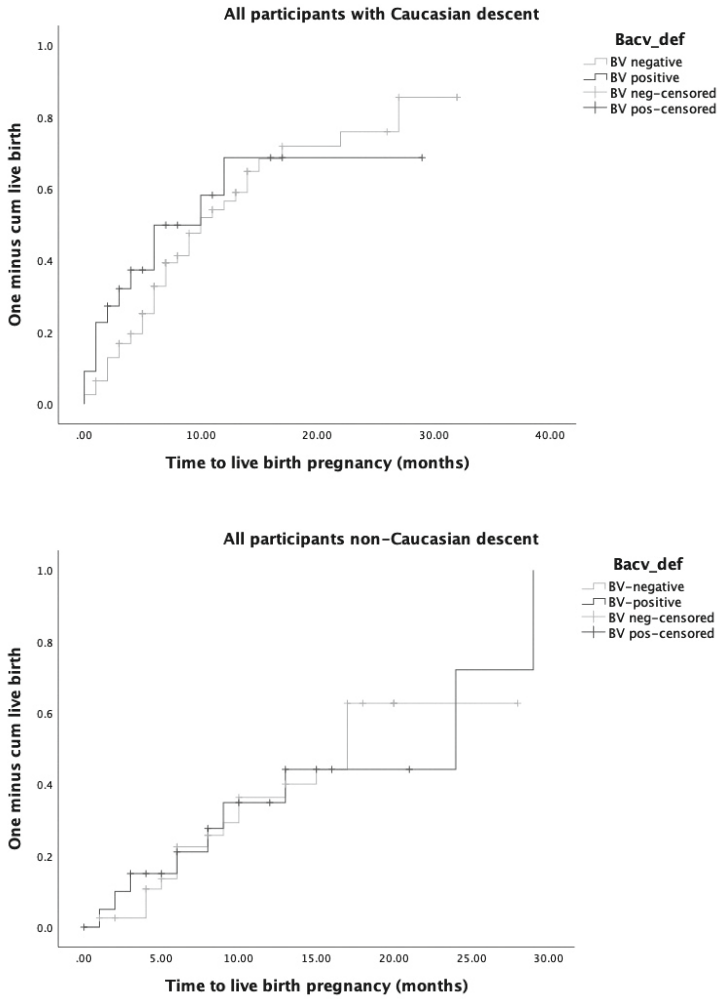

Fig. 4 Kaplan Meier curves for time to live birth pregnancy by BV qPCR for all participants at IFA with (a) Caucasian descent and (b) non-Caucasian descent

6

PREVIOUS CAESAREAN SECTION IS ASSOCIATED WITH LOWER SUBSEQUENT IN VITRO FERTILIZATION LIVE BIRTH RATES

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Abstract

This retrospective cohort study examines the association between previous mode of delivery and subsequent live birth rate in women who become pregnant after in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) after their first delivery. The study included 112 women with a previous caesarean section and 418 women with a previous vaginal delivery, and a total of 1588 embryo transfers between January 2005 and June 2016 (Leiden University Medical Center, the Netherlands). The mean age was 35 years and mean number of embryos transferred per attempt, 1.18. The study population included a total of 429 pregnancies resulting in 296 live births. The crude odds ratio for a subsequent live birth per embryo transfer was 0.60 (CI; 0.44 to 0.83, p=0.002) in women with a previous caesarean section compared to women with a previous vaginal delivery. After adjustment for age, fresh/frozen-thawed embryo transfer and quality of the embryo, the odds ratio was 0.64 (CI; 0.46 to 0.89, p=0.01). It was concluded that in subfertile women trying to achieve a subsequent pregnancy with IVF or ICSI, a history of caesarean section was associated with a reduced live birth rate per embryo transfer compared to women a history of one previous vaginal delivery.

Introduction

The caesarean section (CS) rate has been rising globally, mainly because of labour management choices [1]. In the Netherlands the CS-rate increased slightly from 14% in 2000 to nearly 17% in 2010 [2]. There are few studies on the effect of CS on live birth rate in subsequent pregnancies, with outcomes varying from a negative to no effect in the population [3–6]. The meta-analysis by Gurol-Urganci et al. (2014) showed a reduced fertility (live birth rate) of 11% after a CS. However, the studies were heterogeneous in study design and quality.

Incomplete uterine healing after a CS, called a 'niche' can have effect on abnormal menstrual bleeding and subfertility [7,8]. It is hypothesized that fluid from the caesarean scar is retained in the uterine cavity. This fluid may lead to a failure of embryo implantation due to the embryotoxic environment; a mechanism similar to that proposed for a hydrosalpinx [9].

Three studies on the effect of CS on pregnancies established by IVF or ICSI have recently been published. These indicated that embryo transfer (ET), a crucial part of IVF and ICSI, is more difficult in women with a caesarean scar [10]. Of the three studies, two reported no significant decrease in fertility, while one found a 14% decrease in pregnancy rate [10–12].

It is hypothesized that if a history of CS in fertile couples lead to a reduced live birth rate, the live birth rate will also be reduced in couples with a history of CS undergoing IVF or ICSI. Study aim: To discover if previous mode of delivery influences subsequent IVF/ICSI live birth rates.

Materials & Methods

This was a retrospective cohort study conducted in the Leiden University Medical Center (LUMC), The Netherlands.

Study participants and data retrieval

The medical records between January 2005 and June 2016 were analysed and women with a previous live birth (after an IVF or ICSI induced pregnancy) who returned for a second child using IVF/ICSI and had at least one (fresh or frozen-thawed) embryo transfer were selected for the study. These women were divided in two groups: (i) previous CS or (ii) vaginal delivery (including vacuum extraction). All fresh ETs and/or frozen-thawed ETs of the included study participants after their first live birth were analysed until the end of treatment or second live birth.

Exclusion criteria were history of multiple caesareans or vaginal deliveries, fetal death at first delivery (gestational age >16 weeks), missing delivery mode of first live birth or history of spontaneous pregnancy (a history of spontaneous pregnancy indicates that such women are more fertile than those who never achieved a spontaneous pregnancy and were therefore excluded to reduce bias).

In our study population, the age limit of study participants undergoing an oocyte pick up was 43 years, and for embryo transfer, 45 years. 'Age' was based on age at the last ET. Body mass index (BMI) and smoking characteristics of the study participants were extracted from the patient records mostly at the start of treatment. The cause of subfertility before first IVF treatment (before first delivery) was used to compare both groups. The quality of the embryos was graded using an adapted standard classification system. A class one (best quality) was defined as an embryo consisting of 8 cells (on day three) or 16, 32 or 64 cells (on day four to six). A class two was defined as an embryo of 7 or 9 - 15 cells (on day three) and a class three were defined as an embryo of less than 7 cells (on day three). This classification is based on that of Stylianou, et al, (2012) and the results of the LUMC IVF clinic [13].

IVF and ICSI protocol

Ovarian stimulation protocols were individually adapted to a long (GnRH agonist) or a short protocol (agonist or antagonist and urinary or recombinant FSH). The most frequent was a short protocol with GnRH agonist. ET of frozen-thawed embryos took place in natural cycles, or artificial cycles with administration of oestradiol and progesterone. Information on ultrasound findings such as a niche or uterine lining before embryo transfer was not collected.

All embryo transfers were performed in dorsal lithotomy position. Single ET was the standard protocol, but depending on age and medical history two embryos were sometimes transferred. The twin pregnancy rate in this centre varied between 2,8% - 6% of all IVF/ICSI pregnancies over ten years. Fresh embryos were mainly transferred or frozen on the third day after oocyte pick up, based on patient characteristics, with only a minority (<20%) transferred or frozen on day two or four. Frozen embryos were thawed four days after the LH-surge (measured in blood or urine) or five days after hCG administration. Embryos were transferred on day zero or day one after the thawing procedure. All ETs were included in the study. Luteal support was started two days before fresh embryo transfer until seven weeks of pregnancy.

In both groups, a Wallace® catheter was used for ET. In the 'caesarean' cohort, a Wallace Sureview® catheter (Smiths Medical) was used from 2010. The catheter was used to place the embryo at a distance of 15 mm from the endometrial fundus in the sagittal plane under abdominal ultrasound guidance. In the 'vaginal delivery' cohort, ET was performed with the fixed distance method. The embryos were placed at a distance of 60 mm from the external cervical ostium with a Wallace® catheter. The Wallace® and Wallace Sureview® catheters are identical, except that the Sureview is echogenic, and both were used in >95% of all ETs. If ET failed with the Wallace® catheter, a TDT®-catheter (Irvine Scientific) was used.

Outcomes

Primary outcome: a second live birth following IVF/ICSI in women with a history of CS compared with vaginal delivery [14].

Secondary outcome: biochemical pregnancy rate (β-hCG >50U/L at 15 days or more after oocyte retrieval), miscarriage or ectopic pregnancy and ongoing pregnancy rate (as defined by more than 16-weeks gestational age) in women with a history of CS compared to a history of vaginal delivery.

Statistical analysis

Independent t-test analysis was used for equally distributed parameters of the continuous data. Chi-squared tests/Fisher's exact tests were used for categorical data. Logistic regression with repeated measures (Generalized Estimated Equations, GEE) was used to assess the association corrected for number of embryo transfers on the main outcome measures: live birth rate, pregnancy, miscarriage. This GEE-model was also used to correct for confounding variables (age, fresh/frozen transfer, quality of embryos, cause of subfertility). Confounding variables with a p-value ≤0.05 were retained in the adjusted model. A p-value of ≤0.05 was considered significant. Statistical calculations were performed with IBM SPSS version 23.

Ethical approval

This retrospective cohort study was approved by the Medical Ethics Committee of the LUMC.

Results

Inclusion criteria (Figure 1) were met by 530 study participants. A total of 112 women had a previous CS, and 418 women had a previous vaginal delivery (spontaneous or vacuum extraction).

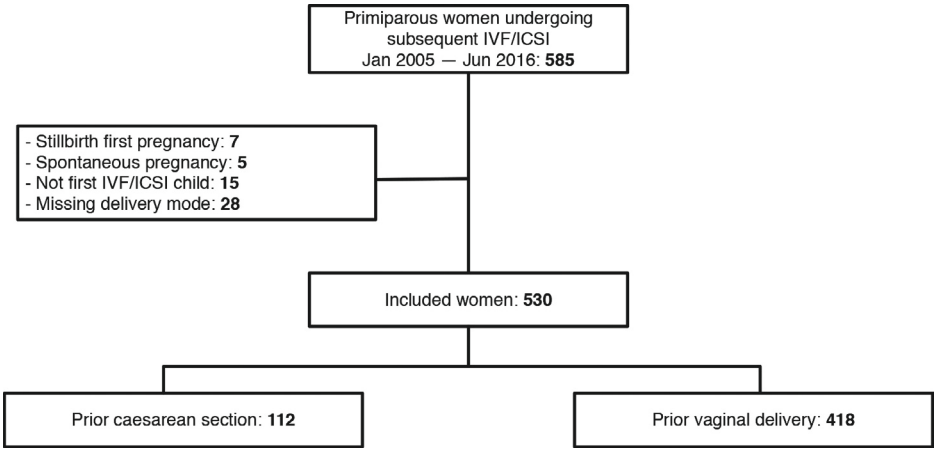

Fig. 1 Flowchart included study participants

The characteristics of both groups are represented in Table 1. BMI of the CS-group tended to be higher than the vaginal delivery group, however not significantly. For 244 women treated by LUMC, BMI was available (46%). Transfer ease was not tabulated in Table 1 because of the low count of classes other than 'easy' (<10 women with class 'medium' or 'hard').

Table 1 Group characteristics

	Caesarean section	Vaginal delivery	p-value
Participants	112	418	
Age at last attempt yr (SD)	35.4 (3.99)	34.5(3.93)	**0.02***
BMI mean (SD) ***	25.3 (4.38)	23.9 (3.76)	0.051
Tobacco use n(%) ***	7/57 (12)	36/225 (16)	0.49
Cause of subfertility n(%)			**<0.01***
Male factor	47 (42)	247 (59)	
Tubal factor	15 (13)	25 (6)	
Hormonal	9 (8)	25 (6)	
Endometriosis	10 (9)	14 (3)	
Unexplained	21 (19)	78 (19)	
Other (uterus anomaly)	1 (1)	2 (0.5)	
Missing	9 (8)	27 (6,5)	
Embryo transfers	390	1291	
Oocytes/OPU** n(SD)	9.79 (5.51)	10.24 (5.49)	0.30
Embryos /transfer	1.19 (0.40)	1.18 (0.39)	0.53
Fresh ET n(%)	118 (30)	435 (34)	0.21
Frozen-thawed ET n(%)	272 (70)	856 (66)	
Ultrasound guided ET n(%)	188 (48)	26 (2)	**<0.001***
Quality of embryos n(%)			0.19
Class 1	136 (35)	461 (36)	
Class 2	103 (26)	390 (30)	
Class 3	151 (39)	440 (34)	

p ≤ 0.05 considered significant ** OPU = Oocyte Pick Up * missing data*

In the study population 296 subsequent live births out of 1681 embryo transfers were documented (Table 2). Study participants underwent an average of three embryo transfers (minimum of one and maximum of 19 transfers).

CS was associated with a significantly lower live birth rate per ET compared to vaginal delivery (Table 3; OR 0.60, (0.44-0.83)). When adjusted for age, fresh or frozen-thawed ET and quality of the embryos, the analysis still showed a significantly lower live birth rate per ET (aOR 0.64 (0.46-0.89)). The adjusting variables were not confounders (less than 10 % influence on OR), but had a significant effect to remain in the model (p<0.001). BMI, smoking and cause of subfertility had no significant influence on the live birth rate. Because there was a significant difference in the cause of subfertility in both groups (Table 1), a second adjusted OR for the primary outcome was included in Table 3 (aOR 0.65 (0.46-0.93)).

Ongoing pregnancy rate was not analysed separately from live birth rate, because only one single stillbirth occurred in the vaginal delivery group.

Table 2 Outcome overview

Outcome	Caesarean delivery	Vaginal delivery	p-value
Embryo transfers (n)	390	1291	
Live birth (n, (%/ET))	50 (13%)	246 (19%)	*
Pregnancy (n, (%/ET))	83 (21%)	347 (27%)	*
Miscarriage (n, (%/pregnancy))	33 (40%)	92 (27%)	*
Ectopic pregnancy (n, (%/pregnancy))	0	8 (2%)	0.21
Stillbirth (n, (%/pregnancy))	0	1 (0.3%)	1

*see Table 3 for analysis

Table 3 Main outcomes per embryo transfer and miscarriage ratio per pregnancy; CS compared to vaginal delivery

Outcome	Odds ratio	CI (95%)	p-value
Outcome/embryo transfer			
Live birth (crude)	0.60	0.44-0.83	**0.002***
Live birth (adjusted)**	0.64	0.46-0.89	**<0.01***
Live birth (adjusted)***	0.65	0.46-0.93	**0.02***
Pregnancy (crude)	0.73	0.56-0.96	**0.02***
Pregnancy (adjusted)**	0.77	0.59-1.00	**0.05***
Miscarriage (crude)	1.16	0.78-1.72	0.47
Miscarriage (adjusted) **	1.16	0.77-1.75	0.47
Outcome/pregnancy			
Miscarriage (crude)	1.78	1.11-2.84	**0.02***
Miscarriage (adjusted)**	1.86	1.09-3.16	**0.02***

*p ≤0.05 considered significant, CI: confidence interval

** adjusted for age, fresh/frozen-thawed transfer, embryo quality

*** adjusted for age, fresh/frozen-thawed transfer, embryo quality and cause of subfertility

Discussion

Our study suggests that a previous live birth by CS following IVF/ICSI could lead to a reduced live birth rate among women embarking on subsequent IVF/ICSI. The miscarriage rate once pregnancy was achieved was significantly higher in the CS-group. Current literature also describes a slightly higher incidence of miscarriage after CS [15].

The main strength of our study is the higher number of study participants included than in previous studies. Our findings show a more distinct difference between both groups than reported by other literature [10–12,16]. A literature search revealed three IVF/ICSI studies which only monitored outcomes of one (fresh) embryo transfer [10–12] and two studies which included women with one or more CS's or vaginal deliveries [10,12]. The mode of conception of the first live birth was not mentioned and there was a difference in subfertility period in the study of Zhang [11]. Factors such as a previous spontaneous or IVF pregnancy, duration of subfertility, and number of IVF-cycles influence the live birth rate according to Templeton's model [17]. It could be that all these factors reduced the association compared with our study in which only study participants with one previous delivery were included.

Our hospital has a fixed group of experienced individuals who carry out ETs. The analysis used repeated measures, and outcomes were measured per ET. This lowers the chance of bias when performing more ETs until success is achieved. There is also less bias towards couples who give up after a few attempts of IVF because of age or traumatic delivery. Traumatic delivery could be more frequent in the group of CS (which possibly were more often in an emergency setting), which could lead to less desire to have a second child. In population studies it is difficult to study decreased family size because the cause might focus on maternal choice, instead of a physiological effect of an impaired implantation [18]. All study participants in our study tried to achieve a second pregnancy in contrast to population-based studies, so that maternal choice did not influence the outcomes.

Due to incomplete data on BMI, smoking ultrasound guidance during transfer and cause of subfertility, the present study cannot rule out that these factors as confounders. All other data for study participants were complete and were used to adjust outcome measures. The database did not contain information about ultrasound findings such as a niche or uterine lining before embryo transfer, the type of scar (classical or transverse incision) or surgical techniques of uterine closure (associated with niches) and there was no information on whether women had a niche or complaints of bleeding problems after their CS. Visible endometrial fluid during IVF stimulation is known to impair outcomes in women with tubal factor [19] and there are reports on endoscopic correction of the niche which resolve abnormal menstrual bleeding, and may have a (positive) effect on fertility problems [9,20].

The CS-group had a significantly higher age (Table 1). Higher age is correlated with a greater risk of CS and reduced IVF/ICSI success rates. Data were corrected for age because it is significantly related to the outcome measure [17]. The CS group more often had non-male factors as a cause of subfertility, including endometriosis, which is linked to a higher risk of having a CS [21]. The cause of subfertility had no influence on the main outcome measure in the GEE model (see Table 3). The CS group tended to have a higher BMI, which approached significance (p=0.051). Data on BMI were only available in 48% of the study participants and were mostly measured before the first pregnancy. Increased BMI is linked to a greater risk of CS [22]. This difference in baseline characteristics could lead to confounding by indication. Data were not corrected for BMI or smoking in our study, because they had no significant effect on the model. However, this could be unreliable because of missing values. Moreover, BMI might not necessarily be correlated with live birth rate [23].

Ovarian stimulation protocols differed between study participants. We have no reason to believe that stimulation protocols were not distributed equally between groups or would influence outcomes. In the last few years of the study period, the CS-group had an echogenic catheter and ultrasound for the embryo transfer, as shown in Table 1. Currently there is little evidence that ultrasound-guided embryo transfer improves pregnancy outcomes [24]. Separate analysis of live birth rate did not show a significant difference between the two periods (data not shown).

Counselling women carefully for a CS by obstetricians is an important element in reducing the number of caesarean sections worldwide. Women with a first ongoing pregnancy after IVF or ICSI are more likely to have a CS, even after matching for the most prominent confounders, notably, age [25]. The implication of our results is that a higher frequency of CS could reduce the chances of a second live birth through IVF or ICSI and could be more pronounced in certain groups of CS-women, for example those with a niche. We presume that this CS-effect is applicable to the rest of the population, i.e., to women without fertility problems though to a lesser extent. It is essential that further research on this subject be carried out. Preferably this would consist of prospective research with a larger number of women with more data available on BMI, smoking, endometrial cavity, and the criteria for IVF. With an extended study size, it would be possible to distinguish more reliably between problems with implantation or with the conceptus reaching full-term.

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7

THE IMPACT OF CAESAREAN SCAR NICHE ON FERTILITY - A SYSTEMATIC REVIEW

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Abstract

Purpose

The trend of increasing caesarean section (CS) rates brings up questions related to subfertility. Research regarding the influence of CS on assisted reproduction techniques (ART) is conflicting. A potential mechanism behind CS-induced subfertility is intrauterine fluid resulting from a caesarean scar defect or niche. The vaginal microbiome has been repeatedly connected to negative ART outcomes, but it is unknown if the microbiome is changed in relation to a niche.

Methods

This systematic review describes literature investigating the effect of a niche on live birth rates after assisted reproduction. Furthermore, studies investigating a difference in microbial composition in subfertile persons with a niche compared to no niche are evaluated. Pubmed, Embase and Web of Science were searched on March 2023 for comparative studies on both study questions. Inclusion criteria were i.e. English language, human-only studies, availability of the full article and presence of comparative pregnancy data on a niche. The quality of the included studies and their risk of bias were assessed using the Newcastle-Ottawa scale for cohort studies. The results were graphically displayed in a forest plot.

Results

Six retrospective cohort studies could be included on fertility outcomes, with a total of 1083 persons with a niche and 3987 without a niche. The overall direction of effect shows a negative impact of a niche on the live birth rate (pooled aOR 0.58, 95% CI 0.48-0.69) with low-grade evidence. Three studies comparing the microbiome between persons with and without a CS could be identified.

Conclusion

There is low-grade evidence to conclude that the presence of a niche reduces live birth rates when compared to persons without a niche. The theory that a caesarean has a negative impact on pregnancy outcomes because of dysbiosis promoted by the niche is interesting, but there is no sufficient literature about this.

Introduction

The global average caesarean section (CS) rate increased by 19 percent from 1990 to 2018, with the WHO projecting continued growth up to 2030 [1]. The trend of increasing CS rates brings up questions related to its sequelae, amongst others, subfertility. Two systematic reviews, including meta-analysis, reported that persons post-CS take longer to achieve a subsequent pregnancy compared to vaginal delivery, with lower live birth rates (LBR), and are at a 14% increased risk of subfertility [1][2]. Both authors advise cautious interpretation of these results due to large heterogeneity in methodology and study quality, as well as unknown factors that play a role in the decision to perform a CS, such as couple's personal views, expectations and experiences. These reviews, in tandem with an expected 28.5% share of CS in global births by 2030, indicate a concerning trend in reproductive health [3].

Research regarding the influence of CS on assisted reproduction techniques (ART) is conflicting. Two recent reviews investigate the impact of a CS in ART, yielding contradictory results on pregnancy rates [4,5]. Several potential mechanisms behind a CS-induced reduction in fertility have been proposed. Principal amongst them is the presence of pelvic adhesions post-CS, disturbed foetal implantation, and the presence of a caesarean section defect or niche [2,6]. The presence of a niche facilitates intrauterine fluid and mucus accumulation, promotes local inflammation, disturbs physiological uterine contractions, and may hamper oocyte pick-up and embryo transfer during ART. The accumulation of blood in the niche could lead to degradation of haemoglobin and result in higher iron exposure, which could promote certain bacteria [6]. A systematic review found the prevalence of a transvaginal ultrasound ascertained niche to be 24%-70% in persons with a history of CS [7]. Studies show that delivery via CS substantially raises the risk of a repeat CS [8]. With each consecutive CS the risk of incomplete scar healing and niche rises, up to 45-100% after a third CS [7,9]. Recently, a panel of experts defined the symptoms arising from a niche, an endometrial indentation at the site of a CS with a depth of at least 2 mm determined by ultrasound, as a Caesarean Scar Disorder [10,11].

The vaginal microbiome has been repeatedly connected to negative ART outcomes [12–14]. The sterile uterus hypothesis is now largely rejected but precise knowledge of what constitutes a physiologic uterine microbiome is lacking. Consensus has been achieved that the vaginal and endometrial microbiome form a continuum [15]. Furthermore, there is early evidence that the presence of not only *Lactobacillus* in the vagina but also in the endometrium influences live birth rates [13,14].

These studies raise the question if the reason for secondary subfertility after a CS is specifically caused by a niche. And if so, maybe the mucus accumulation in the niche

could promote an abnormal microbiome. In this systematic review we evaluate the current studies about fertility outcomes during assisted reproduction techniques (ART) in the context of a niche, as well as the microbial composition following CS.

Methods

Data sources and main outcomes

This systematic review was conducted following the PRISMA Guidelines. This review was not registered upfront in a database, and had no protocol published. Ethical approval was not applicable. The PICO of the first study question was whether persons undergoing ART with a caesarean have different pregnancy outcomes compared to persons undergoing ART without a caesarean (or a niche). Pubmed, Embase and Web of Science were searched in November 2022 for articles using the keywords "caesarean" and "embryo transfer" to identify all relevant articles on the niche fertility search. Two authors (SvdS; MvdT) reviewed independently the title and abstract of each article for relevancy. In some cases, full text documents were also reviewed to make the final decision. In cases of a disagreement, a third person was responsible for making the final decision (KB). Inclusion criteria were English language, human-only studies, availability of the full article and presence of comparative pregnancy data on a niche. Exclusion criteria were conference abstracts, questionnaires, and reviews.

The main outcome for this review was live birth, secondary outcomes were clinical pregnancy, ectopic pregnancy, miscarriage, multiple pregnancy rate and ongoing pregnancy rate. Included articles were searched independently for data by two authors (SvdS; MvdT) on description of the niche, patient characteristics such as infertility duration, number of deliveries, intervention characteristics such as in vitro fertilization (IVF) procedure, fresh/frozen embryo transfer, single or double embryo transfer (SET or DET) and statistical analysis.

The PICO of the second study question was whether subfertile persons with a caesarean have a different microbiome compared to subfertile persons without a caesarean (or niche). A second search using the keywords "bacteria", "caesarean" and "fertility" was used to identify articles regarding microbial composition of a niche in March 2023. Further papers were identified via the snowballing method, using the reference lists of included papers as a starting point. Again two separate authors (SvdS; MvdT) reviewed independently the title and abstract of each article for relevancy. The authors were able to reach consensus in all cases. Inclusion criteria were English language, human-only studies, availability of the full article and presence of data on caesarean delivery. Exclusion criteria were conference abstracts and reviews.

Most important outcome for this part of the review was microbial composition. Secondary outcomes were pregnancy rates. Included articles were sought for a description of the niche, patient characteristics such as subfertility duration, number of deliveries, method of obtaining cervical or endometrial microbial samples and microbial analysis.

Full search strings of both searches are provided in Appendix 1.

Data collection and analysis

The quality of the included studies and their risk of bias were assessed using the Newcastle-Ottawa scale (NOS) for cohort studies. The NOS scores the studies on three categories: the selection and comparability of the exposed and non-exposed, and the ascertainment of outcome. With a maximum score of 9, one point can be awarded per subheading of the selection and outcome categories, with comparability being worth up to two points. Two authors scored the articles independently (SvdS; MvdT), when there was a disagreement, a third person determined the final score (KB).

The extraction forms were explicitly designed for this systematic review (see Appendix 4). Odds ratios (OR) with 95% CI were extracted from the papers or calculated from the reported results in the papers. The results were graphically displayed in a forest plot. If heterogeneity between studies was low, odds ratios would be formally pooled in a random effect meta-analysis. Publication bias was assessed when a review contained more than 10 studies. R 4.3.1 was used to make the forest plot and meta-analysis. Quality of obtained evidence is scored by Grading of Recommendations, Assessment, Development and Evaluation (GRADE) system.

Results

General characteristics

A flowchart detailing the screening process of the two searches can be seen in Figure 1. The niche fertility search identified 323 search results. Title and abstract screening identified 41 studies, and after screening the full article, six studies were included with complete (statistical) data about the niche and ART outcomes [16–21]. The article by Vissers et al., which only mentioned an effect of a niche in their discussion, was excluded because of incomplete data [22]. The study of Lawrenz et al. was excluded because of incomplete data and insufficient details in statistical analysis (only p-values of chi-square and t-test, no data on odds ratios) [23].

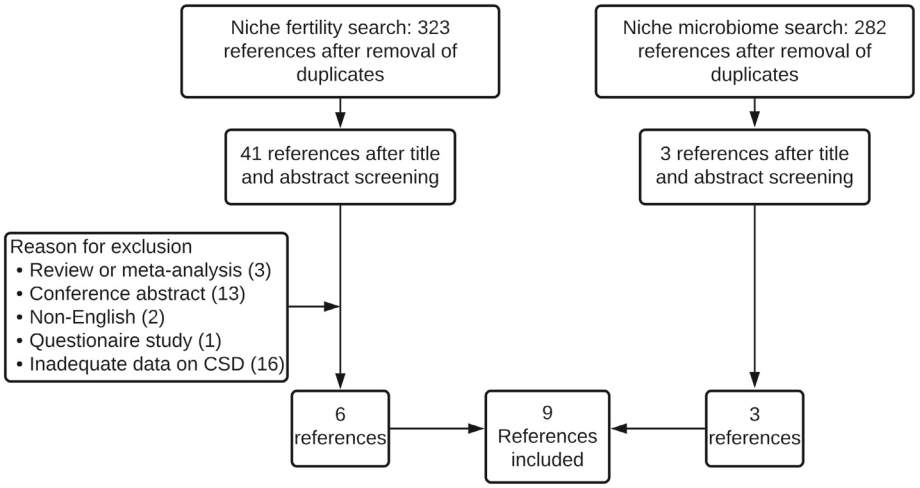

Fig. 1 Flowchart of included studies

Of the included six papers, four were published after the most recent reviews by Riemma and Zhao et al. [4,5]. All studies were retrospective cohort studies. In all six studies only the first cycle of an ART (IVF or intracytoplasmic sperm injection (ICSI)) regimen was analysed. All studies reported on live birth rates (LBR). In total 1083 persons with a niche were analysed.

In the niche microbiome search, 282 papers were identified (Figure 1). Three papers about the microbiome in the context of a niche were included after title and abstract screening [24–26]. Two studies were case-control studies that utilised cervical and endometrial 16s PCR sequencing [24,26]. The other study was a prospective cohort study and utilised bacterial cultures of the niche and endocervix [25]. In total 138 persons with a niche were included.

Appendix 2 and 3 shows an overview of all articles screened.

Five studies received funding for their studies [17,20,21,24,26]. No study reported any conflict of interest. An overview of the included studies of both searches is shown in Table 1.

Overall quality assessment

Overall, the selected studies were heterogenous in their assessed susceptibility to bias. With exception of the study by Huang et al. (8 points) and Wang et al. (9 points), all the studies ranged from poor to medium in NOS scores. Wang et al. analysed only single embryo transfers (SET) and clearly described the methodology used, which resulted in the lowest assessed risk of bias of all studies [21]. Because of the small number, the overall poor

quality of the studies, and the heterogeneity in study population and niche evaluation, only a meta-analysis on the primary outcome of live birth was performed.

All three microbiome studies were scored as having poor quality (2 points for the studies of Yang and 4 points for the study of Hsu). The complete score table on the NOS is included in the Appendix 5. None of the microbiome studies provided any pregnancy outcomes after initial microbiome analysis was performed. Publication bias for both subjects was not assessed because of the low number of articles found.

Niche and pregnancy outcomes

Study population

All selected six studies included secondary infertile participants and analysed fertility outcomes during ART. Treatment strategies of ART were different between the studies. Two studies performed only SET [16,21]. Two studies had much higher proportions of double embryo transfer (DET) compared to others and did not provide reasons as to how SET or DET were assigned [19,20]. In the study of Diao et al. there was also a high percentage of selective reduction of twin pregnancies [19]. One study only analysed fresh cycles [19]. Two studies performed no fresh embryo transfer if any endometrial cavity fluid was seen [16,17]. Two studies only performed frozen embryo transfers [16,20]. An overview of details of the studies is shown in Table 1. Furthermore, some studies only included pre-implantation genetic tested embryos for aneuploidy (PGTa) [16], some had both PGTa and non PGTa [18], and some excluded those [17,21].

Table 1 Overview of included studies

First Author	Year of publication	Study design	Location and years of inclusion	Study objective	Participants	Main outcome	Secondary outcomes	Embryo transfer	Women with a niche	CS without a niche	Vaginal delivery
Pregnancy and CSD											
Asoglu, MR	2020	Retrospective cohort	Turkey, 2017-2019	Effect of a niche (>1mm) on ICSI	vaginal, CS and niche	LBR	CPR, Miscarriage, MPR, DETR	SET/DET FR/FZ	75	75	
Friedenthal, J	2021	Retrospective cohort	USA, 2012-2020	Effect of previous delivery mode on IVF/ICSI	CSD vs vaginal	IR	Ongoing pregnancy, LBR	SET FZ	81		325
Diao, J	2021	Retrospective cohort	China, 2015-2019	Effect of previous delivery mode on IVF/ICSI	Niche vs vaginal	LBR	IR, CPR, early abortion, ectopic pregnancy, PTB, mode of delivery	SET/DET FR	74		401
Huang, L	2022	Retrospective cohort	China, 2013-2019	Effect of niche (>2mm) on IVF/ICSI	Niche vs CS without niche	LBR	CPR, ICF, Miscarriage, caesarean scar pregnancy, placenta praevia rate, MPR	SET/DET FR/FZ	215	1323	
Wang, L	2022	Retrospective cohort	China, 2015-2019	Effect of previous delivery mode on IVF, niche (>2mm)	Niche vs CS without niche	LBR	CPR, BP, Miscarriage, ectopic pregnancy, MPR, PTB	SET FR/ FZ	509	1570	
Zhang, Y	2022	Retrospective cohort	China, 2014-2020	Effect of previous delivery mode on IVF, niche (≤2mm)	Niche vs vaginal	LBR	CPR, ectopic pregnancy, MPR, Miscarriage, PTB,	SET/DET FZ	129		293

Table 1 Overview of included studies (continued)

First Author	Year of publication	Study design	Location and years of inclusion	Study objective	Participants	Main outcome	Secondary outcomes	Embryo transfer	Women with a niche	CS without a niche	Vaginal delivery
Microbiome and CSD											
Yang, X	2021	Case control	China, 2019	PCR comparison of paired endometrial and vaginal samples	Niche vs vaginal	Microbial diversity and composition	Inflammatory cytokine counts		9		7
Hsu, I	2022	Prospective cohort	Taiwan, 2008-2013	Culture-based comparison	Vaginal, CS and niche	Bacterial Presence			101	71	191
Yang, X	2022	Case control	China, 2021	Cervical Microbiome, metabolome and endometrial transcriptome comparison	Niche vs vaginal	Microbial diversity and composition			28		24

IVF In-Vitro-Fertilisation, ICSI Intra cytoplasmic sperm injection, CS Caesarean section, VD Vaginal delivery, IPR implantation rate, CPR clinical pregnancy rate, MPR multiple pregnancy rates, DETR difficult embryo transfer rates, BP Biochemical pregnancy, PTB Preterm birth, LBR live birth rate, SET Single embry transfer, DET double embryo transfer, FR Fresh embryo transfer, FZ Frozen embryo transfer

Niche evaluation

Three studies differed in their definition of a niche from that of the European niche taskforce (>2mm indentation) and two of them used subjective criteria [16,18,19]. All studies determined retrospectively whether there was a niche, using various methods to ascertain its presence. One study checked ultrasound pictures made during the day of oocyte retrieval [17], three studies checked multiple ultrasound pictures by two separate researchers [16,20,21].

Sample sizes

Three studies performed a power analysis before starting the study [16,18,19]. The other three studies had a high number of participants, with more than 120 having a niche, included [17,20,21]. Two out of six studies reached the number of included participants calculated in their power analysis [16,18].

Comparability between studies

Three studies compared vaginal delivery with caesarean delivery as the main study question and conducted a subanalysis on participants with a niche [16,19,20]. Two studies included persons with more than one delivery [18,21]. Three studies had, as their main study question, the comparison between a niche and without a niche following a caesarean delivery [17,18,21]. Baseline characteristics were in some studies significantly different [16,19,20], but these studies adjusted for the baseline differences in the final analysis. Asoglu et al. had an age-matched cohort but did not describe how these controls were chosen [18].

Multiple pregnancy rates differed in the studies, with the highest rates in the study of Diao et al. and Zhang et al. (23% and 28% in delivery control group). Diao et al. did not report any multiple pregnancies in the niche group. However, in two cases in the niche group, a selective embryo reduction was performed, compared to ten cases in the vaginal delivery control group.

Live birth

All six studies reported on live births and had adequate follow-up until birth. A forest plot for the odds ratios (OR) on the live birth rates (LBR) in those with and without a niche of all six studies is shown in Figure 2 and Table 2. All six studies found a negative effect on LBR, for 5 of the 6 studies the effect was statistically significant. One study observed no significant effect [18]. The pooled adjusted OR was 0.58 (95% confidence interval: 0.48-0.69) (heterogeneity p=0.61, $I^2 = 0\%$).

Zhang et al. performed a separate analysis for the SET and DET group with an aOR of 0.59 (0.24-1.43) in the SET subgroup and aOR 0.39 (0.21-0.72) in the DET subgroup. Wang et al. found that the size of the niche had an effect on pregnancy outcomes: the larger the niche, the more pronounced the effect, leading to a greater odds ratio. Huang et al. found no significant effect in frozen cycles (aOR 0.69, 0.45-1.08), but a significant effect in fresh embryo transfers (aOR 0.5, 0.29- 0.87).

Intracavitary fluid

Two studies performed a separate analysis to see if intracavitary fluid (ICF) was influencing pregnancy outcomes. In the study of Diao et al., the presence of ICF (n= 25) in relation to a niche did not significantly alter pregnancy outcomes [19]. However, Huang et al. performed a comparison in the niche and frozen-thawed ET arm of the study between uterine cavity with (n=35) and without ICF (n=90) and showed a significantly lower LBR in the presence of ICF (aOR 0.27, 95% CI 0.08-0.94) [17].

Table 2 Pregnancy outcomes for included studies

First Author	Niche	CS without a niche	Vaginal delivery	OR	95% CI	aOR	95% CI
Live Birth	Live birth/participants						
Asoglu	33 / 75	35 / 75		0.89	0.47-1.71	0.81	0.39-1.66
Friedenthal	37 / 81		192 / 325	Data not provided	Data not provided	0.51	0.30-0.89
Diao	16 / 74		146 / 401	0.48	0.26-0.86	0.50	0.27-0.95
Huang*	51 / 215	448 / 1323		0.61	0.44-0.85	0.63	0.45-0.88
Wang	121 / 509	581 / 1570		0.53	0.42-0.67	0.61	0.48-0.78
Zhang	31 / 129		137 / 293	0.36	0.23-0.57	0.41	0.25-0.66
Clinical pregnancy rate							
Asoglu	37/75	38/75		1.12	0.57-2.18	1.16	0.54-2.51
Diao	22/74		189/401	0.47	0.27-0.81	0.59	0.33-1.06
Huang *	69/215	594/1323		0.58	0.43- 0.79	0.59	0.43-0.80
Wang	191/509	748/1570		0.66	0.59-0.81	0.78	0.62-0.97
Zhang	40/129		167/293	0.34	0.22-0.53	0.39	0.25-0.62
Miscarriage rates							
Asoglu	6	3		2.09	0.50-8.67	2.57	0.58-11.34
Diao	4		24	0.89	0.30-2.66	1.25	0.40-3.91
Huang *	17	121		1.25	0.70-2.22	1.28	0.70-2.33
Wang	66	154		1.37	1.01-1.86	1.41	1.03-1.92
Zhang	9		27	0.74	0.34-1.62	0.89	0.4-1.99

* OR's were inverted to allign with the reference groups chosen in the other studies.

Study	Live birth with niche	Total with niche	Live birth no niche	Total no niche	aOR
Asoglu, 2020	33	75	35	75	0.81 [0.39-1.66]
Diao, 2021	16	74	146	401	0.50 [0.27-0.95]
Friedenthal, 2021	37	81	192	325	0.51 [0.30-0.89]
Huang, 2022	51	215	448	1323	0.63 [0.45-0.88]
Wang, 2022	121	509	581	1570	0.61 [0.48-0.78]
Zhang, 2022	31	129	137	293	0.41 [0.25-0.66]
Summary					**0.58 [0.48-0.69]**

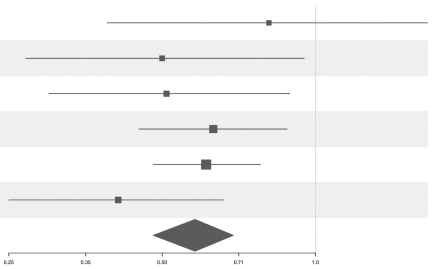

Fig. 2 Live birth rate with niche compared to no defect

Study	Clinical pregnancy with niche	Total with niche	Clinical pregnancy no niche	Total no niche	aOR
Asoglu, 2020	37	75	38	75	1.16 [0.54-2.51]
Diao, 2021	22	74	189	401	0.59 [0.33-1.06]
Huang, 2022	69	215	594	1323	0.59 [0.43-0.80]
Wang, 2022	191	509	748	1570	0.78 [0.62-0.97]
Zhang, 2022	40	129	167	293	0.39 [0.25-0.62]

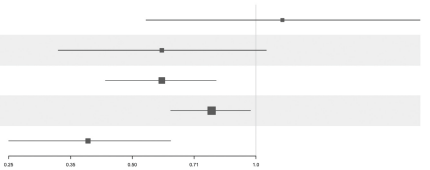

Fig. 3 Clinical pregnancy rate with niche compared to no defect.

Study	Miscarriage with niche	Total pregnant with niche	Miscarriage no niche	Total pregnant no niche	aOR
Asoglu, 2020	6	37	3	38	2.57 [0.58-11.34]
Diao, 2021	4	13	24	119	1.25 [0.40-3.91]
Huang, 2022	17	69	121	594	1.28 [0.70-2.33]
Wang, 2022	66	191	154	748	1.41 [1.03-1.92]
Zhang, 2022	9	40	27	167	0.89 [0.40-1.99]

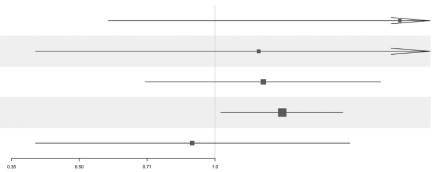

Fig. 4 Miscarriage rate with niche compared to no defect.

Early pregnancy
Five studies reported clinical pregnancy rates. Odds ratios were in line with those of LBR (Table 2, Figure 3). Friedenthal et al. did not report clinical pregnancy rates, it was the only study reporting ongoing pregnancy rate, which was the same as LBR [16]. Four studies reported ectopic pregnancy rate, with in total seven ectopic pregnancies in the niche group and 20 in the control group [18–21].

Five studies reported miscarriage rates. Only Wang et al. found a just significant higher miscarriage rate in persons with a niche, other studies did not find a significant difference. Numbers in all studies were small, in which Wang et al. had the largest numbers (Table 2, Figure 4).

Niche and microbiome
Two studies examined the microbiome in secondary infertile persons with a niche to those with vaginal delivery [24,26]. One study analysed microbial composition in secondary infertile participants with a niche compared to participants with a caesarean without a niche [25]. No follow up was performed to see if participants conceived, hence no data about fertility rates could be extracted. None of the three studies performed any power analysis or sample size calculation.

Two out of three microbiome studies originated from the same research group, with both studies having different inclusion periods. Both studies lacked relevant data on the participants, such as infertility duration, socio-economic status, smoking, and comorbidities. No clear explanation was given how the control group was determined. In both studies there is no definition of a niche given (only described as seen at hysteroscopy). Samples were collected during hysteroscopy for cases with a niche and controls, and they were subsequently analysed using 16s rDNA sequencing. They found a decrease of *Lactobacillus* abundance in the cervix of CS participants, and an increase in the abundance of certain other bacteria (*Proteobacteria, Neisseriaceae, Staphylococcaceae, Sphingomonas, Sediminbacterium,* and *Ralstonia*) [24,26].

Hsu et al. had a more clearly defined prospective cohort (>1 year infertile) and definition of a niche (> 2,5mm indentation). However, this study also lacked relevant population-level data (such as socio-economic status, smoking, comorbidities etc.). Cultures were used to assess microbial composition. Samples were taken with ultrasound guidance during gynaecological examination with a speculum. More bacterial colonies (more Gram-positive cocci, Gram-negative rods (such as *Pseudomonas*)) were found in participants with a niche compared to those without a niche.

Table 3 Concluding evidence of searches

	No. of studies	Study design	No. of patients with a niche	No. of patients without a niche	Effect	Certainty of evidence (GRADE)
Live birth rate	6	observational	1083	3987	aOR 0.58, CI 0.48-0.69	⊕⊕◯◯ LOW
Microbiome	3	observational	138	222	N.A.	⊕◯◯◯ VERY LOW

Discussion

There is low-grade evidence to conclude that the presence of a niche reduces the live birth rates during ART when compared to persons without a niche (Table 3). In the two studies with lowest risk of bias (Wang et al. and Huang et al.), the live birth rate was significantly lower in the niche group. There is insufficient literature on the topic of dysbiosis or intracavitary fluid promoted by the niche influencing ART outcomes.

The previous general review by Riemma et al. did not reveal a lower live birth rate after a CS, possibly because the included studies did not account for the presence of a niche. Since then, more research has come to light reserving a role for the niche in fertility outcomes. One strength of this review is its focus on investigating the impact of the uterine niche, addressing the question of which individuals may experience difficulties conceiving after a cesarean section. We also broadened our scope to include intrauterine fluid and microbiome of individuals with a uterine niche. Additional prospective research is needed to investigate the effects of a niche to determine which persons with a CS may experience lower fertility rates, with a particular focus on intracavitary fluid.

A limitation of our review is the quality of evidence. The heterogeneity among the included studies was high. All studies on fertility outcomes were retrospective cohort studies, which may introduce bias. Preferably, results should be compared between participants with a niche and those with a CS without a niche, rather than comparing participants with a niche to vaginal delivery. The definition of a niche varied among studies, and this is important for comparing results. The definition of >2 mm indentation should be adhered to in future prospective studies. To reduce confounding, focus should also be on single embryo transfers (SET). Studies with a high proportion of DET resulted in a significant number of embryo reductions, which affects the live birth rates. A more complete description of patient characteristics is also essential for adjusting for confounding factors, such as smoking, socio-economic status, ethnicity, body mass index etcetera.

In addition to the heterogeneity of the studies, another limitation could be that the majority of the included studies were conducted in Asian countries. The microbiome is influenced, amongst other factors, by ethnicity, which makes extrapolating the results to another population difficult. Furthermore, the CS rate in China is high, at 35% [27]. This can also introduce bias when comparing our results to other countries with lower CS rates.

In future studies, the description of intracavitary fluid (ICF) should be included, as this could be the primary reason for a lower pregnancy rate. Several studies investigating ICF after CS have suggested that ICF might be the cause of low implantation rates, which is also suggested by the included study by Huang et al. It has been proposed that during frozen cycles, there is less ICF [17,23,28,29]. However, the smaller included study by Diao et al. did not find an impaired pregnancy rate in the presence of ICF [19]. The findings so far are too heterogeneous to provide a clear answer regarding the separate impact of a niche compared to ICF.

Microbiome studies in the IVF population have shown a negative impact of the microbiome on fertility rates [13,14]. If a dysbiotic microbiome is more often encountered in persons with a niche, it could be the reason for lower fertility rates. This review demonstrates a similar association between bacterial counts, specifically lower *Lactobacilli* and an increase in other bacteria such as *Pseudomonas* and *Streptococcus,* as observed in studies about fertility and the microbiome. Therefore, this could be an important factor in understanding lower fertility rates after a CS (with a niche). The changed microbiome could also possibly be linked to the immune system, as the study by Nobuta et al. describes more chronic endometritis in persons with cesarean scar syndrome [30]. Another theory could be that a dysbiotic microbiome (or the microbiome as a proxy for a certain lifestyle) influences the mode of delivery and the wound healing after a CS, increasing the risk of a niche. Future studies should be performed with high-quality design and preferably with objective testing methods for the vaginal or cervical microbiome, such as qPCR or 16s RNA sequencing. None of the included niche microbiome investigating studies reported fertility outcomes. However, this information is key for understanding the potential effects of the microbial environment on fertility and pregnancy outcomes.

Many questions will remain unanswered until further research is conducted. If the issue lies in the niche and the accumulated fluid, a niche repair (either laparoscopically or hysteroscopically) could potentially be beneficial [31]. If the problem is related to an abnormal microbiome, antibiotics, probiotics or lifestyle interventions might prove successful. An interesting hypothesis is that flushing the niche or uterine cavity during

hysterosalpingogram or hysteroscopy could lead to improved outcomes [32]. All treatments should only be offered within the context of a clinical trial to bring us closer to treating subfertility.

However, it is essential to bear in mind that if the first CS could be prevented through effective pre-labour counselling, subsequent pregnancies have fewer complications and lower maternal morbidity. Reducing the caesarean section rate may also contribute to preventing subfertility issues.

Conclusion

There is low-grade evidence to conclude that the presence of a niche reduces the live birth rates when compared to persons without a niche (Table 3). The overall direction of effect in all studies indicates a negative impact of a niche on the live birth rate (pooled aOR 0.58, 0.48-0.69). The theory that this could be attributed to dysbiosis or the intracavitary fluid promoted by the niche is interesting, but there is insufficient literature on this topic.

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Appendix 1

Search string Caesarean niche and IVF fertility

Pubmed
(Isthmocele*[tw] OR "cesarean section"[majr] OR cesarean*[ti] OR caesarean*[ti] OR postcesa*[ti] OR "C-section*"[ti] OR "sectio"[ti] OR "sectios"[ti]) AND ("Fertilization in Vitro"[mesh] OR "Fertilization in Vitro"[tw] OR "Fertilisation in Vitro"[tw] OR "in Vitro Fertili*"[tw] OR "inVitro Fertili*"[tw] OR IVF[tiab] OR "embryo transfer*"[tw] OR "transfer embryo*"[tw] OR "transferring embryo*"[tw] OR "transfered embryo*"[tw] OR ICSI[tiab] OR ((intracytoplasm*[tw] OR (intra[tw] AND cytoplasm*[tw])) AND sperm*[tw] AND inject*[tw]))

175 results on 14 November 2022

Web of Science
title
(Isthmocele* OR "cesarean section" OR cesarean* OR caesarean* OR postcesa* OR "C-section*" OR "sectio" OR "sectios")
topic
("Fertilization in Vitro"[mesh] OR "Fertilization in Vitro" OR "Fertilisation in Vitro" OR "in Vitro Fertili*" OR "inVitro Fertili*" OR IVF[tiab] OR "embryo transfer*" OR "transfer embryo*" OR "transferring embryo*" OR "transfered embryo*" OR ICSI OR ((intracytoplasm* OR (intra AND cytoplasm*)) AND sperm* AND inject*))
108 (31 mrt 2022)

123 results on 14 November 2022

Embase
(Isthmocele*.mp. OR exp *"cesarean section"/ OR cesarean*.ti. OR caesarean*.ti. OR postcesa*.ti. OR "C-section*".ti. OR "sectio".ti. OR "sectios".ti.) AND (exp *"in vitro fertilization"/ OR "Fertilization in Vitro".mp. OR "Fertilisation in Vitro".mp. OR "in Vitro Fertili*".mp. OR "inVitro Fertili*".mp. OR IVF.mp. OR "embryo transfer*".mp. OR "transfer embryo*".mp. OR "transferring embryo*".mp. OR "transfered embryo*".mp. OR ICSI.mp. OR ((intracytoplasm*.mp. OR (intra.mp. AND cytoplasm*.mp.)) AND sperm*.mp. AND inject*.mp.))
79 (31 mrt 2022)

166 results on 14 November 2022 (with option "remove medline records")

Search string Caesarean niche and microbiome

Pubmed
("Microbiology"[Mesh] OR "microbiology" [Subheading] OR microbio*[tw] OR microbia*[tw] OR bacteri*[tw]) AND ("Cesarean Section"[Mesh] OR Cesarean*[tw] OR Caesarean*[tw] OR postcesar*[tw] OR postcaesar*[tw] OR sectio[tw] OR sectios[tw]) AND ("Fertility"[Mesh] OR "Infertility"[Mesh] OR fertilit*[tw] OR infertilit*[tw] OR fecundit*[tw] OR multipara[tw] OR "Reproduction"[Mesh:noexp] OR reproduc*[tiab])

127 results on 7 March 2023

Embase
(exp microbiology/ OR microbio*.mp. OR microbia*.mp. OR bacteri*.mp.) AND (exp cesarean section/ OR Cesarean*.mp. OR Caesarean*.mp. OR postcesar*.mp. OR postcaesar*.mp. OR sectio.mp. OR sectios.mp.) AND (exp fertility/ OR exp infertility/ OR fertilit*.mp. OR infertilit*.mp. OR fecundit*.mp. OR multipara.mp. OR reproduction/ OR reproduct*.ti,ab.)

191 results on 7 March 2023

Web of Science
TS=(microbio* OR microbia* OR bacteri*) AND TS=(Cesarean* OR Caesarean* OR postcesar* OR postcaesar* OR sectio OR sectios) AND TS=(fertilit* OR infertilit* OR fecundit* OR multipara OR reproduct*)

61 results on 7 March 2023

Appendix 2 and 3 not provided, you can inquire about them at the author.

Appendix 4

Extraction form

Author(s)
Journal
Year of Publication
Title
PMID/DOI
Type of research *(prospective/retrospective)*
Country
Study question
Inclusion dates
Included participants cases/controls *(number of deliveries, infertility duration, age, BMI, smoking)*
Definition of a niche in cases and what method to confirm niche
Type of controls *(vaginal/caesarean)*
Type of embryo transfer included *(IVF/ICSI, SET/DET, fresh/frozen, what protocol on intra cavitary fluid)*
For microbiome studies: type of microbiome/bacterial analysis
Inclusion – exclusion criteria
Poweranalysis
Primary outcome
Secondary outcomes
Results – type of statistical test
Adjusted variables
Differences in Table 1
Follow – up
Funding data

Appendix 5 Newcastle-Ottawa scale

Note: A study can be awarded a maximum of one star for each numbered item within the Selection and Exposure categories. A maximum of two stars can be given for Comparability.

		Selection				Comparability	Outcome			Score overall
First Author	Year	Representativeness of the exposed cohort	Selection of the non-exposed cohort	Ascertainment of exposure	Demonstration that the outcome of interest was not present at the start of the study	Comparability of cohorts on the basis of the design or analysis	Assessment of the outcome	Was follow-up enough for outcomes to occur	Adequacy of follow-up of cohorts	
Asoglu	2020	X			X	X	X	X	X	**6**
Friedenthal	2021	X	X		X		X	X	X	**6**
Diao	2021		X		X		X	x	X	**5**
Huang	2022	X	X	X	X	X	X	X	X	**8**
Wang	2022	X	X	X	X	XX	X	X	X	**9**
Zhang	2022		X	X	X	X		X	X	**6**
Hsu	2022	X	X	X	X					**4**
Yang	2021	X					X			**2**
Yang	2022	X					X			**2**

8

SUMMARY AND DISCUSSION

Summary

This thesis aimed to assess the impact of bacterial vaginosis (BV) and caesarean section scars on subfertility. ***Chapter 1*** gave a general introduction on BV, caesarean section scars and fertility treatments in the Netherlands. In ***chapter 2*** the relationship between (asymptomatic) bacterial vaginosis and pregnancy rates during intrauterine insemination (IUI) or in vitro fertilization (IVF) treatment is investigated. This chapter reveals a higher incidence of miscarriages among participants testing positive for bacterial vaginosis, with an odds ratio of 4.22 (1.10-16.21). Although no significant differences were observed in ongoing pregnancy rates and live birth rates, there was a tendency for these rates to be lower in qPCR BV positive participants. Of all 133 attempts, the incidence of asymptomatic bacterial vaginosis was 26%. Additionally, this study compared qPCR results with the microbiome sequencing results, which were in line with each other. qPCR is a less extensive and expensive method to analyse the presence of bacterial vaginosis compared to microbiome 16s rRNA sequencing. ***Chapter 3*** showed that a simple pH-measurement could accurately predict if asymptomatic subfertile participants have a qPCR BV positive swab. It could reduce costs to perform more extensive testing only when the vaginal pH is 4.7 or above. During fertility treatments, the vaginal microbiome of participants changes, as shown in ***chapter 4***. Overall, the number of *Lactobacilli* decreased during fertility treatment. Switching to BV positivity was associated with a slightly reduced number of *Lactobacilli* already at intake, which decreased below 50% during fertility treatment in 17% of participants. The hypothesis posits that the exogenous hormones and stress during fertility treatment alter the balance of the microbiome. The only three cases in which BV positivity was reversed to BV negativity, possibly resulted from lifestyle interventions. To assess if testing for BV at initial fertility assessment has an added value, ***chapter 5*** delves deeper into the time to pregnancy of all participants at initial fertility assessment. No difference was observed in time to pregnancy based on BV-status in the whole group. However, participants with a lower chance to conceive (e.g. unexplained subfertility or necessity to start fertility treatment) tended to have a longer time to pregnancy if they were BV positive.

Chapter 6 shows that participants with a previous caesarean delivery had significant lower live birth rates during IVF treatment compared to a previous vaginal delivery, with an adjusted odds ratio of 0.60 (0.46-0.89). This was a retrospective study in which data from 1588 embryo transfers was analysed. Unfortunately, no data regarding a niche could be collected. ***Chapter 7*** reviewed literature specifically on pregnancy outcome data in persons with a niche. The review showed an adverse effect on live birth rates compared to those who had undergone a caesarean section in general (without niche) or had a previous vaginal delivery. However, the quality of the included studies was low due to study design and heterogeneity, resulting in low-grade evidence of the adverse effect.

For the second hypothesis of this review concerning the microbiome of the fluid inside the niche, only three poorly designed studies could be included. A potential relationship between abnormal microbiome (or BV) and niche (fluid) formation could not be assessed. The three studies did describe the same kind of bacteria, such as *Proteobacteria*, in a niche as those bacteria associated with poor IVF pregnancy results.

Discussion

The bacterial jungle

The normal vaginal microbiome is characterised by a high abundance of *Lactobacilli*. In contrast to the gut microbiome, which benefits from diversity, the vaginal microbiome is ideally less diverse. *Lactobacilli* play a crucial role by producing lactic acid, maintaining the vagina's acidic environment at a pH-level of 3.5-4.5, thereby inhibiting the growth of pathogenic microorganisms. The production of lactic acid is under control of the oestrogen levels present, and therefore the vaginal microbiome changes throughout the woman's life cycle. An increase in diversity of bacteria in the vaginal microbiome can lead to dysbiosis or BV. BV-related bacteria have shown to induce immune activation and elevate levels of pro inflammatory cytokines, resulting in mucosal inflammation of the genital tract [1,2].

Impact on fertility

This thesis shows that BV has an impact on fertility treatment outcomes, with higher miscarriage rates and longer time to pregnancy. Testing for BV at fertility intake has an informative value for couples and fertility experts on time to pregnancy for persons starting with fertility treatment (IUI and IVF) (***chapter 5***). During fertility treatment with IUI or IVF, it is shown BV could be a reason for miscarriages and has a slight influence on ongoing pregnancy (***chapter 2,*** BIFI study). Both studies of chapter 2 and 5 are one of the first studies on bacterial vaginosis and pregnancy results combining longitudinal treatment data of multiple attempts of IUI and IVF until live birth. These results should be interpreted with caution due to the limited number of included participants. More significance might have been achieved with a larger number of participants, as a trend towards a negative effect is observed. Not reaching the intended number of participants as calculated by the power analysis for this study was primarily attributed to the low inclusion rates during the COVID pandemic and individuals being less willing to participate into the study. Further research on the impact of BV on pregnancy outcomes in fertility treatment should follow, with its focus on IVF. The latest review on this topic in IVF treatments did show a negative effect on early pregnancy loss and clinical pregnancy but did not find a significant decrease in live birth rate [3]. Our study, combining IUI and IVF, has a less

pronounced correlation than earlier studies [4,5] because participants starting IUI had probably a better prognosis. The influence of BV is therefore probably more pronounced in the IVF treatment group, who have a poorer prognosis.

Test methods

A strength of our studies is the combined pH, qPCR, and microbiome analysis, to show the highly comparative results between the different testing methods. There is no consensus on how to measure bacterial vaginosis or an abnormal vaginal microbiome. Skafte-Holm also encouraged to combine different testing methods to make comparability between studies better [3]. At this point, to combine data of different studies is therefore challenging. We believe DNA based measurements are more precise with less intraobserver bias than microscopic analysis with, for example, the Nugent score. However, different kits of qPCR have different levels of thresholds, so consensus on which threshold is the best to define a qPCR BV positive would make research more comparable. qPCR analysis is less expensive than complete 16s rRNA sequence analysis, and ***chapter 2*** showed that they have comparable results. If testing the vaginal microbiome becomes an added value in the fertility work-up, only an indicative pH measurement or qPCR could be sufficient as a cost-effective initial measurement ***(chapter 3).***

Influence of lifestyle and fertility treatments

Our prospective collected data showed a slightly higher body mass index (BMI), lower socio-economic status (SES) and more smokers in BV positive groups. This was not reported in earlier studies investigating fertility outcomes. Other studies did not document SES, or only reported smoking as 'ever smoked' [4,5]. Lifestyle is an important issue to consider as confounding factor. Treatment of BV with antibiotics or probiotics has not yet been shown to have a long-term effect, and it may not necessarily influence the poorer fertility outcomes without improving lifestyle. It is important to thoroughly document lifestyle factors during future studies as well, to gain a better understanding of the causality between poor fertility outcomes and BV and their underlying factors.

Chapter 4 presented three cases in which the microbiome improved along the way from initial fertility assessment to fertility treatment. The only distinguishing factors from other individuals were their attempts to lose weight or quit smoking. There is currently too little evidence to support that lifestyle intervention for obese persons could improve the vaginal microbiome [6]. A general decrease of the beneficial *Lactobacilli* during fertility treatments was shown in ***Chapter 4***. Due to this decrease, a negative shift towards BV in the microbiome appeared in nine cases, however, Shannon Diversity Index was not significantly altered. This suggests that individuals with an already divers colonised microbiome were more prone to switch to BV. Fertility treatments, including most of them

with hormonal medications and hormonal shifts in the body, could pose great stress on the psyche and on the body. Bacterial vaginosis is therefore potentially not a symptom on its own, but a proxy for the inner balance or immune system of a person. When this balance is disrupted, non-beneficial bacteria can overgrow and lead to harmful side effects.

To test or not to test

Testing for an abnormal microbiome should be discouraged at this point, as there is no evidence-based treatment option for BV which improves pregnancy results. The WHO's screening principles (Wilson and Jungner criteria) describe that screening for a disease should only be conducted when treatment options are available. Some studies also test the endometrial microbiome, however this is more invasive and shows similar results with vaginal microbiome, so this should be abandoned [7,8]. The only randomised controlled study about treating asymptomatic BV on pregnancy results did not show an effect, and only one randomised controlled study in a general group of participants undergoing frozen embryo transfers showed a possible effect of probiotic treatment [9–11]. If persons have discharge complaints and are symptomatic for BV, these complaints should be taken seriously and should be treated by antibiotics and probiotics combined [12].

The future of microbiome testing

Vaginal microbiome testing gives detailed information on the number of different types of bacteria. Microbiome testing could give an answer, in contrast to qPCR, if someone belongs to for example the 'medium' prognosis group of community state type III (dominated by *L. iners*). Not enough is known why *L. iners* dominated persons have poorer outcomes than other *Lactobacilli* dominated persons (***chapter 2 & 5***) [5]. The only possible way to 'treat' *L. iners* dominance could be by transplantation of the more beneficial *L. crispatus*. Vaginal microbiome transplantation (VMT) is still in a very early stage being researched. Wrønding et al. describe the procedure of VMT as collecting samples from several donors with a menstrual cup, followed by processing the microbiome and cryopreserving it. The donor sample is then matched to the patient in an in vitro competition assay to assess whether the graft can inhibit the growth of the patient's dysbiotic bacteria. The VMT is introduced through an intrauterine insemination catheter into the patient's vagina. This is a highly personalised procedure and, therefore, very time consuming [13,14].

Scary caesarean sections

The issue of subfertility after a caesarean section is often overlooked by healthcare professionals. However, couples who experienced a quick conception for their first child, delivered by caesarean, inquire about a link for causality between this caesarean and their subfertility. Current literature is still inconclusive about the exact influence of caesarean sections. A review and meta-analysis of Zhao et al. about caesarean delivery compared to

vaginal delivery found a negative effect on live birth (including our SCAR study of ***chapter 6***) [15]. Another recent review and meta-analysis by Riemma et al. did not show a significant negative effect on live birth rates in IVF. Riemma included more recent studies than Zhao, as well as our SCAR study of ***chapter 6*** [16]. An earlier Delphi study did confirm that caesarean scar disorder (CSDi) includes subfertility as a symptom of a niche, however they made a consensus on little evidence-based literature [17]. ***Chapter 7*** reviews all current literature on comparing pregnancy results of persons with a niche during IVF with those without a niche. Our review shows a negative association of a niche and subfertility, and therefore evidence of a possible 'dose response' negative effect after a caesarean section. The more complicated the recovery after a caesarean section, the larger the niche, the higher the chance it will influence fertility [18]. Subfertile individuals with a caesarean scar, and especially with a niche, and wishing to conceive should be informed that the previous caesarean section could potentially contribute to their subfertility.

The microbiome after a caesarean section

Intracavitary fluid is more often encountered during hormonal stimulation when persons have a niche. Strategies about transferring an embryo when intracavitary fluid is present are different between studies and hospitals [19]. This intracavitary fluid has an embryotoxic effect on implantation similar to that of a hydrosalpinx [20]. Our review (***chapter 7)*** included three studies of poor quality assessing which bacteria are present in persons with and without a niche. The same kind of bacteria were described in a niche as those bacteria associated with poor IVF pregnancy results. They showed fewer *Lactobacilli* and increased presence of other types of bacteria, such as *Proteobacteria*. The hypothesis that fluid inside a niche contains or promotes an abnormal microbiome should be further researched.

To this day, a comprehensive understanding of the reasons behind subfertility and the niche (or its fluid) remains elusive. There is limited evidence that hysteroscopic or laparoscopic repair for a niche could improve pregnancy outcomes [21]. Only if persons have postmenstrual spotting, surgical options are proven effective [22].

Future research and perspectives

The niche

Prospective studies are necessary to compare IVF pregnancy results between persons with a caesarean section with and without a niche, to confirm the negative influence of the niche. Ideally, also the microbiome at cervical and vaginal level of these persons should be considered. The niche should be well-described, and studies should adhere to the definition of a >2mm indentation. This will provide more detailed information on the potential reasons for the lower pregnancy rates following a caesarean section. Research

on different surgical techniques for preventing a niche and niche related problems could show a positive effect on subfertility in the future.

Treatment of BV

High-quality research, such as randomised controlled trials, should be conducted to analyse if lifestyle interventions, treatment with antibiotics or probiotics or vaginal microbiome transplantation could improve pregnancy outcomes in BV positive persons. The randomised controlled trial of Haahr et al. (treating BV with antibiotics and/or probiotics) did not show any improvement yet [10,11]. These studies should first be performed in the IVF population, as the effect of BV is most pronounced in that population. Afterward, it should be investigated whether the treatment of BV can also improve time to pregnancy in an IUI or unexplained subfertility population. Our studies and previous literature show that it is key for future studies to include BMI, smoking status, socio economic status and ethnicity in their assessment [23]. Only by taking all these factors into account the exact causality between BV and negative live birth outcomes can be revealed.

Outcome measures

Both the SCAR and BIFI study included multiple (IUI and) IVF attempts in their statistical analysis, which we consider a major strength of both studies. The majority of fertility studies uses only one single attempt of IUI or IVF as outcome measure. We think it is important to account for time to pregnancy in fertility studies. This could be accomplished in different ways, for example, by including multiple attempts and analysis with Generalised Estimated Equations analysis (same as chapter 2 and 6) or with survival analysis (as in chapter 5). This approach should be used in future research because most fertility treatments do not consist of one attempt. Secondly, studies must report live birth rates together with ongoing pregnancy rates as this represents the most important outcome.

The best solution

Because BV could be a proxy for the overall health of a person, more effect could be expected of randomised controlled lifestyle trials. Fertility treatments are expensive, and if success rates could be improved by lifestyle adjustments, this could reduce the number of treatments needed and therefore the costs and burden to society [24–26]. If lifestyle interventions are effective for BV and pregnancy results, testing for BV could more easily convince persons to follow a healthier lifestyle after fertility intake. When couples can control their fertility potential, they are more motivated to make lasting changes to their lifestyle. This could result in a positive cascade with healthier pregnancies, less caesarean sections, healthier mothers, and following generations, in a world currently struggling with a growing amount of lifestyle associated diseases.

Take home message of this thesis

This thesis shows the adverse effects of bacterial vaginosis and caesarean sections on fertility treatment. Bacterial vaginosis may present with minimal or no noticeable discharge complaints but can contribute to significant consequences. The growing prevalence of caesarean deliveries has faced criticism for its implications on future pregnancies but could also potentially affect a person's fertility. This thesis aims to contribute a component to the larger puzzle why certain persons struggle to conceive, especially when the subfertility remains unexplained. However, the research conducted thus far has not identified the exact causality, and consequently, a cure for these problems has not been determined. Adopting a healthy lifestyle could be a crucial factor in preventing bacterial vaginosis or obstructed labour. Exercising a bit more patience in our society, such as postponing an IVF treatment until a more favourable microbiome is achieved or refraining from hastily proceeding to the operation room for a caesarean section, could potentially prevent negative subsequent outcomes. Additionally, accurate pre-labour counselling for a caesarean section could provide couples with more insight into the potential negative consequences of the procedure. Having a holistic view of the whole woman, considering her past and future, can facilitate shared decision-making and serve as a foundation for more personalised medicine in fertility treatment.

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9

NEDERLANDSE SAMENVATTING

Nederlandse samenvatting

Onvruchtbaarheid, ook wel subfertiliteit, is een toenemend probleem in de maatschappij, voornamelijk door de steeds hogere leeftijd waarop vrouwen hun eerste kind krijgen. Er mag worden gesproken van subfertiliteit als er na één jaar gerichte gemeenschap geen spontane zwangerschap is opgetreden. Bij het oriënterend fertiliteitsonderzoek, dat wordt verricht bij subfertiele stellen, wordt onderzocht wat de oorzaak kan zijn van de subfertiliteit. Duidelijke oorzaken zijn een onregelmatige menstruatiecyclus, verminderde spermakwaliteit of afgesloten eileiders. Hier kan een vruchtbaarheidsbehandeling voor ingesteld worden zoals ovulatie-inductie, intra-uteriene inseminatie of in-vitrofertilisatie (IVF). Echter, bij 30% van de subfertiele stellen wordt er geen duidelijke oorzaak voor de subfertiliteit gevonden en wordt vervolgens vaak eerst een aantal maanden afwachtend beleid afgesproken. Gezien de toename van subfertiliteit, het aantal vruchtbaarheidsbehandelingen, en daarmee de stijgende kosten voor de gezondheidszorg, is het belangrijk om ook andere factoren die mogelijk een ongunstige invloed hebben op de fertiliteit verder te onderzoeken.

In dit proefschrift wordt de impact van twee factoren op (sub)fertiliteit onderzocht; bacteriële vaginose en het keizersnedelitteken. Bacteriële vaginose (BV) is een disbalans in het vaginale microbioom, waarbij er een verminderd aantal *Lactobacillen* in de vagina aanwezig zijn. Dit kan vaginale afscheidingsklachten geven en een riekende geur, maar is in de helft van de gevallen asymptomatisch. Bacteriële vaginose wordt beïnvloed door factoren als obesitas, roken en het gebruik van hormonen. Het is al vaker in verband gebracht met vroeggeboortes. Er zijn verschillende manieren om te testen of iemand BV heeft, zoals de Amsel criteria (met pH meting), Nugent score, qPCR en 16s rRNA sequencing van het gehele microbioom. Het behandelen van BV is echter lastig, na behandeling met alleen antibiotica komen bij veel mensen de klachten na enkele weken terug.

In het eerste deel van dit proefschrift wordt het vaginale microbioom van subfertiele vrouwen onderzocht. Eerdere studies die dit onderzochten keken voornamelijk naar het microbioom tijdens een ivf-behandeling, in dit proefschrift wordt het breder getrokken naar de gehele subfertiele populatie.

In ***hoofdstuk 2*** wordt het verband tussen asymptomatische bacteriële vaginose (BV) en zwangerschapsuitkomsten bij intra-uteriene inseminatie (IUI) en in-vitrofertilisatie (IVF) beschreven. Van alle 133 pogingen IUI en IVF die worden beschreven, was de incidentie van BV 26%. Ons onderzoek toont een hogere incidentie van miskramen bij IUI en IVF wanneer er positief werd getest op BV (odds ratio van 4.22 (1.10-16.21)). Er lijkt een tendens te zijn voor een lager aantal levendgeborenen bij BV positieve deelnemers, echter was dit

verschil niet significant. Tevens onderzocht deze studie de gelijkenis tussen qPCR analyse en microbioom sequencing voor het aantonen van BV. qPCR is een goedkopere en minder uitgebreide methode om BV te diagnosticeren dan 16s rRNA microbioom sequencing. In ***hoofdstuk 3*** onderzoeken we of een eenvoudige pH meting een alternatief kan zijn voor de duurdere qPCR bepaling van BV. We tonen aan dat een eenvoudige pH meting accuraat kan voorspellen of een asymptomatische subfertiele vrouw een positieve qPCR BV test heeft. Door het uitvoeren van een simpele pH test kunnen kosten bespaard worden wanneer enkel een qPCR wordt verricht bij een pH boven of gelijk aan 4.7. Tijdens een vruchtbaarheidsbehandeling kan het vaginale microbioom van deelnemers veranderen, wat wordt gedemonstreerd in ***hoofdstuk 4***. Het aantal *Lactobacillen* daalt gedurende een vruchtbaarheidsbehandeling. Deelnemers die tijdens deze behandeling van qPCR BV negatief naar qPCR BV positief veranderden, 17% van het totaal aantal deelnemers, hadden al een licht verlaagd aantal *Lactobacillen* bij hun intake. De hypothese is dat exogene hormonen en stress tijdens een vruchtbaarheidsbehandeling de balans van het microbioom verstoort. In drie gevallen werd er een verbetering van het microbioom gezien, welke mogelijk toe te schrijven is aan leefstijlveranderingen van deze deelnemers. Om te bepalen of testen op BV zinvol is bij het oriënterend fertiliteitsonderzoek, onderzoeken we in ***hoofdstuk 5*** wat de tijd tot zwangerschap is bij verschillende groepen deelnemers gebaseerd op hun BV-status. Er werd geen verschil gezien in tijd tot zwangerschap gebaseerd op BV-status in de gehele groep. Echter, deelnemers met een lage kans op spontane zwangerschap (zoals een directe indicatie voor vruchtbaarheidsbehandeling of bij onverklaarde subfertiliteit) leken wel een langere tijd tot zwangerschap te hebben als zij BV positief waren ten opzichte van qPCR BV negatieve deelnemers.

In het tweede deel van dit proefschrift wordt dieper ingegaan op het keizersnedelitteken, en de implicaties daarvan op de fertiliteit. Het aantal bevallingen per keizersnede stijgt de laatste decennia, in sommige landen tot wel 40% van het totale aantal bevallingen. Dit wordt toegeschreven aan onder andere de toename van obesitas, de hogere maternale leeftijd en de toegenomen risico aversie van de maatschappij. Keizersnedelittekens geven echter een hoger risico op zwangerschapscomplicaties in een volgende zwangerschap, en worden ook in verband gebracht met subfertiliteit. Een deel van de problemen na een keizersnede ligt mogelijk aan een niche. Een niche is een inkeping in de baarmoederwand ter plaatse van het litteken van ten minste 2mm, welke soms gevuld is met vocht of bloed.

Hoofdstuk 6 laat zien dat er significant minder levendgeborenen zijn bij ivf-behandeling bij personen die een keizersnede hebben gehad ten opzichte van personen die vaginaal zijn bevallen (gecorrigeerde odds ratio van 0.60 (0.46-0.89)). Dit was een retrospectief onderzoek, waarbij data van 1588 embryo terugplaatsingen is geanalyseerd. Helaas bevatte deze data geen informatie of er een niche ter plaatse van het keizersnedelitteken

zat, maar toonde wel de ongunstige invloed van een keizersnedelitteken aan. ***Hoofdstuk 7*** vat bestaande wetenschappelijke literatuur samen waarbij specifiek is gekeken naar de zwangerschapsuitkomsten van vrouwen met een niche. Deze review laat zien dat de niche een negatief effect heeft op het aantal levendgeborenen, vergeleken met vrouwen die vaginaal zijn bevallen of een keizersnedelitteken hebben zonder niche. Echter, de kwaliteit van het bewijs was laag op basis van de wisselende studieopzet en heterogeniteit van de geïncludeerde personen in de verschillende studies. Een tweede hypothese die werd onderzocht in deze review was of het vocht dat in de niche aanwezig is een abnormaal microbioom bevat. Dit werd helaas maar in drie studies van lage kwaliteit onderzocht. In deze studies werden dezelfde soort bacteriën beschreven als in de studies bij vrouwen die IVF ondergaan waar deze zijn geassocieerd met slechtere zwangerschapsuitkomsten. Een causaal verband tussen een abnormaal microbioom en een niche kon echter niet worden gelegd vanwege het lage aantal gevonden studies.

Concluderend, laat dit proefschrift zien dat bacteriële vaginose en een keizersnedelitteken (en dan voornamelijk met een niche) een negatieve invloed hebben op zwangerschapskansen bij subfertiele vrouwen. De uitkomsten van dit proefschrift worden bediscussieerd in ***hoofdstuk 8***. Allereerst is het belangrijk om in de toekomst een vaste definitie voor BV of een abnormaal microbioom te hebben, zodat er op een gelijkwaardige manier gemeten wordt en onderzoeken goed vergelijkbaar zijn. Tot op heden is nog niet aangetoond dat het behandelen van BV met antibiotica of probiotica helpt om de zwangerschapsuitkomsten te verbeteren in deze subfertiele groep. Om deze reden moet bij deze groep vrouwen alleen worden getest op BV in onderzoeksverband. Mogelijk is in de behandeling van BV, naast antibiotica of probiotica, ook een rol weggelegd voor leefstijl van de subfertiele vrouw tijdens het oriënterend fertiliteitsonderzoek. Daarnaast lijkt behandeling middels vaginale microbioomtransplantatie veelbelovend, maar dit is nog niet grootschalig toegepast en onderzocht. Tot op heden is nog onvoldoende bekend of en hoe een niche de zwangerschapskansen vermindert, bovendien zijn er onvoldoende aanwijzingen dat een (operatieve) behandeling van een niche de kansen zou verbeteren. Ook de intrigerende hypothese dat mogelijk het vocht, danwel het microbioom, in de niche zorgt voor de verminderde kans op zwangerschap vraagt om verder wetenschappelijk onderzoek.

Dit proefschrift laat zien dat (asymptomatische) bacteriële vaginose en een keizersnede in de voorgeschiedenis beide een rol hebben in (onverklaarde) subfertiliteit bij vrouwen die een vruchtbaarheidsbehandeling ondergaan. Voor beide factoren, bacteriële vaginose en keizersnede, geldt: voorkomen is beter dan genezen!

10

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Curriculum Vitae

Marjolein werd op 19 januari 1992 thuis geboren te Gouda. Zij slaagde in 2010 cum laude aan het Coornhert Gymnasium te Gouda. Tijdens de laatste twee jaar op de middelbare school heeft zij tevens het programma bij het Pre-University College in Leiden gevolgd. Door dit traject kwam zij al vroeg in aanraking met het doen van wetenschappelijk onderzoek.

In 2010 startte Marjolein met de opleiding Geneeskunde aan de Universiteit Leiden, die zij in 2016 afrondde. Zij volgde tevens het Honours College in de bachelorfase en het Leiden Leadership Programme in haar masterfase aan de Universiteit Leiden. In 2012 ging zij ter verbreding van haar studie onderzoek doen bij prof. Frank Willem Jansen op de gynaecologie-afdeling. Na een verloskundestage in het Mount Meru Regional Referral Hospital in Arusha, Tanzania (2013), was zij verkocht aan de Gynaecologie. De wetenschapsstage van Geneeskunde voerde zij uit bij de fertiliteitsafdeling van het LUMC, wat het begin werd van dit promotieonderzoek.

Zij werkte na haar studie een jaar als ANIOS Obstetrie en Gynaecologie in het HMC Bronovo. In deze periode startte zij de BIFI-studie voor dit proefschrift onder leiding van dr. K.E. Boers. Zij begon met haar opleiding tot gynaecoloog in 2018 in het HMC (met als opleiders dr. M.J. Kagie/dr. W. Hermes) en in het LUMC (opleiders prof. dr. J.J.M. van Lith/ dr. M. Sueters). Vanaf het vierde jaar van haar opleiding tot gynaecoloog kreeg zij een wetenschapsbeurs van het HMC, waarna ze vol toewijding haar promotieonderzoek naast haar opleiding heeft voltooid. De voorliefde voor fertiliteit heeft zich geuit in haar verdere differentiatie tot gynaecoloog bij de Voortplantingsgeneeskunde bij het Reinier de Graaf Gasthuis (opleider dr. K. Kapiteijn) en Erasmus Medisch Centrum (onder supervisie van prof. dr. J.S.E. Laven). Voor de differentiatie benigne gynaecologie liep zij een stage in het Mount Sinai Hospital in Toronto, Canada, onder supervisie van dr. A. Murji en dr. J.M. Solnik (mei 2024). Dit jaar rondt Marjolein haar opleiding tot gynaecoloog af.

Marjolein woont met haar man Vincent in Den Haag.

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